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The Role of ATM in the Regulation of Interferon-γ Responses

by

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Declaration of Originality

Unless otherwise stated in the text, the present thesis is the result of my own work.

Abstract

Interferon (IFN)-γ is a key cytokine in the modulation of immune responses necessary for elimination of viral infections and some cancer cells. IFN-γ signals through the Janus kinase (JAK)/ signal transducers and activators of transcription (STAT) signalling pathways. Ataxia telangiectasia mutated (ATM) was identified by our group as a regulator of IFN-γ responses. Within DNA double-strand break (DSB) repair, ATM controls chromatin remodelling, which is also indispensable for transcription. We hypothesised, therefore, that ATM modulated IFN-γ responses at the transcriptional level by regulating STAT1 chromatin binding and/or chromatin-modifying proteins. ATM silencing impaired to varying degrees IFN-γ-induced expression of several IFN-stimulated genes (ISGs) at the mRNA and protein levels and delayed STAT1 serine 727 phosphorylation within the nucleus. These effects were independent of ATM enzymatic activity. ATM knockdown impaired IFN-γ-induced binding of STAT1 to class II transactivator (CIITA) and human leucocyte antigen (HLA)-DRα promoters, but not to IFN regulatory factor (IRF)-1 and antigen peptide transporter (TAP) 1 promoters. This could account for HLA Class II expression being more severely impaired than that of HLA Class I, even though both are classical IFN-γ target genes. ATM, however, bound to the promoters of all these ISGs. ATM and STAT1 interacted under basal conditions, with STAT1 becoming tyrosine 701 phosphorylated upon IFN-γ treatment. The siATM phenotype regarding HLA class II expression was partially or completely recapitulated by silencing of histone deacetylase (HDAC) 1 and 3, respectively, in contrast to phenotypes induced by RNAi against HDAC2 and Kruppel-associated box (KRAB) domain-associated protein 1 (KAP1). KAP1 also acted, directly or indirectly, as a negative regulator of IFN-γ-induced STAT1 phosphorylation and, consequently, of HLA class I and II HLA expression. Interestingly, KAP1 interaction with STAT1 decreased after IFN-γ treatment. Overall our data indicates that ATM plays an important role as a modulator of IFN-γ-mediated transcription probably by interacting with STAT1, regulating its binding to the promoters of a specific subset of ISGs and regulating chromatin-modifying proteins such as KAP1, HDAC1, HDAC2 and HDCA3. Understanding how ATM regulates IFN-γ signalling may provide new insights into important immune-mediated mechanisms, such as those involved in tumour suppression and thus have therapeutic value.

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Abbreviations

A_{2A}AR	A_{2A} adenosine receptor
AK	Adenylate kinase
APCs	Antigen-presenting cells
APS	Ammonium persulphate
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia- and Rad3-related
BRCA	Breast cancer
BRD	Bromodomain-containing protein
BRG1	Brahma-related gene 1
BSA	Bovine serum albumin
CaMK	Calcium-calmodulin-dependent kinase
CBP	CREB binding protein
CCD	Coiled-coil domain
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
CHK	Checkpoint kinase
CIITA	Class II transactivator
CIS	Cytokine-inducible SH2-containing protein
CK	Casein kinase
Co-IP	Co-Immunoprecipitation
CREB	cAMP response element-binding
CRM1	Chromosome region maintenance 1
CTL	Cytotoxic T lymphocytes
CXCL9	CXC ligand 9
DAPI	4’,6-diamidino-2-phenylindole dihydrochloride
DBD	DNA-binding domain
DDR	DNA damage repair
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DNA-PKCcs	DNA-dependent protein kinase catalytic subunit
DSB	Double-strand break
DTT	Dithiothreitol
EBNA1	Epstein-Barr nuclear antigen 1
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EMCV	Encephalomyocarditis virus
ERK	Extracellular-signal-regulated kinases
FAT	FRAP-ATM-TRRAP
FATC	FAT-C-terminal
FC	Flow cytometry
FCS	Foetal calf serum
FERM	Four-point-one, ezrin, radixin and moesin
FITC	Fluorescein isothiocyanate
FRAP	FKBP-rapamycin-associated protein
GAS	Gamma-activated sequence
GBP	Guanylate-binding protein
GFP	Green fluorescent protein
GH	Growth hormone
GM-CSF	Colony-stimulating factor
GRIM19	Gene associated with retinoid-IFN-induced mortality 19
hBRM	Human Brahma
HDAC	Histone deacetylase
HEAT	Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A and TOR1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2	Epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
HK	Hexokinase
HLA	Human leucocyte antigen

HMGB1	Histone H1-high-mobility group B
HP1	Heterochromatin protein 1
hPIV	Human parainfluenza virus
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICS	IFN consensus sequence
IEG	Immediate early gene
IF	Immunofluorescence
IFIT2	ISG interferon-induced protein with tetratricopeptide repeats 2
IFN	Interferon
IFNGR	IFN-γ receptor
IFNGR1	IFN-γ receptor α chain
IFNGR2	IFN-γ receptor β chain
IgG	Immunoglobulin G
Ii	Invariant chain
IKK	Inhibitor of kB kinase
IL	Interleukin
IP10	IFN-γ-induced protein 10
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
JAK	Janus kinase
JH	JAK homology
KAP1	Kruppel-associated box (KRAB) domain-associated protein 1
KD	Kinase domain
kDa	Kilodaltons
KU-55933	2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one
LCMV	Lymphocytic Choriomeningitis Virus
LD	Linker domain
LMP	Low molecular mass polypeptide
MAPK	Mitogen-activated protein kinase
MCL	Myeloid cell leukemia
MCM	Minichromosome maintenance complex component
MDC	Mediator of DNA damage checkpoint protein

MEFs	Mouse embryonic fibroblasts
MEKK	Mitogen-activated protein kinase kinase kinase
MET	Hepatocyte growth factor receptor
$MgCl_2$	Magnesium chloride
MHC	Major histocompatibility complex
MRAS	Muscle RAS oncogene homolog
MRN	MRE11-RAD50-NBS1
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NES	Nuclear export signal
NF-κB	Nuclear factor-κB
NFY	Nuclear factor Y
NK	Natural killer
NKG2D	Natural-killer group 2, member D
NLS	Nuclear localisation signal
NP-40	Nonidet P-40
NRTKs	Non-receptor tyrosine kinases
NS	Nonspecific
ns	Nonsignificant
NSCLC	Non-small cell lung cancer
NTD	N-terminal domain
OAS	2’,5’-oligoadenylate synthase
OSM	Oncostatin M
p	Promoter
p21	Protein 21
p53	Protein 53
PARP1	Poly[adenosine diphosphate-ribose] polymerase-1
PBS	Phosphate buffered saline
PDLIM	LIM domain
PE	Phycoerithrin
PFA	Para-formaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIAS	Protein inhibitors against STATs

PIKKs	Phosphatidylinositol-3 kinase-related kinases
PKA	Protein kinase A
PKC-δ	Protein kinase C-δ
PKR	Double-stranded RNA-activated protein kinase
PMSF	Phenylmethylsulfonyl fluoride
PMSP	Proline-Methionine-Serine-Proline
PRD	PIKK-regulatory domain
PRKWNK2	WNK lysine deficient protein kinse 2
PRMT1	N-methyltransferase 1
PTMs	Post-translational modifications
PTP	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
PYK2	Proline-rich tyrosine kinase 2
qPCR	Real time quantitative-PCR
RFX	Regulatory factor X
RIG-I	Retinoic acid-inducible gene I
RIPA	Radioimmunoprecipitation assay
RNAi	RNA interference
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SCFR	Stem cell growth factor receptor
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	SRC homology
SH2D	SRC homology 2 (SH2) domain
SHP	SH2-domain containing tyrosine phosphatase
SMG-1	Suppressor of morphogenesis in genitalia
SMRT	Silencing mediator for retinoic acid receptor and thyroid hormone receptor
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription

SV	Simian virus
SWI/SNF	SWItch/Sucrose NonFermentable
TAD	Transactivation domain
TAF1	TATA binding protein-associated factor 1
TAF1L	TAF1-like
TAP	Antigen peptide transporter
TEMED	N,N,N,N-tetrametiletilenodiamine
T_H1	T helper cells type 1
T_H2	T helper cells type 2
Tip60	TAT-interacting protein 60
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Tris-base	Tris(hydroxymethyl)aminomethane
TRRAP	Transformation/transcription domain-associated protein
TSA	Trichostatin A
TSS	Transcription start site
TTBK	τ tubulin kinase
TYK2	Tyrosine kinase 2
Ubc8	Ubiquitin conjugating enzyme 8
UBP43	Ubiquitin-specific protease 43
USF	Upstream stimulatory factor
VSV	Vesicular stomatitis virus
WB	Western blot
WT	Wild-type

CHAPTER 1

Introduction

1.1. Janus kinase (JAK)/ signal transducers and activators of transcription (STAT) signalling pathways and Interferon (IFN)-γ

The major biological responses to Interferon (IFN)-γ, including antiviral responses, immune modulation and tumour suppression, are mediated through well-known canonical Janus kinase (JAK)/ signal transducers and activator of transcription (STAT) signalling pathways (Bach *et al*., 1997; Santos and Costa-Pereira, 2011; Schindler and Darnell, 1995; Stark *et al*., 1998). In addition to this type II IFN, type I IFNs and a considerable number of other cytokines and growth factors also commonly use the JAK/STAT pathway to regulate not only the immune system, but also biological processes such as cell growth, cell differentiation and apoptosis (Stark *et al*., 1998), which are often deregulated in cancer. Signal specificity is achieved through different combinations of members of the JAK and STAT families in response to each individual stimulus. Presently, 26 members are known to belong to the JAK/STAT pathway in mammals, which include different JAKs, STATs and negative regulators, such as the SH2-domain containing tyrosine phosphatase (SHP) proteins, protein inhibitors against STATs (PIAS) and members of the suppressor of cytokine signaling (SOCS) family (Liongue *et al*., 2012). A brief description is given below regarding the most relevant characteristics of the JAK and the STAT families, the tyrosine kinases and the transducers and transcription factors of the JAK/STAT pathway, respectively, in addition to the most common mechanisms used by negative regulators to modulate and control the responses mediated by this signalling pathway. A more detailed emphasis is given to JAK1, JAK2 and STAT1 since these are the specific proteins responsible for mediating IFN-γ responses.

1.1.1. The JAK family of tyrosine kinases

The JAK family of non-receptor tyrosine kinases (NRTKs) includes four members, namely JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). JAKs are found constitutively associated with the cytoplasmic tails of cytokine receptors that do not have intrinsic kinase activity and are, therefore, necessary to propagate the signal. After becoming activated as a consequence of cytokine binding to the receptor,

JAKs are responsible for mediating STATs recruitment to the receptor and for phosphorylating these transcription factors. This enables the STATs to translocate as phosphorylated dimers into the nucleus and to initiate cytokine-induced transcription. All of these kinases share a common protein structure with seven conserved structural JAK homology (JH) domains, grouped into four distinct functional domains. The N-terminal four-point-one, ezrin, radixin and moesin (FERM) domain is formed by the JH7 to JH5 domains and part of the JH4 domain. The SRC homology (SH) 2-like domain is constituted by part of the JH4 domain and the JH3 domain. The regulatory pseudo-kinase domain consists solely of the JH2 domain, while the C-terminal tyrosine kinase domain corresponds to the JH1 domain (Chen *et al*., 1997; Kisseleva *et al*., 2002) (**Fig.1.1**).

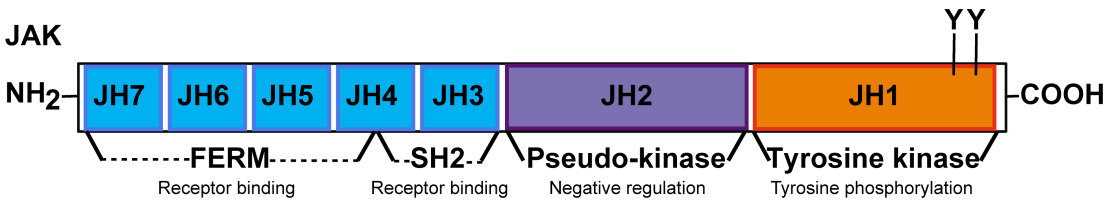


Figure 1.1. Structural and functional organisation of JAK proteins. The members of the JAK family of tyrosine kinases share a common structure, which is composed by seven conserved JAK homology domains (JH1 to JH7). These JH domains are grouped into four different functional domains: the FERM domain, a potential SH2 domain, followed by a pseudokinase domain and the C-terminal tyrosine kinase domain. The two tyrosine (Y) residues critical for either releasing the blockage to the catalytic site by the pseudokinase or for activation of the JAKs enzymatic activity are depicted in the C-terminal JH1 domain.

The FERM domain has been shown to mediate the stable interaction between JAKs and receptors (Hilkens *et al*., 2001; Zhou *et al*., 2001), while the SH2-like domain is thought to also participate in this interaction, independently of a SH2 'classical function' of binding to phosphorylated tyrosine residues (Radtke *et al*., 2005). The JH1 domain is responsible for the catalytic activity of these tyrosine kinases, while the pseudo-kinase JH2 domain, blocks the catalytic site on JH1 until cytokine binding to the receptor induces the phosphorylation of critical tyrosines residues closely located in JH1 in the activation loop (Chen *et al*., 2000; Heinrich *et al*., 2003; Saharinen *et al*., 2000). While phosphorylation of one of these residues releases the negative regulation exerted by the pseudo-kinase

domain, phosphorylation of a second residue is necessary for JAKs to become enzymatically active. In JAK1 these two phosphorylation sites correspond to the tyrosine residues 1022 and 1023, while for JAK2 these are at positions 1007 and 1008 (Feng *et al*., 1997; Liu *et al*., 1997).

1.1.2. The STAT family

1.1.2.1. STATs structural and functional organisation

Seven members of the signal transducer and activator of transcription (STAT) family are expressed from seven different genes in mammals: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. The STAT proteins are 750-850 amino acids long and share a common conserved structure of six functional domains. This organisational structure comprises an N-terminal domain (NTD), which is followed by a coiled-coil domain (CCD), the DNA-binding domain (DBD), the α-linker domain (LD), the SH2 domain (SH2D) and finally a transactivation domain (TAD) located at the C-terminal (Darnell, 1997; Darnell *et al*., 1994; Ihle and Kerr, 1995; Stark *et al*., 1998) (**Fig. 1.2**).

The NTD has been shown to play a crucial role in the formation of unphosphorylated STAT dimers, as well as in the formation of tetramers involving all STATs with exception of STAT2 and STAT6. These tetramers of activated/ phosphorylated STATs, which are able to bind to the promoters of genes where at least two tandem STAT binding sites exist (John *et al*., 1999; Vinkemeier *et al*., 1996; Vinkemeier *et al*., 1998; Xu *et al*., 1996; Zhang and Darnell, 2001), have also been implicated in the transcription of genes that present weak recognition non-consensus binding sites for STATs (Xu *et al*., 1996). In addition, numerous proteins necessary for STAT1-induced transcription, as is the case of the cAMP response element-binding (CREB) binding protein (CBP)/ p300, interact with STATs *via* the NTD (Zhang *et al*., 1996; Zhang *et al*., 1999b). The N-terminal domain has in fact been shown to be important for STAT4 binding of CBP, while STAT6 did not interact with this co-activator of transcription. This may prove important to understand the opposing role of these transcription factors in the development of T helper cells type 1 (T_H1) and type 2 (T_H2) and in T_H1 and T_H2

cytokine profiles (Zhang *et al*., 2011b). This domain has been implicated in STATs nuclear translocation and is required for both activation and deactivation of STATs, regarding tyrosine phosphorylation and dephosphorylation, respectively (Mertens *et al*., 2006) (Murphy *et al*., 2000). The NTD was, in addition, proven crucial for nuclear import of tyrosine phosphorylated dimeric STAT5B (Zeng *et al*., 2002). Interestingly, tyrosine dephosphorylation was shown to involve the alteration of STAT1 homodimers spatial conformation from a parallel to an antiparallel orientation mediated by the association of both STATs NTD, exposing the phosphorylated tyrosines to the action of phosphatases (Mertens *et al*., 2006). Work done by Wang and colleagues (2011) has also determined that while STAT1 and STAT2 activation by IFN-α/β resulted in viral clearance, activation of STAT3 by these type I IFNs, on the contrary, inhibited the antiviral response in an NTD-dependent way that was unrelated to STAT3's transcriptional activity

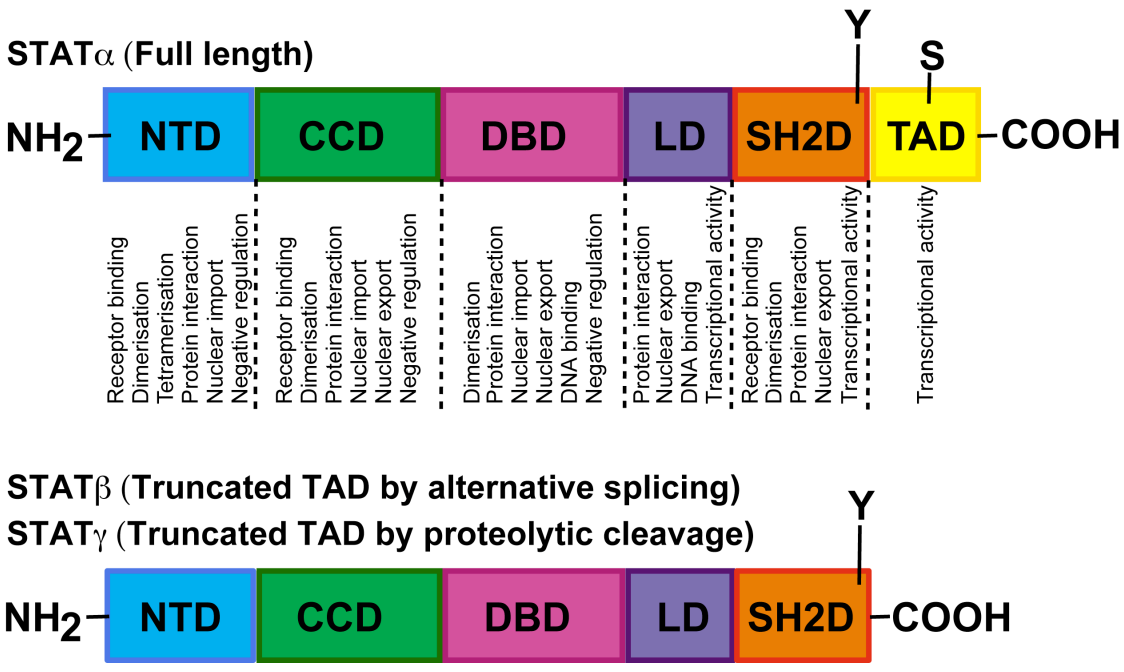


Figure 1.2. Structural and functional domains of STAT proteins. STAT proteins structure can be divided in six distinct functional domains, which include the N-terminal domain (NTD), the coil-coiled domain (CCD), the linker domain (LD), the SH2 domain (SH2D), and a C-terminal transactivation domain (TAD). The activating tyrosine residue, the phosphorylation of which determines the formation of STAT1-phosphorylated dimers in a parallel configuration is located just immediately before the TAD. In addition to this tyrosine, a serine residue present in the TAD is also depicted, the phosphorylation of which is necessary for maximal transcriptional activity. The full-length STAT proteins are designated by STATα isoforms, while the truncated proteins generated

by alternative splicing or by proteolytic processing are named STATβ and STATγ isoforms, respectively. These truncated isoforms are often considered as dominant-negative regulators of the long STAT isoforms.

The CCD, as the NTD, functions as a region of protein-protein interaction. IRF-9 and c-JUN terminal kinase are examples of proteins known to associate with STAT1 (Horvath *et al*., 1996) and with STAT3 (Zhang *et al*., 1999b), respectively, through the CCD. A leucine-rich repeat sequence, located in the coiled-coil domain in STATs, was shown to function as a nuclear export signal (NES) and to promote STAT1 translocation in the context of cytokine signalling from the nucleus back into the cytoplasm through the export receptor chromosome region maintenance 1 (CRM1, or exportin-1) (Begitt *et al*., 2000). A similar leucine-rich sequence on STAT5B CCD could also be the one responsible for the CRM1-mediated export of this transcription factor. A region was also identified in the CCD necessary for the nuclear import of unphosphorylated monomeric STAT5B (Zeng *et al*., 2002). Down-regulation of STATs transcriptional activity may occur by recruitment of negative regulators *via* the CCD, as happens with STAT5 and the nuclear co-repressor silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) (Nakajima *et al*., 2001). Post-translational events mediated by the CCD domain may also modulate STAT1 protein levels (Hoshino *et al*., 2006). Finally, interleukin (IL)-6 and epidermal growth factor (EGF) receptors mediate both STAT3 recruitment and binding (Zhang *et al*., 2000) and STAT3 nuclear translocation through its CCD (Ma *et al*., 2003).

The DBD is a highly conserved domain amongst STAT proteins and the incredible specificity regarding binding and transcription of different genes by each individual STAT is achieved just by minor dissimilarities within this domain (Boucheron *et al*., 1998). Work done by Mcbride *et al*. (2002), has determined that the STAT1 DBD contains a sequence that resembles a NES, inaccessible for CRM1 perception while STAT1 is tyrosine phosphorylated and bound to DNA, but which, once STAT1 is release from DNA and dephosphorylated, mediates STAT1 nuclear exit (McBride *et al*., 2000). In addition to STAT1, tyrosine phosphorylated dimerised STAT3 also requires the DBD for nuclear translocation (Ma *et al*., 2003; Meyer *et al*., 2002). The elegant work developed by Chen and co-workers and by the

Darnell group has helped to clarify how different conformational orientations adopted by dimerised STAT1 molecules, dependent on their STAT1 tyrosine phosphorylation status, influence DNA binding and DNA release (Chen *et al*., 1998; Mertens *et al*., 2006). DNA binding by STAT1 homodimers requires these transcription factors to adopt a parallel structure, involving the reciprocal association of the SH2 domain of one STAT1 monomer with the phosphorylated tyrosine residue of the other STAT1 molecule (Chen *et al*., 1998; Mertens *et al*., 2006; Zhong *et al*., 2005). In contrast, when STAT1 tyrosine-phosphorylated dimers get released from DNA, they assume a parallel conformation mediated through their N-terminal domains, which exposes and interrupts the phospho-tyrosine-SH2 interaction. After this event the reciprocal association of each STAT1 molecule DBD and CCD, following spatial re-orientation, allows dephosphorylation to occur and leads to an antiparallel structure (Zhong *et al*., 2005).

The LD is a highly conserved region that bridges the DBD and the SH2D. The Darnell group established this LD to be essential for IFN-γ-induced STAT1 transcriptional activity and showed that it was not required for IFN-α signalling (Yang *et al*., 1999). Taking into consideration that neither STAT1 phosphorylation nor this protein's nuclear translocation and binding to DNA were disturbed by mutation of the LD, it has been suggested that this structural region may be instead important for recruitment and association of additional factors required for transcription (Yang *et al*., 1999). Indeed, the LD participates in protein-protein interactions and STAT3 association with the gene associated with retinoid-IFN-induced mortality 19 (GRIM19), a known transcriptional repressor, is one such example (Lufei *et al*., 2003; Zhang *et al*., 2003). Furthermore, this domain also interacts with proteins of the nuclear pore complex, possibly sustaining the permanent shuttling of unphosphorylated STATs between the cytoplasmic and nuclear compartments in resting cells (Marg *et al*., 2004).

The SH2D is considered to be one of the domains with the highest degree of homology between the different STAT family members and it is crucial for both the recruitment and binding of STATs to the cytoplasmic tails of the receptors, as well as for establishing reciprocal interactions between one STAT monomer with the phosphotyrosine of another monomer. In addition, tyrosine 701 phosphorylation of

STAT1 and homodimerisation of this transcription factor driven by cytokine stimulation are dependent on the arginine 602 residue of the SH2D (McBride *et al*., 2002; Shuai *et al*., 1993b), whilst STAT2 tyrosine phosphorylation and dimer formation with STAT1 has been shown to be dependent of proline 630 within the SH2D (Gamero *et al*., 2004). Several mutational studies have shed light regarding the importance, and sometimes opposing action, that certain residues within the SH2D have. While mutation of proline 630 impaired IFN-α-induced STAT2 nuclear translocation and transcription (Gamero *et al*., 2004), mutation of the adjacent residue tyrosine 631 sustained STAT1 phosphorylation and activity, showing that very close residues within the same domain may either induce resistance or increase the efficacy of IFN-α treatment in leukaemia (Scarzello *et al*., 2007). The possibility of targeting arginine 656 and asparagine 658 located on the SH2D of STAT1 is of potential therapeutic importance to overcome resistance to IFNs and augment IFN-γ-induced STAT1-mediated transcription and viral clearance (Lim and Cao, 2006; Poat *et al*., 2010).

The C-terminal domain is, of all structural domains, the one with most variability in the amino acid sequence (Levy and Darnell, 2002). This likely determines the specificity of each STAT regarding recruitment of distinct co-activators and differential gene transcription, and, as a consequence, of a particular stimulus in a given cellular context. The importance of this domain resides in the shared location amongst all STATs of a conserved activating tyrosine and of a TAD conserved serine residue within a conserved mitogen-activated protein kinase (MAPK) consensus site, proline-methionine-serine-proline (PMSP), present on STAT1, STAT3 and STAT4, or within a proline-leucine-serine-proline (PLSP), in the case of STAT5 *(Wen et al*., 1995). As already mentioned, phosphorylation of the C-terminal located tyrosine mediates stimulus-induced dimerization of STATs in a particular conformation engaged by reciprocal interaction with the SH2D (Shuai *et al*., 1994), allows active translocation of STAT parallel dimers through the nuclear pore complex, DNA binding to the promoters of inducible genes containing STAT recognition sequences and transcriptional function (Darnell, 1997).

Phosphorylation of the conserved TAD correlates with maximal transcriptional activity of the STATs and seems to be indispensable for the expression of only certain genes, although these seem to vary according to the cellular context (Kovarik *et al*., 2001; Morinobu *et al*., 2002; Wen *et al*., 1995; Zhang *et al*., 1995). Besides interacting with the NTD, CBP/ p300 has also been shown to bind to the TAD (Bhattacharya *et al*., 1996; O'Sullivan *et al*., 2004; Pfitzner *et al*., 1998; Schuringa *et al*., 2001; Zhang *et al*., 1996), which in the particular case of STAT1 is independent of phosphorylation status of the serine 727 residue, but dependent on the PMSP motif where this residue lies. Binding of CBP/ p300 by STAT1 TAD was suggested to function as a mechanism of IFN-induced viral elimination, by preventing the recruitment of this co-activator by viral pathogens (Shuai *et al*., 1993b; Zhang *et al*., 1996). With regards to STAT3, CBP/ p300 was reported to acetylate lysine 685 residue located at the C-terminus and this post-translational modification (PTM) increased STAT3 DNA binding rate and STAT3-mediated transcription (Wang *et al*., 2005).

Other proteins have been described to bind the TAD, such as the minichromosome maintenance complex component (MCM) 5. The association of MCM5 with STAT1 was investigated by the Darnell and the Zhang groups and was implicated in STAT1-mediated transcriptional activity, including within the context of IFN-γ stimulation (DaFonseca *et al*., 2001; Snyder *et al*., 2005; Zhang *et al*., 1998c). Breast cancer (BRCA) 1 was also found to bind STAT1 TAD and this was shown to be critical for the selective transcription of certain ISGs upon IFN-γ treatment, such as protein 21 (p21) (Ouchi *et al*., 2000). In contrast, the expression of other molecules such as IRF-1 was independent of BRCA1-induced transcriptional synergy with STAT1 (Ouchi *et al*., 2000). Work done by this group additionally identified the 2,5 oligoadenylate synthetase (OAS), an antiviral protein and a mediator of apoptosis, as another ISG transcribed in response to IFN-γ, which was dependent on the concerted action of STAT1 and BRCA1 (Mullan *et al*., 2005). Moreover, BRCA1 was shown to act as a regulator of the JAK/STAT pathway in a human prostate cancer cell line through induction of JAK1 and JAK2 activation by direct interaction with these kinases, which led to STAT3 constitutive activation and promoted cell survival (Gao *et al*., 2001).

1.1.2.2. STAT isoforms generated by alternative splicing and proteolytic processing

STAT proteins are subjected to regulation by alternative splicing and, therefore, are found either as a full-length protein (α isoform), or as a truncated protein lacking the C-terminal TAD (β isoform) (**Fig.1.2**). These truncated β isoforms, so far identified for STAT1, STAT3 and STAT5, have been considered as functional dominant-negative proteins of the longer α isoforms (Caldenhoven *et al*., 1996; Moriggl *et al*., 1996; Mui *et al*., 1996; Schaefer *et al*., 1997; Shuai *et al*., 1993a; Wang *et al*., 1996).

The functional features of the β isoforms can be exemplified by the STAT1 alternative splicing isoform STAT1β (Schindler *et al*., 1992) (**Fig.1.2**). Despite truncation of the TAD serine 727 residue, needed for STAT1α maximal transcriptional activity for a subset of genes, STAT1β can still be tyrosine phosphorylated upon adequate stimulation and bind either to another STAT1β molecule or to a STAT1α isoform, forming homodimers or heterodimers, respectively (Vinkemeier *et al*., 1996). STAT1α is vital for IFN-γ and IFN-α/β induced transcription, either as an homodimer or as an heterodimer together with STAT2α (Stark *et al*., 1998). Formation of heterodimers between both full-length and truncated isoforms of STAT1 or DNA binding of STAT1β instead of STAT1α has been suggested as a mechanism by which the truncated isoform may negatively regulate the cytokine-inducible activity of the long isoform of STAT1.

Although STAT1α and STAT1β isoforms differ in terms of activation, transcriptional ability and functions, a fact that prompted STAT1α to be considered as the active isoform and the truncated isoform simply as a dominant-negative of the first one (Sironi and Ouchi, 2004), this notion is being revised in light of new reports. Against the established idea that STAT1β is merely a negative regulator of STAT1α, the truncated isoform has in fact a functionally active role in inducing cell death that is mechanistically different from STAT1α-mediated apoptosis (Baran-Marszak *et al*., 2004; Najjar *et al*., 2008; Zakharova *et al*., 2003). Several other groups have reported that STAT3 and STAT4 short isoforms are also biologically

active and, importantly, exert specific and distinct functions from the long isoforms through the transcription of particular sets of genes, despite the lack of a TAD. This likely involves recruitment of co-activators different than the ones that associate with the transactivation domain of the full-length isoforms (Hoey *et al*., 2003; Maritano *et al*., 2004; Yoo *et al*., 2002).

In addition to alternative splicing, STAT regulation can likewise occur through truncated forms generated by proteolytic processing of the C-terminus (Azam *et al*., 1997) (**Fig. 1.2**). The proteolytically cleaved STATs are designated as STATγ isoforms and, so far, these have only been detected for STAT3, STAT5 and STAT6, and always in a cellular context where truncated isoforms generated by alternative splicing are absent (Hendry and John, 2004). Proteolytic cleavage of STATs is performed by serine proteases, although the cysteine protease named calpain has also been shown to be able do it (Oda *et al*., 2002; Suzuki *et al*., 2003). These proteolytic STATγ isoforms differ from the STATβ isoforms and evidence so far suggests that they function only as dominant-negative forms of the unprocessed proteins (Ilaria *et al., 1999;* Piazza *et al*., 2000; Xia *et al*., 2001).

1.1.2.3. Dimerisation and functional differences between unphosphorylated and phosphorylated STATs

Signalling through the JAK/STAT pathway has been explained for many years in a very attractive way. Given its simplicity, this model became a paradigm in the field, despite the existence of contradicting evidence. According to this model, STATs exist as unphosphorylated monomers restricted to the cytoplasm of resting cells. After ligand engagement of the receptor, conformational changes of the receptor complex allow the associated JAKs to become activated by trans- and auto-phosphorylation. The activated JAKs then phosphorylate tyrosine residues in the cytoplasmic tails of the receptor, which serve as docking sites to recruit the latent and inactive cytoplasmic STATs monomers *via* their SH2D. After phosphorylation of the STATs C-terminus activating tyrosine residue, these monomers dissociate from the receptor and dimerise *via* reciprocal interactions involving the SH2D of one monomer with the phosphorylated tyrosine of the other molecule. Stimulus-

induced dimerisation allows translocation into the nucleus, binding to specific DNA recognition sequences and initiation of transcription-based responses.

In recent years, this concept has been reconsidered after a growing body of evidence provided by different studies contradicting three important aspects of this simple model. These studies have established that unphosphorylated STATs are not simply retained in the cytoplasm, but permanently shuttle between the cytoplasmic and nuclear compartments in resting cells. It has also been shown that STATs exist as dimers independently of stimulation, with stimulus-induced tyrosine phosphorylation determining a change in the orientation of the dimerised monomers. In addition, both unphosphorylated and phosphorylated STATs are known to exert distinct biological functions. Work done by several groups over more than ten years ago has indeed confirmed the existence of unphosphorylated STAT1, STAT3 and STAT5 dimers, as well as the occurrence of cytokine-independent continuous bi-directional shuttling of STATs across the nuclear membrane (Meyer and Vinkemeier, 2004). Moreover, unphosphorylated STATs are not only transcriptionally active, but can additionally perform other functions independently of gene expression (Adach-Kilon *et al*., 2011; Chatterjee-Kishore *et al*., 1998; Chatterjee-Kishore *et al*., 2000; Cheon and Stark, 2009; Gough *et al*., 2009; White *et al*., 1996; Yang *et al*., 2007).

Thus, an even more elegant model regarding JAK/STAT signalling pathway has emerged that integrates and conciliates the existence of active unphosphorylated STAT dimers *versus* functionally distinct tyrosine phosphorylated dimers. This model initially formulated by Darnell and colleagues (Zhong *et al*., 2005) based on work done regarding STAT1 in particular, suggested that STAT1 monomers dimerise independently of stimulation, but adopt different conformations depending on the status of tyrosine phosphorylation. Moreover, the pre-association of two unphosphorylated STAT monomers allows them to be simultaneously recruited to the ligand-engaged receptor and phosphorylated, which determines dimers to re-orient into a parallel conformation by establishing reciprocal interactions between each monomer SH2D and the phosphorylated tyrosines on the C-terminus (Wenta *et al*., 2008; Zhong *et al*., 2005).

The new conformation allows the active nuclear import of these phosphorylated dimers through importin-α5, which is followed by binding to specific DNA recognition sequences. DNA dissociation is a two-phase process that first requires the N-terminal domains of both monomers to associate, while the SH2-phosphotyrosine interactions are terminated (Mertens *et al*., 2006). This allows STAT1 dimers to re-orientate the monomers C-terminal domains in opposing directions by rotating them into an antiparallel conformation. These dimers are linked by interactions established between the coiled-coil and DNA-binding domains that allow phosphatases to access and remove the phosphor group of the tyrosine residue (Mertens *et al*., 2006; Zhong *et al*., 2005).

The constitutive shuttling of unphosphorylated STATs between the cytoplasmic and nuclear compartments is mediated by their interaction with nucleoporins (Meyer *et al*., 2002; Meyer and Vinkemeier, 2004). Curiously, transcription and *de novo* synthesis of unphosphorylated STAT1 is induced by STAT1 tyrosine phosphorylated protein, either when homodimerised in response to IFN-γ or in a multiprotein complex with phosphotyrosine-STAT2 and IRF-1 upon stimulation with type II IFNs. Similarly, unphosphorylated STAT3 expression is also increased following tyrosine phosphorylation of STAT3 dimers after stimulation with cytokines of the IL-6 family. Both unphosphorylated STAT1 and STAT3 proteins have been shown to be transcriptionally active, though they induce the expression of a distinct subset of genes comparatively to the phosphorylated dimers by resorting to distinct mechanisms (Chatterjee-Kishore *et al*., 1998; Yang *et al*., 2007). STAT3-induced transcription independently of tyrosine phosphorylation is responsible for augmenting the expression of the hepatocyte growth factor receptor (MET) and muscle RAS oncogene homolog (MRAS) (Yang *et al*., 2005), while the presence of unphosphorylated STAT3 molecules has been shown to be necessary for RAS-mediated transformation (Gough *et al*., 2009).

Basal expression of the low molecular mass polypeptide (LMP) 2 gene by unphosphorylated STAT1 is another example of unphosphorylated STATs being functionally active proteins. Although unphosphorylated STAT1 is able to promote constitutive expression of LMP2 *via* a transcriptional complex formed together with

IRF-1 and an additional element, this complex is displaced by IFN-γ-induced tyrosine phosphorylated STAT1 homodimers. This suggests that replacement of unphosphorylated STAT1 dimers by tyrosine phosphorylated ones is necessary to achieve a higher level of transcription of LMP2 in a situation that involves, for instance, IFN-γ production by immune cells in response to a viral infection (Chatterjee-Kishore *et al*., 1998; Chatterjee-Kishore *et al*., 2000; White *et al*., 1996). Furthermore, IFN-γ-driven expression of regulatory genes in the context of immune responses is sustained by unphosphorylated STAT1 (Cheon and Stark, 2009; Gough *et al*., 2009), which can also promote constitutive transcription of pro-survival genes in glioma cells, thereby potentially exerting an oncogenic role that is usually attributed to STAT3 activity (Adach-Kilon *et al*., 2011).

1.1.2.4. Dynamics of STATs cellular movement and translocation

It is now obvious that STATs are highly dynamic molecules and are constantly cycling between the cytoplasm and the nucleus, either in resting cells or in response to a particular stimulus, although the associated mechanisms of translocation differ. In the particular case of IFN-γ stimulation, signalling occurs through STAT1 homodimers, which quickly become phosphorylated on tyrosine 701 in response to the cytokine. These adopt a parallel conformation and translocate into the nucleus (Fagerlund *et al*., 2002; Lillemeier *et al*., 2001; McBride *et al*., 2002; Sekimoto *et al*., 1997; Wenta *et al*., 2008). This translocation occurs by active transport mediated by importin-α5, which identifies a bipartite-motif, different from a classical NLS, formed by an arginine/leucine-rich sequence present on DBD of both tyrosine phosphorylated STAT monomers, (Fagerlund *et al*., 2002; McBride *et al*., 2002; Melen *et al*., 2001).

Previous studies have considered the kinetics of this movement and have determined that IFN-γ-induced STAT1 accumulates in the nuclear compartment within few minutes of activation. Interestingly, this accumulation reaches a maximum around thirty minutes after stimulation and, remarkably, can be detected for several hours (Haspel *et al*., 1996). Although almost all the entire pool of STAT1 molecules becomes tyrosine phosphorylated upon IFN-γ treatment, the

amount that is effectively retained in the nucleus corresponds to approximately a quarter of STAT1 total amount (Haspel *et al*., 1996). Despite the fact that STAT1 protein has a half-life of about twenty-four hours *(Lee et al*., 1997; Levy and Garcia-Sastre, 2001), the half-life of tyrosine phosphorylated STAT1 has been estimated to be only around 15 minutes (Haspel *et al*., 1996). Therefore, STAT1 is continuously being tyrosine phosphorylated by the JAKs, shuttling into the nucleus where it transiently binds DNA, becoming dephosphorylated by nuclear phosphatase (PTP) TC45, and subsequently exported back to the cytoplasmic compartment (Meyer *et al*., 2004; ten Hoeve *et al*., 2002). STAT1 nuclear export is dependent on previous tyrosine dephosphorylation, as this reveals a NES in its DBD recognized by CRM1 (McBride *et al*., 2000).

The most accepted model regarding the way STATs traffic through the cytoplasmic and nuclear compartments and gain access to the nuclear membrane is that of a 'random walk'. The elegant work done by the Kerr group detected a diffusional movement for phosphorylated STAT1 upon both IFN-α and IFN-γ treatment. (Lillemeier *et al*., 2001). This fast and non-directional type of movement was observed in the cytoplasm and also in the nucleus, where STAT1 rapidly becomes associated with DNA. Intriguingly, it was shown to occur independently of the cytoskeleton or microtubules network. Oncostatin M (OSM)-activated STAT3 was also shown to follow a diffusional random walk model of movement (Kermorgant and Parker, 2008).

Other authors have found evidence for interactions between STATs and the microtubules network, for instance STAT5B in response to growth hormone (GH) treatment (Phung-Koskas *et al*., 2005) and STAT3 downstream of hepatocyte growth factor (HGF) (Kermorgant and Parker, 2008). It has been hypothesised that these associations may happen, for instance, in order to protect phosphorylated STATs in response to a weak stimulus from phosphatase activity, or the need of directing these proteins to a particular subcellular compartment. It looks feasible that STATs may move either by rapid random walks in response to strong activating stimuli or by using the slower, but oriented, cytoskeleton and microtubules network (Kerr *et al*., 2003).

1.1.2.5. Modulation and regulation of STAT activity

Cytokines and growth factors that signal through the JAK/STAT pathway control several important cellular processes from differentiation to proliferation and apoptosis. As a consequence, these cytokines activities must be tightly regulated, especially regarding the intensity and duration of STAT proteins activation. Several PTMs, involving tyrosine and serine residues phosphorylation/ dephosphorylation, acetylation, methylation, in addition to modulation by negative regulators of the pathway, cross-talk and trans-activation of other signalling pathways, are all examples of how the regulation of JAK/STAT pathways is achieved (Ginter *et al*., 2012; Kerr *et al*., 2003; Levy and Darnell, 2002; Stark *et al*., 1998).

1.1.2.5.1. STAT phosphorylation/ dephosphorylation and nuclear translocation

Stimuli-induced transcriptional activation of STATs is mediated by an initial post-translational event, consisting of the phosphorylation of the activating tyrosine residue located at the C-terminus of the protein by the receptor-associated JAKs. This is frequently followed by a second phosphorylation of the TAD serine residue, which is believed to be carried out by several different serine kinases. This is the case for STAT1-induced activation by either IFN-α/β or IFN-γ. As previously mentioned, phosphorylation of tyrosine 701 residue on both STAT1 molecules of antiparallel STAT1 homodimers forces these homodimers to rotate. As a consequence, the concentration of tyrosine phosphorylated parallel STAT1 dimers increases (Wenta *et al*., 2008). Phosphorylation of the serine residue present in STATs TAD has for long been considered as necessary for maximal transcriptional activity to be obtained. Indeed, clearance of viral infections, as well as proliferation arrest, induced by IFN-γ *in vivo* models, was found to be dependent of STAT1 serine 727 residue phosphorylation, which is only present on the full-length STAT1α (Varinou *et al*., 2003). A leucine residue on position 724, proximal to serine 727 residue of STAT1, also appears necessary for STAT1-induced transcription (Sun *et al*., 2005a).

The groups of Darnell, Kovarik and Decker, have studied extensively the biological implications of this serine residue PTM. Darnell and colleagues first confirmed the occurrence *in vivo* of serine 727 phosphorylation, located within the PMSP motif of STAT1. These researchers then determined *in vitro* that IFNγ-induced transcription of IRF-1 was dependent of serine 727 phosphorylation, with IRF-1 mRNA levels being approximately reduced by 80% in cells where this residue was mutated. STAT3 transcriptional activity was also shown to be dependent of this PTM, as assessed using a reporter assay for IRF-1 (Wen *et al*., 1995). Studies conducted by the Kovarik group, using fibroblasts obtained from STAT1-deficient mice complemented either with wild type STAT1 or with STAT1 mutated on serine 727 (S727A), established that IFN-γ-induced phosphorylation of this residue can only occur after the tyrosine 701 residue has been phosphorylated. This depended on the SH2 domain that is involved in reciprocal interactions with this phospho-tyrosine in STAT1 dimers being unaltered (Kovarik *et al*., 2001). Furthermore, this work established that IFN-γ-induced serine 727 phosphorylation takes place in the nuclear compartment instead of in the cytoplasm, as previously believed (Zhu *et al*., 1997), and only after association of tyrosine phosphorylated STAT1 molecules with the chromatin (Sadzak *et al*., 2008).

Strong evidence supports a selective transcriptional dependence of certain ISGs on serine phosphorylation, although no explanation has been provided for this transcriptional difference. Nonetheless, the results from studies that have addressed this topic have varied regarding which genes are dependent and the degree of dependence. It may be that these differences simply reflect the different cellular backgrounds of the working models used. According to the results obtained by Kovarik and colleagues, IFN-γ-inducible expression of guanylate-binding protein (GBP) 1 and TAP1 genes was extremely dependent, while IRF-1 expression was only relatively conditioned by STAT1 serine 727 phosphorylation. From the *in vivo* studies performed by the Decker group using mice where STAT1 on serine 727 phosphorylation has been prevented by mutation, both GBP1 and IRF-1 expression, were profoundly dependent on this PTM, while transcription of both TAP1 and the negative regulator SOCS 1 was only slightly impaired. Expression of the mouse major histocompatibility complex (MHC) class I and II

were found to be temporarily affected, with prolonged stimulation overcoming that delay (Varinou *et al*., 2003).

A more recent study using transfection of human salivary glands cells with STAT1 mutants, either unable to be phosphorylated at tyrosine 701 or at serine 727, reinforced the idea that IFN-γ-induced STAT1 serine 727 phosphorylation is essential for the expression of IRF-1, in addition to the expression of IFN-γ-induced protein 10 (IP10) and FAS, which occurred even in the absence of tyrosine phosphorylation (Tsuboi *et al*., 2011). The Sthephanou group has additionally shown the existence of an interaction between STAT1 and cyclin D1, which was negatively regulated upon IFN-γ treatment. This involved proteosomal degradation of cyclin D1 and was, therefore, mediated independently of STAT1 transcriptional activity, although the STAT1 serine 727 phosphorylation site, but not the tyrosine phosphorylation one, was involved (Dimco *et al*., 2010).

In addition, work done by Perwitasari and colleagues (2011) using a mouse model has shown that in the context of West Nile virus infection, it is the phosphorylation of STAT1 serine 708, by the inhibitor of kB kinase (IKK) ε, and not the phosphorylation of the 727 residue, that is relevant for expression of the ISG IFIT2 needed for innate immunity-mediated viral clearance. Interestingly, STAT1 serine 708 phosphorylation was dependent on both the dephosphorylation of tyrosine 701 and nuclear export of STAT1 *via* CRM1. Serine 708 phosphorylation of STAT1 thus appears to be a cytoplasmic event, whereas STAT1 serine 727 phosphorylation takes place in the nucleus (Perwitasari *et al*., 2011). It has been, in fact, already suggested many times that phosphorylation of critical tyrosine and serine residues may be independently regulated. This appears particularly obvious since serine phosphorylation induced by several growth factors can occur without tyrosine phosphorylation (Ceresa and Pessin, 1996; Chung *et al*., 1997b; Gotoh *et al*., 1996; Jain *et al*., 1999; Kovarik *et al*., 2001; Lim and Cao, 1999; Ng and Cantrell, 1997; Yamashita *et al*., 1998; Zhu *et al*., 1997).

Constitutive STAT activation through phosphorylation of the critical tyrosine residue can be promoted by persistent signalling of upstream kinases and it has

been strongly correlated with tumour development and progression. The same may be true in the case of serine 727. Indeed, persistent phosphorylation of this residue on STAT1, probably due to casein kinase (CK) 2 activity, was found to turn STAT1, usually considered to be a tumour suppressor, into a pro-survival factor for Wilms' tumour cells capable of up-regulating myeloid cell leukaemia (MCL) 1 and heat shock protein (HSP) 27 (Timofeeva *et al*., 2006). Recently, the mast/ stem cell growth factor receptor (SCFR), also known as tyrosine-protein kinase KIT, was shown to physically interact with STAT1, STAT3 and STAT5 and promote tyrosine phosphorylation of all three proteins. Although phosphorylation of STAT1 serine residue was constitutive and non-inducible by KIT, STAT3 and STAT5 serine phosphorylation was attributed to this kinase. Curiously, and despite the phosphorylation of these transcription factors, with exception of STAT5, they were not transcriptional active, providing further evidence that STATs and their phosphorylation have biological functions besides gene expression (Chaix *et al*., 2011). Indeed, this may be the case for RAS-mediated transformation sustained by mitochondrial STAT3, where it was found to promote oxidative phosphorylation by increasing the electron transport chain activity independently of its nuclear transcriptional activity (Gough *et al*., 2009; Wegrzyn *et al*., 2009). Curiously, STAT1 serine 727 residue, seems to be required, independently of IFN-induced tyrosine phosphorylation, for STAT1-mediated programed cell death (Kumar *et al*., 1997; Stephanou et al., 2001), as well as for sumoylation of this transcription factor (Vanhatupa *et al*., 2008). Koromilas and co-workers have also reported that STAT1 may suppress breast tumour development independently of both tyrosine and serine phosphorylation (Raven *et al*., 2011).

Although different kinases have been pointed as being the one responsible for serine phosphorylation of the STAT family members, it is currently accepted that rather than one specific serine kinase, several kinases can perform this function depending on both the cellular context and the particular cytokine or growth factor with which the cells are stimulated with [reviewed in (Decker and Kovarik, 2000)]. While the initial reports suggested p38, from the MAPK family, to be the kinase carrying out STAT serine phosphorylation events (Bhattacharjee *et al*., 2011; Goh *et al*., 1999; Uddin *et al*., 2000), since then, kinases such as JAK2 (Zhu *et al*., 1997) and proline-rich tyrosine kinase 2 (PYK2) (Takaoka *et al*., 1999) have also

been shown to phosphorylate this residue. In addition, double-stranded RNA-activated protein kinase (PKR) (Deb *et al*., 2001), calcium-calmodulin-dependent kinase (CaMK) II (Nair *et al*., 2002), as well as protein kinase C (PKC)-δ (Deb *et al*., 2003; Uddin *et al*., 2002) and phosphatidylinositol 3-kinase (PI3K) (Nguyen *et al*., 2001), have all been shown to be capable of mediating such PTM. It seems likely that several more kinases will be recognised to phosphorylate the serine residue of STAT proteins, and, therefore, to modulate the transcriptional activity of these transcription factors. In fact, it has been suggested by a RNA interference (RNAi) screen performed by the Costa-Pereira group, that numerous additional kinases modulate IFN-γ signalling (Watling *et al*., 2008) and, by inference, this may occur through PTMs involving the phosphorylation of serine residues on the STATs.

1.1.2.5.2. STAT regulation by ubiquitination, ISGylation and arginine methylation

The ubiquitin conjugation system usually directs short-lived or obsolete regulatory proteins involved in a diversity of cellular processes, such as cell cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, and endocytosis, for proteasomal degradation (Hershko and Ciechanover, 1998). This system of attachment of ubiquitin polypeptides to proteins is also a regulatory process controlling the biological activity of several cytokines that use the JAK/STAT. All three proteins E3 ubiquitin ligase PDZ, LIM domain (PDLIM) 2 and the A_{2A} adenosine receptor (A_{2A}AR) have been shown to promote STAT1 ubiquitination, targeting it for proteasomal-mediated degradation (Tanaka *et al*., 2005) The V proteins of simian virus (SV) 5 and of human parainfluenza virus (hPIV) type 2 have been shown to target STAT1 and STAT2, respectively, for proteasome-mediated degradation (Precious *et al*., 2005). Infection with SV5 resulted in a total absence of STAT1 protein and prevented IFN-α and IFN-γ-mediated anti-viral response (Andrejeva *et al*., 2002). It thus seems that certain viruses have developed strategies to avoid STATs antiviral action by promoting the degradation of these proteins by ubiquitination.

The term ISGylation is employed to define a particular conjugation process of ISG15 molecules, distinguished by the fact that it is triggered upon stimulation. Work done by Liu and co-workers (Andrejeva *et al*., 2002; Liu *et al*., 2003), as well as by Malakhov and colleagues (Malakhov et al., 2003) have excluded the possibility of ISGylated proteins being targeted for degradation *via* the proteosomal pathway as happens with the normal ubiquitylation process. The importance of ISGylation in the context of innate immunity results from the fact that the components of the ISGylation system are IFN-inducible proteins, or are up-regulated by viral and bacterial infections or even by genotoxic stress. ISG15, ubiquitin-activating enzyme-E1-like (UBE1L), ubiquitin-specific protease 43 (UBP43) and ubiquitin conjugating enzyme 8 (Ubc8), have all been identified as components of this system (Kim *et al*., 2004). UBE1L promotes ISGylation, while UBP43 is the ISG15-specific protease responsible for de-ISGylation (Zhang and Zhang, 2011).

The role of ISGylation is somewhat controversial, since hyper-ISGylation of IFN-α/β-induced JAK/STAT signalling pathway members has been detected in UBP43-deficient mice (Kim and Zhang, 2005), but this was later attributed to UBP43 itself and not to augmented accumulation of ISGylation (Kim *et al*., 2006). Moreover, IFN-α/β-induced ISGylation by ISG15 was found irrelevant for elimination of both vesicular stomatitis virus (VSV) and lymphocytic choriomeningitis virus (LCMV) infections (Kim *et al*., 2006), but it was shown to play a role in antiviral responses against influenza, herpes simplex and spleen necrosis viruses (Jeon *et al*., 2010). In addition, a role for ISGylation as a stabiliser of IRF-3 and, therefore, as a positive regulator of immune responses, has been suggested. This ubiquitin-like conjugation process has also been shown to target, and negatively regulate, retinoic acid-inducible gene (RIG)-I in response to IFN-α (Kim *et al*., 2008). More recently, ISG15 was shown to be highly expressed in dengue and West Nile virus infected mouse cells, with subsequent increase of ISGylation levels and improved the antiviral responses (Dai *et al*., 2011). ISGylation thus seems to regulate IFN responses in different manners, and its importance to be conditioned by the viral pathogen, the infected cell type and the animal model used. For the time being, the only relation between ISG15 and type II IFN stems from the fact that ISG15

increases IFN-γ expression by T cells in order to facilitate clonal expansion and activity of effector cytotoxic natural killer cells (D'Cunha *et al*., 1996).

Although arginine methylation of STATs has been recognised for many years, as being necessary, in addition to tyrosine and serine phosphorylation, for IFN-mediated transcription, this is still a poorly studied topic. STATs arginine methylation occurs at their NTD and the protein arginine N-methyltransferase 1 (PRMT1) has been found to interact with the IFN-α/β receptor, (Abramovich et al., 1997) and to target STAT1 in response to IFN-α/β stimulation, thereby positively modulating transcription (Mowen *et al*., 2001). It has been suggested that arginine methylation protects STAT1 from PIAS1 binding and inhibition, as well as from TCP45 association, thus allowing STAT1 binding to the DNA and protecting it from nuclear dephosphorylation (Mowen *et al., 2001;* Zhu *et al*., 2002). The Vinkemeier and the Behrmann groups, however, have questioned the role attributed to methylation of STATs (Komyod *et al*., 2005; Meissner *et al*., 2004). Regarding IFN-γ-induced methylation of STAT1, only one article was found that addressed this subject. PRMT1 and PIAS1 were shown to regulate the expression of a particular group of ISGs, with PIAS1 binding to the promoters of STAT1-transcribed genes and preventing these ISGs transcription, while PRMT1-mediated methylation of PIAS1 unbound PIAS1. However, this article has been recently retracted and this data thus invalidated (Weber *et al*., 2009).

1.1.3. SOCS, SHP and PIAS: negative regulators of the JAK/STAT pathway

As previously mentioned, the magnitude and duration of cytokine-induced biological responses must be controlled, since inappropriate activation may lead to development of allergies, autoimmunity, inflammation and cancer. The JAK/STAT pathway is additionally regulated by members of the SOCS, SHP and PIAS families (Wormald and Hilton, 2004).

The SOCS family of negative regulators includes eight members: SOCS1 to SOCS7 and cytokine-inducible SH2-containing protein (CIS), all of which have a non-conserved N-terminal domain, a central SH2 domain and a C-

terminal SOCS box domain in their protein structure [reviewed in (Hilton *et al*., 1998)]. Since SOCS proteins expression is cytokine-induced *via* STAT-mediated transcription, they serve as an example of a negative feedback mechanism for cytokine signalling (Endo *et al*., 1997; Matsumoto *et al*., 1997; Naka *et al*., 1997; Starr *et al*., 1997). SOCS proteins modulate JAK/STAT-mediated cytokine signalling in three different ways, namely by binding to the cytokine receptors tyrosine phosphorylated residues and thus preventing STATs recruitment to the receptor; through binding to the receptor associated JAKs, or by promoting ubiquitin-mediated STATs proteasomal degradation *via* the SOCS-box (Cohney *et al*., 1999; Kamura *et al*., 1998; Narazaki *et al*., 1998; Nicholson *et al*., 1999; Yasukawa *et al*., 1999; Zhang *et al*., 1999a).

Studies conducted so far have identified the SOCS family members SOCS1 and SOCS3 as important down-regulators of IFN-γ signalling, whilst SOCS1 has also been recognised as a regulator of lymphocyte differentiation (Kovarik *et al*., 2005; Marine *et al*., 1999; Naka *et al*., 1998; Song and Shuai, 1998; Starr *et al*., 1998). SOCS1 inhibitory effect *in vitro* has been shown to be exerted by direct interaction with JAKs (Narazaki *et al*., 1998; Nicholson *et al*., 1999; Yasukawa *et al*., 1999), while in mice SOCS1 was found to inhibit IFN-γ signalling through binding to IFNGR1 tyrosine 440, thus preventing STAT1 recruitment to the receptor (Qing *et al*., 2005; Starr *et al*., 2009). As a consequence of SOCS1 negative regulation, STAT1 tyrosine phosphorylation and phosphorylation-dependent DNA binding, and CIITA and HLA class II transcription were impaired (O'Keefe *et al*., 2001). Moreover, IFN-γ-induced SOCS1 and SOCS3 protein transcription is quite low, but maximal inhibition of IFN-γ-activated STAT1 can be achieved by a small amount of these negative regulators (Song and Shuai, 1998). In addition, SOCS1 exhibited a much stronger inhibitory activity towards the activation of STAT1 than SOCS3. These results suggest that SOCS1 and SOCS3 are inhibitors of IFN-mediated JAK/STAT activated signalling pathways. It has also been suggested that SOCS3 acts as a negative regulator of IL-6 signalling (Croker *et al*., 2003; Lang *et al*., 2003). This has been disputed by data showing that SOCS3 is essential to suppress STAT1 activation and an IFN-γ-like response, which occurs upon IL-6 treatment in the absence of STAT3 in mouse embryonic fibroblasts (MEFs)

(Costa-Pereira *et al*., 2002). SOCS2 was involved in GH and insulin-like growth factor (IGF) I signalling and in postnatal growth regulation (Metcalf *et al*., 2000). The biological role of other SOCS proteins remains to be fully determined, but several studies have suggested the relevance of SOCS1, SOCS3, SOCS5 and SOCS7 in counteracting STAT-driven tumourigenesis and other malignancies (Cooper *et al*., 2010; Francipane *et al*., 2009; He *et al*., 2003; Kovarik *et al*., 2005; Marine *et al*., 1999; Sasi *et al*., 2010; Starr *et al*., 2009; Starr *et al*., 1998; Tomita *et al*., 2011; Ying *et al*., 2010).

SHP2 is a tyrosine phosphatase responsible for regulating the JAK/STAT pathway activation in response to both IFN-α and IFN-γ (Tonks and Neel, 2001; You *et al*., 1999). Of the utmost importance was the finding that SHP2 interacted with STAT1 in response to EGF or IFN-γ stimulation and that, although STAT1 tyrosine and serine phosphorylation may occur as independent events, SHP2 was able to promote dephosphorylation of both residues (Wu *et al*., 2002). Targeting SHP2 has been suggested as a possible approach for bladder cancer treatment, since ablation of IFN-γ signalling could be the result of human epidermal growth factor receptor 2 (HER-2)-mediated increased expression of SHP2 (Su *et al*., 2007). In contrast to SHP2's suppressor function, SHP1 has been shown to promote EGF- and IFN-γ-mediated STAT1 activation and DNA binding (You and Zhao, 1997). Nonetheless, in Leishmania infection, IFN-γ pro-inflammatory effects were prevented by dephosphorylation and thus inactivation of JAK2, which was mediated by SHP1 tyrosine phosphatase activation by the parasite (Blanchette *et al*., 1999). PTP1B can supress IFN-γ signalling through association with phosphorylated JAK1, while TC-PTP has been shown to dephosphorylate JAK1, JAK3, STAT1, STAT3 and STAT5 (Bourdeau *et al*., 2007; Simoncic *et al*., 2002; ten Hoeve *et al*., 2002). TC-PTP-mediated tyrosine dephosphorylation of IFN-γ-activated STAT1 further involved proteins of both the actin cytoskeleton and the extracellular matrix and it was thus proposed that IFN-γ may participate in the elimination phase of cancer immunosurveillance by inducing anoikis of non-adherent metastatic tumour cells (Chen *et al*., 2011).

Whilst expression of SOCS proteins is cytokine induced, the PIAS are constitutively expressed nuclear proteins. PIAS are able to inhibit STATs activity, but they are quite promiscuous regarding the number of proteins they can interact with and regulate (Shuai, 2006). The human family of PIAS proteins includes five members, of which PIAS3 is recognised to inhibit STAT3 activity (Chung *et al*., 1997a), while PIAS1 and PIASy have a certain degree of specificity for STAT1 (Liu *et al*., 1998). Recent work done by Kubota *et al*., has shown that suppression of type I IFN-signalling by PIASy in the context of viral infection relies on SUMO E2 enzyme UBC9, but not on PIASy-mediated recruitment of SUMO E3 activity (Kubota *et al*., 2011). Several mechanisms of action have been suggested for PIAS-mediated suppression of cytokine-induced transcription besides sumoylation. PIAS may bind to tyrosine phosphorylated STATS and, therefore, prevent STATS binding to DNA (Chung *et al*., 1997a; Liu *et al*., 1998), or alter STATs subcellular compartmentalisation or even through sumoylation (Ungureanu *et al*., 2003) and recruitment of chromatin-regulatory proteins such as histone deacetylases (HDACs), (Shuai, 2006). STAT1 reversible PIAS-induced sumoylation at lysine 703 was shown to effectively regulate transcription of a particular group of ISGs, possibly by affecting tyrosine phosphorylation induced by IFN-γ (Rogers *et al*., 2003; Ungureanu *et al*., 2003). PIAS1 is able to suppress IFN-γ-mediated antiviral activity and STAT1-driven GBP1 and TAP1 transcription by sumoylation (Liu *et al*., 2004; Ungureanu *et al*., 2005), while PIASy was shown to function as a co-repressor of IFN-γ/ STAT1-transcritpion (Liu *et al*., 2001).

1.2. IFN-γ biological functions and modulation of the immune system

There are three main classes of IFNs: type I, comprising mainly the IFN-α subtypes and IFN-β; type II, comprising IFN-γ; and type III, comprising IFN-λs (Gad *et al*., 2010). IFNs are characterized by their antiviral activity, but also for their immune regulatory and antiproliferative properties, which make them important in tumour-immune system interactions (Dunn *et al*., 2006; Platanias, 2005). IFNs biological functions are dependent on JAK/STAT-induced transcription of genes responsive to these cytokines (Bach *et al*., 1997; Boehm *et al*., 1997; Darnell *et al*., 1994; Decker *et al*., 1997; Stark *et al*., 1998). The analysis of mice

deficient for each one of the different STATs helped to clarify the different functions and specificities of the different combinations of JAKs and STATs and their preferential use by a certain stimulus. For example, *Stat1* knockout animals presented a phenotype characterised by profound deficiencies of the immune system's ability to clear microbial and viral infections, as well as a predisposition for cancer development (Durbin *et al*., 1996; Kaplan *et al*., 1998; Meraz *et al*., 1996). In humans the germline mutations in the STAT1 gene, essential for both type I and type II IFNs-mediated signalling, were accompanied, as for mice, by defective antimicrobial immunity (Dupuis *et al*., 2001).

Transcription of the human IFN-γ gene, located on chromosome 12 (Young and Hardy, 1995), leads to a noncovalent precursor formed by two identical peptides that are further processed by cleavage to obtain the biologically active form of this cytokine (Boehm *et al*., 1997). IFN-γ is produced by several immune cells, such as natural killer (NK) and γδ T cells, both part of the innate adaptive arm of the immune system, and by $CD8^+$ and Th1 $CD4^+$ T cells, which belong to the adaptive arm, and additionally by NKT cells (Boehm *et al*., 1997; Schoenborn and Wilson, 2007; Whitmire *et al*., 2005). IFN-γ is a pro-inflammatory cytokine, the biological functions of which are vital, for instance, for clearance of intracellular pathogens infections, such as virus and parasites (Jankovic *et al*., 2007; Trinchieri, 2001; Wheelock, 1965), and, for maintaining tumour immunesurveillance, both by eliminating tumour cells and preventing the process of metastasis (Brunda *et al*., 1995; Ikeda *et al*., 2002; Kakuta *et al*., 2002; Sadanaga *et al*., 1999; Xu *et al*., 2009; Zhang *et al*., 2011a). Given its pro-inflammatory activity and ability to regulate several distinct groups of T cells, this cytokine has also been implicated as a negative regulator of autoimmune diseases, such as multiple sclerosis, arthritis and diabetes (Berghmans *et al*., 2011; Kelchtermans *et al*., 2005; Lee *et al*., 2012; Lunemann *et al*., 2011; Sobel *et al*., 2002; Wood and Sawitzki, 2006). Therefore, a better understanding of the mechanisms of regulation and of the molecules responsible for modulating this cytokine could prove useful to safely target IFN-γ signalling pathways in a variety of malignancies.

The IFN-γ receptor (IFNGR) is constituted by two polypeptide chains, designated α (IFNGR1) and β (IFNGR2) chains, while the active IFN-γ receptor complex, to which IFN-γ binds, is formed by two IFNGR molecules, and, therefore, by two IFNGR1 and two IFNGR2 chains (Valente *et al*., 1992). This has been discussed before in detail but in simple terms IFN-γ binding to the IFNGR brings the receptor-associated JAKs into close proximity, which promotes their activation. JAK1 is found associated with IFNGR1 (Igarashi *et al*., 1994; Kaplan *et al*., 1996), while JAK2 is bound to IFNGR2 (Bach *et al*., 1996; Kotenko *et al*., 1995; Sakatsume *et al*., 1995). It has been suggested that JAK2 undergoes autophosphorylation and is responsible for the phosphorylation of JAK1, which renders these tyrosine kinases active upon the conformational changes undergone by the receptor induced by ligand binding. The JAKs phosphorylate tyrosine residues in the cytoplasmic chains of the receptors, in particular the tyrosine 440 on IFNGR1, which is attributed to JAK1. These then function as docking sites for monomeric latent STATs. JAK2 phosphorylation of tyrosine 701 on each STAT1 monomer induces a conformational change that allows the formation of STAT dimers *via* reciprocal phosphotyrosine and SH2 domain interactions. Diffusion through the cytoplasm to the periphery of the nuclear pore is followed by active import through the nuclear membrane, binding to specific DNA sequences of ISGs and regulation of gene expression that elicit the biological activities of IFN-γ (Briscoe et al., 1996; Levy and Darnell, 2002; Sakatsume et al., 1995) **(Fig. 1.3)**.

IFNGR is expressed in different cell types and tissues, including several areas of the lymphoid organs, macrophages, diverse cells in lymphoid tissues (Valente *et al*., 1992), in $CD4^+$ T cells upon activation (comprising Th1 and T_H2, T_H17 and regulatory T cells), which are particularly relevant as effectors of the adaptive immune system (Chen and Liu, 2009). The receptor for IFN-γ is also present in $CD8^+$ T cells (Whitmire *et al*., 2005) and was also found expressed in the majority of B cells and hepatocytes, as well as in endothelial cells lining the blood vessels, certain epithelial and endometrial mucosa cells, osteoclasts and in glandular cells, amongst others (Valente *et al*., 1992; Yang *et al*., 2002).

IFN-γ is, therefore, able to affect and modulate a number of cell types and tissues. A recent study, for instance, has shown that IFN-γ is essential to modulate the process of myelopoiesis when initiated in response to infection, promoting the development of monocytes, which are better immune effectors against lymphocytic choriomeningitis viral infection than neutrophils (de Bruin *et al*., 2012).

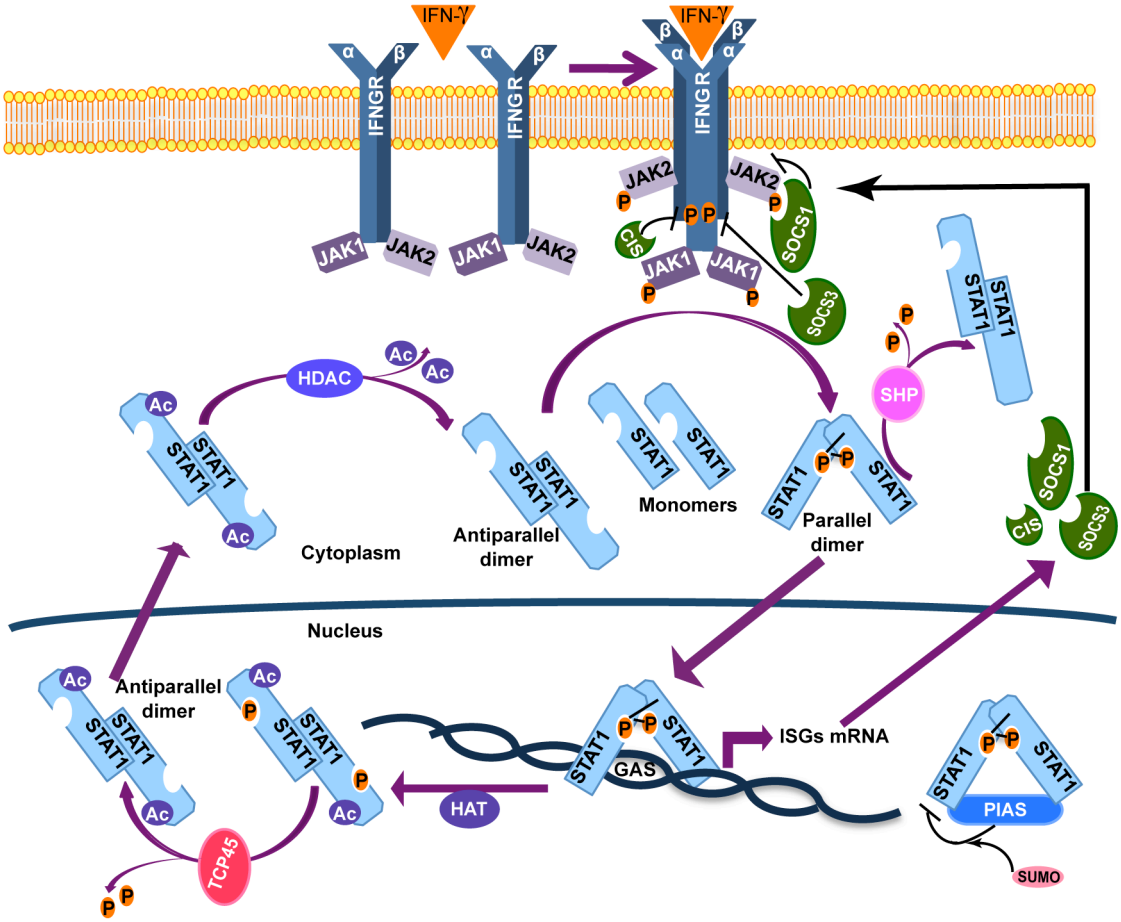


Figure 1.3. IFN-γ signalling *via* the JAK/STAT pathway. IFN-γ binding promotes receptor dimerisation. These conformational changes bring into close proximity the tyrosine kinases JAK1 and JAK2, which are associated with the IFNGR1 and IFNGR2 respectively. This allows the JAKs to become activated by auto- and transphosphorylation. JAKs first phosphorylate the receptor tails to create docking sites for STATs, which bind *via* their SH2 domains and become subsequently phosphorylated on tyrosine 701. STAT1 homodimers undergo a conformational change from an antiparallel structure to adopt a parallel structure *via* reciprocal interactions between the SH2 domain and the phospho-tyrosine of each monomer. These STAT1 dimers are actively translocated into the nucleus, where they bind to GAS sites and induce transcription of ISGs. Amongst these, the SOCSs inhibit STAT1-mediated signalling in a negative feedback loop, by competing with STATs for receptor binding, inhibiting the JAKs or promoting ubiquitin-mediated STATs proteasomal degradation. PIAS proteins negatively regulate STAT1 activity *via* direct

association and STAT1 sumoylation, while SHPs dephosphorylate STAT1 tyrosine and serine residues. The HAT CBP may acetylate STAT1 on lysine residues in the nucleus, which targets STAT1 for dephosphorylation by TCP45, mediated by a conformational alteration of the STAT dimers to an antiparallel structure, which exposes the tyrosine residues to the phosphatase. STAT1 dimers are then exported into the cytoplasmic compartment, where HDAC3 is thought to remove STAT1 acetylation, priming these dimers for a new cycle of IFN-γ-induced activation.

The importance of IFN-γ signalling can be emphasised by the fact that several viruses have developed ways of disrupting this pathway (Blake *et al*., 1997; Moormann *et al*., 2007), as well as by the occurrence of persistent infections caused by bacteria and parasites in individuals where this cytokine or its receptor are found mutated (Mwanda *et al*., 2004). The importance of IFN-γ-mediated T cell responses can also be exemplified by the lack of Epstein-Barr nuclear antigen 1 (EBNA1)-specific T cells in children that suffer with endemic Burkitt's lymphoma. The absence of these IFN-γ-producing T cells has been associated with the emergence of this malignancy, which could be caused by T cell clonal exhaustion provoked by continuous stimulation with the EBNA1 antigen (Moormann *et al*., 2007). IFN-γ deficiency has also been shown to act as an important trigger for other types of lymphoma development (Street *et al*., 2002).

In addition, IFN-γ can modulate the adaptive immune response by polarising $CD4^+$ T helper cells towards a T_H1-cell phenotype (Nishikawa *et al*., 2003). This cytokine, produced by specific T cells, was shown to be extremely important for the elimination of HLA class II-deficient melanoma cells by IFN-γ-stimulated macrophages (Corthay *et al*., 2005). IFN-γ is also involved in the control of autoimmunity either by activating regulatory T cells or by promoting apoptosis of autoreactive T lymphocytes. As an example, this cytokine was shown to be an essential regulator in the context of experimental autoimmune myocarditis (Barin *et al*., 2010). In what experimental autoimmune encephalomyelitis is concerned, a recent report suggests that IFN-γ mediates a protective effect in the central nervous system, independent of the action of infiltrating immune cells (Lee *et al*., 2012).

Numerous effects exerted by IFN-γ over cytotoxic $CD8^+$ T lymphocytes are indirect, since their functional activity is augmented as a consequence of increased expression of the proteasome machinery and TAP proteins in immune cells and up-regulation of HLA class I molecules in cells infected with intracellular pathogens. Indeed, IFN-γ promotes tumour recognition by increasing the activity of the HLA class I pathway (Bui *et al*., 2006; Dunn *et al*., 2006). In contrast to previous reports where IFN-γ direct action over $CD8^+$ T cells was considered to downregulate these cells' activity (Badovinac *et al*., 2000; Ramana *et al*., 2000; Refaeli *et al*., 2002), work done by Whitmire *et al*. (2005), using transgenic mice unable to produce IFN-γ or deficient in the expression of IFN-γ receptor, has demonstrated that this cytokine directly promoted the expansion of lymphocytic choriomeningitis virus-specific $CD8^+$ T cells. With regards to MHC class I, their downregulation is thought to represent an evasion mechanism adopted by tumours to escape the immune system. This can be mediated by the production of TGF-β1 by tumour cells, which prevents IFN-γ-induced expression of these molecules at the surface of malignant cells (Hsiao *et al*., 2008).

In the context of transformation, cancer development and metastasis, IFNs have a relevant role in controlling the interactions between the immune system and tumour cells (Dunn *et al*., 2006). These cytokines are additionally involved in the regulation and coordination of the different components of the innate and the adaptive immune systems, actively participating in the elimination and sculpting phases of a process known as cancer immunoediting (Dunn *et al.*, 2005; Dunn *et al*., 2006). While endogenously produced type I IFNs do not act on tumour cells but rather on host haematopoietic cells responsible for inhibiting tumour growth, type II IFN (IFN-γ) has not only the ability to regulate the activity of immune cells, but can also affect tumour cells directly during the antitumour immune response, inhibiting their proliferation and promoting apoptosis (Tannenbaum and Hamilton, 2000). These effects are strictly dependent on tumour cells being sensitive to this cytokine and expressing the IFN-γ receptor.

Additionally, and in contrast to type I IFNs, IFN-γ can augment the antigen presenting ability of professional antigen-presenting cells (APCs) or render non-

APCs into cells with antigen-presenting capacity by up-regulating the expression and synthesis of HLA class II molecules at the surface of these cells, which enables a more efficient activation of helper $CD4^+$ T cells (Gattoni *et al*., 2006). HLA class II importance is highlighted by the loss of expression of these molecules in several cancers, with HLA-DR expression in tumour cells being recognised as relevant for the process of immune surveillance. As an example, IFN-γ-induced expression of these molecules has been seen impaired or even abrogated in numerous head and neck cancer cell lines (Meissner *et al*., 2008). HLA-DR expression was shown to be absent in and unable to be stimulated by IFN-γ treatment in small cell lung cancer (SCLC), but not in non-small cell lung cancer (NSCLC) cells. IFN-γ-induction of HLA-DR is dependent on the expression of class II transactivator (CIITA) initiated by this cytokine and this molecule was absent in SCLC cell lines (Yazawa *et al*., 1999). Deficiency of HLA class II expression due to lack of IFN-γ inducible expression of CIITA has also been reported in neuroblastoma (Yazawa *et al*., 2002), in Ewing sarcoma (Berghuis *et al*., 2009) and in colorectal and gastric cancer (Satoh *et al*., 2004), further sustaining the relevance of these molecules for tumour evasion from the immune system.

Curiously, Schwinn *et al.*, have shown that IFN-γ down-regulates the expression of ligands of NK group 2, member D (NKG2D) on both melanoma and glioma cells, which impaired the elimination of these cells by NK cells expressing inhibitory NKG2D, a mechanism of tumour immune surveillance extremely important when tumour cells gain the ability to down-regulate HLA class I molecules (Schwinn *et al*., 2009). IFN-γ has also been shown to act as a negative regulator of the metastatic process by increasing the expression of Irf-1 on tumour cells of a pulmonary metastatic mouse model, which was then responsible for recruiting natural killer (NK) cells to infiltrate in the lungs and increasing these cells cytotoxic activity (Ksienzyk *et al*., 2011). A previous study had established NK antimetastatic effect to be mediated by TNF-related apoptosis-inducing ligand (TRAIL) expression on these cells, which was dependent of IL-12-induced synthesis of IFN-γ by NK and NKT cells (Smyth *et al*., 2001).

Although the recognised antitumour properties of the IFNs make their clinical use extremely appealing, several aspects concerning, for instance, the control of local delivery, the identification of IFN-dependent antitumour mechanisms and severe toxic side effects, make this a task difficult to accomplish. At the present, only type I IFNs are clinically used for the treatment of certain cancer patients (Dunn *et al*., 2006), such as those suffering from renal cell cancer and hairy cell leukaemia. The use of IFNs as adjuvants in prophylactic vaccines is being revised, as it could increase efficiency of the antiviral immune response by modulating the T_H1 cellular subset (Kim *et al*., 2000; Toporovski *et al*., 2010).

1.3. Interferon-γ-driven transcription and mechanisms of transcriptional regulation

As mentioned before, the different biological functions triggered by all the different cytokines known to use the JAK/STAT pathway for signalling, is made possible by the activation of different STAT dimers and formation of distinct STAT complexes through recruitment of additional molecules, which allows for the transcription of specific groups of genes depending on the cellular context and stimulus (Costa-Pereira *et al*., 2002; Darnell *et al*., 1994; Friedman *et al*., 1984; Larner *et al*., 1984; Levy and Darnell, 2002; Platanias, 2005; Stark *et al*., 1998). Upon IFN-γ stimulation tyrosine phosphorylated STAT1 homodimers in a parallel conformation bind to the DNA at GAS sequences, defined as TTCNNNGAA, which are recognised by all STATs and initiate transcription of γISGs (Decker *et al*., 1997; Li *et al*., 1996) (**Fig. 1.3**).

IRF-1, for instance, is an immediate early gene (IEG) transcribed by IFN-γ-activated STAT1. IRF-1 functions as a transcription factor, necessary for the expression of other ISGs that are considered to be part of secondary and tertiary phases of IFN-γ-induced transcription. IRF-1 transcriptional activity mediates directly HLA class I expression (Jarosinski and Massa, 2002). In addition, IRF-1 also participates indirectly in the transcription of HLA class II molecules by promoting the transcription of CIITA together with STAT1 and the upstream stimulatory factor (USF)-1, which is permanently associated with CIITA promoter (p) IV. CIITA is considered the master regulator of HLA class II transcription and

its pIV is the one associated with IFN-γ inducible expression of this gene (Kern *et al*., 1995; Masternak *et al*., 2000; Muhlethaler-Mottet *et al*., 1998; Steimle *et al*., 1993; Zhou and Glimcher, 1995; Zhu *et al*., 2000). HLA class I and II expression is differently modulated, with HLA class I being regulated both at the transcriptional and translational levels (Lee *et al*., 1999b), while regulation of HLA class II expression is done essentially at the transcriptional level and considered to be extremely complex (Croce *et al*., 2003; Ting and Trowsdale, 2002). Given the differences between HLA class I and II transcription, assessing the expression of these molecules may provide valuable information regarding the differential role of ataxia telangiectasia mutated (ATM) in the regulation of IFN-γ responses which was the main objective of this PhD research project.

No STAT1 binding site has been identified in the promoter of the HLA class I gene and, therefore, IFN-γ-mediated STAT1 induction of HLA class I transcription is indirect *via* STAT1-induced IRF-1 transcription. IRF-1 binding to an IFN consensus sequence (ICS) determines IFN-γ-induced HLA class I transcription (Lee *et al*., 1999b). Moreover, cell surface expression of HLA class I requires intervention of LMP2 and LMP7, for processing of cytosolic proteins into small peptides, which will be presented as antigenic peptides in association with the HLA molecules (Lee *et al*., 1999b). TAP1 and TAP2, molecules responsible for transporting these processed antigenic peptides into the endoplasmic reticulum where they become associated with the HLA class I heavy chain, are also indispensable for cell surface expression of these to occur. IFN-γ stimulation determines the transcription of both HLA I molecules, as well as of TAP1 and TAP2 accessory molecules (Lee *et al*., 1999b).

The expression of all HLA class II molecules involves the formation of an enhanceosome, through the interaction of the co-activator CIITA and three additional transcription factors, namely the regulatory factor X (RFX), the nuclear factor Y (NFY) and CREB (Cogswell *et al*., 1991; Masternak *et al*., 2000; Zhu *et al*., 2000). Furthermore, transcription of both CIITA and HLA class II molecules is additionally subjected to active repression by an inhibitory protein (Krief *et al*., 1994). Expression of HLA class II molecules at cell surface requires invariant

chain (Ii), also an IFN-γ-inducible gene, which protects the antigen-binding groove from inadequate binding (Magniez *et al*., 2004). Acetylation and methylation of the CIITA pIV have also been reported as additional regulatory events (Morris *et al*., 2002; Zika *et al*., 2003), while CIITA has been described as possessing acetyltransferase activity, necessary for promoting HLA-DRα transcription (Beresford and Boss, 2001).

Several studies have been conducted in the attempt to identify IFN-induced STAT1 binding sites and thus clarify how the composition of STAT transcriptional complexes activated by distinct IFNs can determine binding to specific recognition sequences and differential transcription and regulation of ISGs (Bhinge *et al*., 2007; Hartman *et al*., 2005). A genome-based study conducted by Bhinge and co-workers (2007) detected 381 STAT1 binding sites. For more than half of those genes the binding sites were located within a distance of 50 kb away from the gene transcription start site and a considerably percentage of these located even closer, within 20 kb distance. From the analysis of all these binding sites, 226 genes were found to have a GAS sequence. Assuming that STAT1 binding sites locate between 1 kb upstream and 200 bp downstream the transcription starting site (TSS) of a gene the authors have highlighted 59 STAT1 target genes, one third of which had a GAS sequence within this proximal region.

A genome-wide study performed by Wormald *et al*. (2006) has shown that STAT1 binding sites are localised proximal to numerous genes responsive to IFN-γ, which are regulated at the transcriptional level. Although STAT1 is usually seen as an activator of transcription, this protein also functions as a transcriptional repressor for certain genes (Ramana *et al*., 2000; Sharma and Iozzo, 1998). Some STAT1 binding sites seemed to localise in close proximity to the TSS of ISGs and, curiously, binding of RNA polymerase II was found repressed in resting cells at most of STAT1 binding sites (Wormald *et al*., 2006). These regions may be associated with RNA polymerase II recruitment and subsequent detection of mRNA transcription upon IFN-γ stimulation. The majority of the small number of STAT1 binding sites from where RNA polymerase II was not excluded in unstimulated cells corresponded to IRF-1 and IFNAR1 genes.

In addition to these two genes, which are important effectors of IFN-responses, expression of seven other likely important IFN-γ-target genes was analysed and the distance between STAT1 binding site and the beginning of each gene-coding region studied and found to be quite small. The authors suggested that STAT1 binding sites are located proximal to the start of the coding region of those IFN-γ responsive genes that are crucial for this cytokine's signalling, such as IRF-1. Accordingly, for those IFN-γ-induced genes with the STAT1 DNA binding site and gene coding area separated by a longer distance, transcription may be more tightly regulated and possibly require remodelling of chromatin structure at the level of the promoter. For a considerable number of IFN-γ-responsive genes, STAT1 binding sequences are closely located to genomic regions where the transcription machinery is present and where to RNA polymerase II must be additionally recruited by STAT1 (Wormald *et al*., 2006).

Moreover, different genes may rely on different regulatory mechanisms, through interaction with a variety of transcription co-activators. The MCM family may serve as a good example. MCM5 can be recruited upon IFN-γ stimulation in order to interact and localise with STAT1 at the promoters of ISGs, such as IRF-1, GBP1, TAP1 and CIITA and this association was found to be dependent on STAT1 serine 727 phosphorylation (DaFonseca *et al*., 2001). This interaction with MCM5 may be necessary for maximal transcriptional activation of STAT1, since this DNA helicase was also involved in recruiting RNA polymerase II to the IRF-1 promoter. Likewise, other MCM family members have been shown to accompany the moving RNA polymerase II transcription complex after being recruited to the promoters by IFN-γ-activated STAT1 (DaFonseca *et al*., 2001).

IFN-γ responses are clearly regulated at the transcriptional level and, at least for some ISGs, this regulation also requires higher chromatin reorganisation. The elegant work done by Christova and colleagues revealed that such remodelling induced chromosome loops on the HLA-DRα gene after only 10 minutes of IFN-γ treatment (Christova *et al*., 2007). In the context of innate immune responses, IFN-γ was shown to mediate the recruitment of chromatin remodelling molecules and to increase chromatin relaxation at the level of IL-6 and TNF promoters, in order to

reverse endotoxin exposure-induced tolerance in macrophages. IFN-γ induction facilitated the recruitment of nuclear factor (NF)-κB p65 subunit and C/EBPβ transcription factors, which may also have restored the recruitment of RNA polymerase II to the promoters of these two pro-inflammatory cytokines (Chen and Ivashkiv, 2010).

Indeed, expression of the CXC ligand (CXCL) 9 IFN-γ-responsive chemokine, upon IFN-γ and tumour necrosis factor (TNF)-α stimulation, was shown to be dependent on the recruitment of the histone acetyltransferase (HAT) CBP. This co-activator bound both STAT1 and NF-κB, in addition to promoting RNA polymerase II association to this transcriptional complex, which increased CXCL9 transcription, independently of histone acetylation by CBP (Hiroi and Ohmori, 2003). Different transcriptional proteins may, therefore, be involved in RNA polymerase II recruitment, since IRF-9 has also been shown to do so, but in contrast to the previously mentioned work, histone deacetylation was indispensable for IFN-β mediated transcription by STAT1 and STAT2 heterodimers. Curiously, IRF1 expression mediated by IFN-β-activated STAT1 homodimers, also an early-transcribed gene in response to IFN-γ, was unaffected by inhibition of histone deacetylase (HDAC) activity.

Chromatin structure and organisation must be tightly regulated during important biological processes such as repair of DNA lesions, DNA replication and transcription. Alteration of histones acetylation, methylation or phosphorylation status, are all PTMs able to alter chromatin conformation and accessibility. With regards to acetylation, this is frequently a requirement for chromatin to relax, which results from the concerted action of HATs and/or inhibition of HDACs and ensures transcription can occur by permitting the assembly of a transcription complex involving DNA recruitment of transcription factors and co-activators.

Curiously, HDAC activity has been reported as crucial for IFN-induced transcription (Chang *et al*., 2004; Nusinzon and Horvath, 2003; Sakamoto *et al*., 2004). In fact, IFN-β-stimulated binding of RNA polymerase II to the ISG54 promoter was prevented by trichostatin A (TSA), a pan inhibitor of HDAC activity

(Sakamoto *et al*., 2004). Interestingly, ectopic expression of IRF9 reverses the inhibitory actions of TSA, suggesting that IRF9 functions to recruit RNA polymerase II to the promoter of ISGs (Snyder *et al*., 2005). CBP/ p300 co-activators have also been shown to be involved in IFN-γ-induced recruitment of RNA polymerase II (Darnell, 1997; Hiroi and Ohmori, 2003) and this enzyme has been found association with STAT1 at the promoters of different ISGs (Hiroi and Ohmori, 2003; Snyder *et al*., 2005). Where IFN-γ signalling is concerned, HDAC1 was found to be a positive transcriptional regulator rather than a transcriptional repressor of CXCL10 expression, also known as IP10 (Nusinzon and Horvath, 2003; Guo *et al*., 2006).

These data support the notion that STAT1 transcriptional activity may be mainly mediated by STAT1-induced recruitment of RNA polymerase II in response to IFN-γ treatment (Bhinge *et al*., 2007). While, additional chromatin remodelling is likely required for ISGs where RNA polymerase II binding is suppressed in resting cells or simply inaccessible, for those genes, such as IRF-1, the process would simply increase the constitutive level of transcription, instead of creating all the necessary conditions for the recruitment and assembly of the transcriptional machinery. IFN-induced STAT1-dependent ISG transcription is thus an intricate and complex subject, possibly involving different levels of transcriptional regulation. This may be achieved by varying STAT1 distance to the gene-coding region (Bhinge *et al*., 2007) and/or by a differential recruitment of transcription factors, co-activators and chromatin remodelling proteins to multiple proximal binding sites within the promoter sites (Smith *et al*., 2006).

Tuberculosis infection has been associated with inhibition of IFN-γ signalling in macrophages, with the impairment of CXCL11 and CIITA expression at the transcriptional level being associated with inhibition of RNA polymerase II recruitment to the promoters of these unrelated ISGs. The complexity of IFN-γ-mediated chromatin remodelling was well exemplified by the author's description that inhibition of this cytokine resulted in a distinct pattern of alterations on CIITA and CXCL11 promoters, which plausibly reflect differences regarding the transcriptional machinery needed for these two different genes to be expressed.

While CXCL11 transcription was affected as the result of TATA binding protein being unable to bind the promoter, histone acetylation was impaired at the CIITA promoter (Benson and Ernst, 2009).

Recent data also support the notion that IFN-γ transcriptional responses are highly dependent of chromatin remodelling. IFN-γ-induced CIITA expression, for instance, requires not only the recruitment of STAT1 and newly synthesised IRF-1 to the promoter, but also acetylation of histones 3 and 4 (Morris *et al*., 2002). Impairment of these chromatin remodelling processes at IFN-γ-responsive genes promoters, which are dependent on STAT1 binding for transcription, abrogates this cytokine-induced transcription. Infection of murine macrophages with toxoplasma gondii affected STAT1 binding to GAS sequences on both IFN-γ induced and -repressed genes. The incorrect binding of STAT1 to GAS sequences was considered to be the cause for the lack of recruitment of proteins, such as chromatin remodelling factor brahma-related gene (BRG) 1 and for decreased acetylation at the CIITA pIV (Lang *et al*., 2012).

Besides being a component necessary for the transcriptional activation of HLA class II genes (Mudhasani and Fontes, 2002; Mudhasani and Fontes, 2005), BRG1 may be generally required for transcription of other IFN-γ responsive genes. BRG1 and brahma (BRM) are the ATPase subunits of the switch/sucrose non-fermentable (SWI/SNF) complex, involved in chromatin remodelling at the level of nucleosome location. Work done by Zhang and co-workers (2010) determined that BRM functioned as a co-repressor bound to GAS sequences in resting cells, whilst IFN-γ treatment culminated with the acetylation and release of BRM from the chromatin and its replacement by BRG1. This BRM/BRG1 switch resulted in increased IFN-γ-induced transcriptional activity of the genes at which this exchange was observed. However, this varied depending on the cell type, with CIITA being expressed in Raji cells but not in Jurkat cells. In addition, by performing restriction endonuclease accessibility assays, it was demonstrated that the exchange of BRM by BRG1 upon IFN-γ stimulation, was associated with a more relaxed and open structure of the chromatin (Zhang *et al*., 2010).

1.4. Modulation of the canonical JAK/STAT pathway, other IFN-γ-activated pathways and identification of ATM as a regulator of IFN-γ signalling

The majority of IFN-γ biological responses are mediated by activation of the canonical JAK/STAT pathway. However, as briefly mentioned, IFN-γ also activates a number of other pathways. These pathways may mediate the transcription of different subsets of STAT1-independent ISGs and, therefore, act as modulators of the canonical pathway in order to achieve the full range of IFN-γ biological responses (Ramana *et al*., 2002). Indeed, PYK2 and extracellular-signal-regulated kinase (ERK) 1 and 2 are activated in response to IFN-γ stimulation and were initially thought to regulate STAT1 serine 727 phosphorylation (Goh *et al*., 1999; Takaoka *et al*., 1999). However, subsequent reports excluded the ERKs as the sole kinases responsible for this PTM (Decker and Kovarik, 2000). PYK2 was reported as the kinase responsible for the IFN-γ-induced phosphorylation of mitogen-activated protein kinase kinase kinase (MEKK) 4 in keratinocytes, however the functional relevance of such IFN-γ-induced signalling branch remains unknown (Halfter *et al*., 2005). The NRTK FYN, from the SRC-family, was shown to interact with JAK2 upon IFN-γ signalling (Uddin *et al*., 1997). FYN was later shown to be necessary for the formation of a complex involving STAT5B and PI3K, which was required for IFN-γ-mediated permeability of the intestinal epithelium (Smyth *et al*., 2011). CamKII (Nair *et al*., 2002), PKCδ (Deb *et al*., 2003), PI3K and its effector protein kinase B (PKB) (Nguyen *et al*., 2001; Sadzak *et al*., 2008) can also phosphorylate serine 727 on STAT1 in response to IFN-γ. Another recent study suggests, for example, that PKB/AKT pathway activation by IFNs acts together with and independently of the JAK/STAT pathway to induce the biological effects of these cytokines by promoting mRNA translation of ISGs (Kaur *et al*., 2008). PKB/AKT participation was linked with the regulation of the mammalian target of rapamycin (mTOR) pathway (Kaur *et al*., 2008; Watling *et al*., 2008). Even though the importance of IFN-γ-induced, STAT1-independent, signalling is clear the mechanisms involved are not. Data indicate that additional as yet unidentified pathways also exist (Briscoe *et al*., 1996; Watling *et al*., 2008). Investigating how these STAT1-dependent and -independent pathways are

involved in IFN-γ signalling could provide ways for future therapeutic application of this knowledge in cancer treatment.

Curiously, in the context of non-canonical JAK/STAT signalling, unphosphorylated STAT1 has been reported to bind to heterochromatin in *Drosophila melanogaster*. The authors suggested that this interaction was required for maintaining the heterochromatin structure stably condensed through retention of heterochromatin protein (HP) 1, a known repressor of chromatin opening. JAK-mediated phosphorylation of STAT would result in less unphosphorylated STAT, release HP1 from the heterochromatin thereby affecting heterochromatin stability (Li, 2008; Shi *et al*., 2008). It would be interesting to investigate if this also happens in human resting cells, with IFN-γ signalling resulting in destabilisation of the chromatin structure.

Work performed by the Kerr group has demonstrated that different components of the JAK/ STAT pathway are differentially needed for distinct IFN-γ-induced biological responses (Costa-Pereira *et al*., 2005). The kinase activity of JAK1, which is needed for phosphorylating the receptor in order to create STAT1 docking sites is absolutely required for adequate IFN-γ signalling. However, a kinase-negative mutant of JAK1 was still able to partially elicit cytokine-induced transcription, but it was unable to sustain an antiviral response (Costa-Pereira *et al*., 2005). It has been suggested (Briscoe *et al*., 1996) that JAK2 autophosphorylates and trans-phoshorylates JAK1. This phosphorylation cascade activates STAT1, which is required for the vast majority of IFN-γ responses. Thus, it is not surprising that the activity of this kinase was indispensable for both full transcriptional activation and cytokine-induced viral protection (Briscoe *et al*., 1996). In a similar way to what was observed with a kinase-negative JAK1, IFN-γ-induced transcription was still possible when the key STAT1 docking site on the receptor (IFNGR1) was mutated on tyrosine 440 (Y440F). However, lack of phosphorylation of this residue was shown to affect differentially several ISGs, with IRF1 and GBP1 expression being practically normal, while SOCS1, SOCS3, CIITA and HLA class II were profoundly impaired (Costa-Pereira *et al*., 2005).

A kinome-wide siRNA screen that used flow cytometric detection of IFN-γ-induced HLA Class I and II expression as read-outs, together with IFN-γ-induced antiviral assays against encephalomyocarditis virus (EMCV), was performed by the Costa-Pereira group with the purpose of unravelling additional molecules relevant for IFN-γ signalling (Watling *et al*., 2008). Kinases such as PI3K and CamKII, reported previously by others (Nair *et al*., 2002; Nguyen *et al*., 2001), as well as predictably JAK1 and JAK2 were shown to be required for these IFN-γ responses. Silencing of τ tubulin kinase (TTBK) 1, responsible for phosphorylation of τ proteins, which promote microtubule assembly and stabilise microtubules, affected IFN-γ-responses (Sato *et al*., 2006), as it did the silencing of bromodomain-containing protein (BRD) 2, which localises to the nucleus and plays a role in cell cycle-responsive transcription (Peng *et al*., 2007). Adenylate kinase (AK) 7, a kinase known to mediate the conversion of ADP molecules into ATP and AMP (Dzeja *et al*., 1999), was also identified in this screen as a modulator of IFN-γ signalling, together with hexokinase (HK) 2, protein kinase A (PKA), WNK lysine deficient protein kinase 2 (PRKWNK2), TATA binding protein-associated factor 1 (TAF1) and TAF1-like kinase (TAF1L) (Watling *et al*., 2008).

Ataxia telangiectasia mutated (ATM), a well-known serine/threonine protein kinase involved in DNA double-strand breaks repair was the kinase to retain more interest regarding regulation of IFN-γ biological activity (Watling *et al*., 2008). As far as ATM is concerned, the silencing of this kinase by siRNA impaired Class II HLA induction in response to IFN-γ treatment, but not Ataxia Telangiectasia and Rad3-related (ATR) knockdown. The relevance of molecules involved in Class II HLA induction is demonstrated by a common defect in human tumour cell lines. This defect consists of the incapacity of IFN-γ to promote the expression of Class II transactivator (CIITA) and, consequently, of Class II HLA and invariant chain (Ii) in the tumour cells. This can have obvious implications regarding the appropriated detection and elimination of tumour cells by the immune system (Lu *et al*., 1997).

1.5. ATM as a modulator of IFN-γ-induced JAK/STAT signalling pathways

ATM silencing impaired both HLA class II expression and the antiviral response associated with IFN-γ stimulation, which are known to be dependent on JAK/STAT activation. Therefore, ATM acts as a modulator of the canonical JAK/STAT pathway activated downstream of IFN-γ.

1.5.1. ATM: a member of the phosphoinositide-3-kinase-related protein kinase (PIKK) family

The phosphoinositide-3-kinase-related protein kinase (PIKK) family includes six members: ATM, ataxia- and RAD3-related (ATR), DNA-dependent protein kinase catalytic subunit (DNA-PK), mTOR, suppressor of morphogenesis in genitalia (SMG-1) and transformation/transcription domain-associated protein (TRRAP). These proteins are functionally described as serine/threonine kinases that target protein serine or threonine residues followed by glutamine (SQ/TQ) motifs and are important players in cellular processes involving DNA integrity and RNA quality, such as DNA damage repair (DDR), replication, recombination, transcription and translation, guaranteeing genomic stability and correct expression of genes (Izumi *et al*., 2010).

SMG1 is involved in the process of nonsense-mediated mRNA decay, a mRNA quality control system that degrades anomalous mRNAs with premature termination codons, in order to prevent these from being translated into truncated proteins either non-functional or with altered function (Usuki *et al*., 2006; Yamashita *et al*., 2005). mTOR is an essential regulator of cellular growth and metabolic processes involved in the uptake of nutrients, growth factor signalling, energy levels and in response to stress conditions [reviewed in (Hall, 2008)]. This serine/threonine kinase has also been shown to participate in the initiation of autophagy (Shang and Wang, 2011). TRAPP participates in several DNA-dependent processes, such as transcription, since this protein can act as a cofactor for several HAT complexes, necessary for their recruitment to chromatin [reviewed in (Murr *et al*., 2007)]. Histone acetylation dependent on HAT loading on

the chromatin mediated by TRAPP is necessary for loading repair effectors at DNA DSBs (Murr *et al*., 2006).

ATM, ATR and DNA-PK are known and important orchestrators of DDRs and have been shown to be quickly activated ensuing the occurrence of DNA lesions. While DNA DSBs essentially activate ATM and DNA-PK, ATR is mainly activated by ultraviolet (UV) radiation, single-stranded (ss) DNA gaps and blocked replication forks (Shiloh, 2006). All three kinases are recruited to chromatin through accessory binding proteins and initiate the DDR based on the phosphorylation of downstream effector kinases, which are responsible for the control of cell cycle progression and apoptosis, as well as DNA repair and cellular senescence (Falck *et al*., 2005).

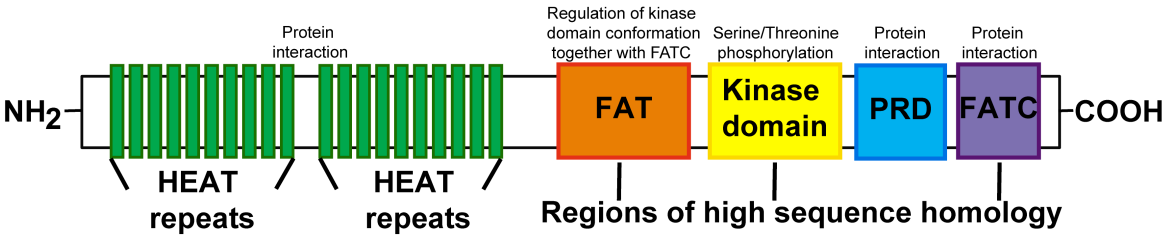
1.5.1.1. The PIKK protein structure and kinase activity regulation

The members of the PIKK family are large proteins, as exemplified by ATM, with a molecular weight of 350 kilodaltons (kDa). PIKKs share a common conserved structure organised in four domains: the FRAP-ATM-TRRAP (FAT) domain, the kinase domain (KD), the PIKK-regulatory domain (PRD) and the FAT-C-terminal (FATC) domain (Bosotti *et al*., 2000; Keith and Schreiber, 1995; Mordes *et al*., 2008) (**Fig.1.4**).

The PIKK kinase domain (KD) has a high sequence homology with the one present in PI3K family members, which is quite different from those of most other eukaryotic protein kinases, the reason why PIKK enzymes are often considered atypical protein kinases (Cantley, 2002; Manning *et al*., 2002). The kinase domain, PRD and FATC domains locate in the C-terminus of the protein. All three PRD, FAT and FATC domains, have been suggested to regulate the activity of the kinase possibly by targeting the activation loop in the KD (Bosotti *et al*., 2000). The FAT domain locates N-terminal of the KD and is formed by Huntingtin, elongation factor 3, subunit A of protein phosphatase 2A and TOR1 (HEAT) repeats. The rest of the structure of these kinases is thought to be involved in interactions with other proteins, which could explain its high variability amongst the PIKK family members, and is made of HEAT repeats, as the one present in the FATC domain

(Adami *et al*., 2007; Llorca *et al*., 2003; Perry and Kleckner, 2003; Rivera-Calzada *et al*., 2005). The FATC domain of ATM, DNA-PK, ATR, and TRRAP proteins was shown to function as a protein-protein interaction region for the HAT TAT-interacting proteins 60 (TIP60). Moreover, TIP60 positively regulated the enzymatic activity of ATM and DNA-PK in response to DNA damage (Jiang *et al*., 2006). Nonetheless, there is still a lot unknown about the precise functions of the different PIKK structural domains.

PIKK structural and functional organization



ATM phosphorylation and acetylation residues

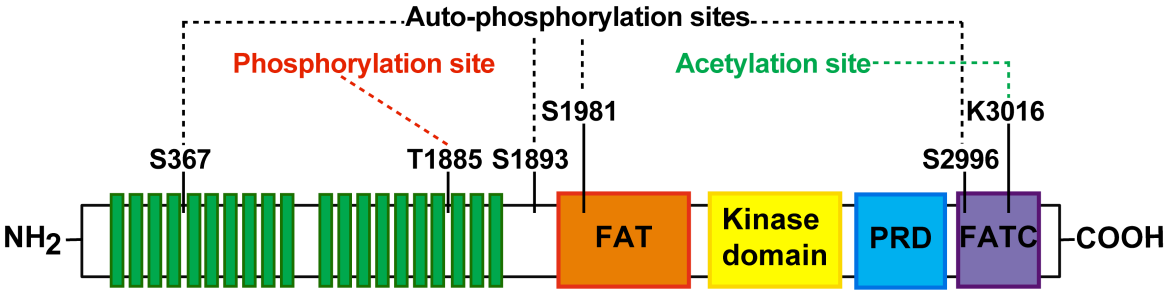


Figure 1.4. Structural and functional organisation of PIKK family members and depiction of ATM's known auto-phosphorylation, phosphorylation and acetylation residues. The kinase domain is located in the C-terminal region, being flanked on its N-terminal side by the FAT domain and on the other side by the regulatory domain PRD, followed by the FATC domain. The N-terminal domains is largely composed by HEAT repeats, which are also present in the FAT domain.

1.5.1.2. ATM known functions and its role in DNA repair

The DNA damage response is a mechanism of tumour suppression that acts by preventing genomic instability (Fan et al., 2006), in which ATM kinase has an essential function. ATM is the gene mutated in the cancer pre-disposing genomic instability syndrome AT, which is an autosomal recessive disorder characterised by a number of defects, including radiosensitivity, cancer predisposition, neurodegeneration and immunodeficiency, among others [reviewed in (Shiloh, 2006)]. ATM serine/threonine kinase is predominantly nuclear (Gately *et al*., 1998).

ATM exists as an inactive dimer, however, upon DNA DSBs formation, auto-phosphorylation of serine 1981 residue of human ATM has been suggested to result in an active monomer. ATM monomers initiate a complex cascade of phosphorylation events, ultimately leading to the repair of DSBs (Bakkenist and Kastan, 2003). The MRE11-RAD50-NBS1 (MRN) complex acts as a sensor for ATM recruitment by accumulating in nuclear foci upon detection of DSBs and is additionally involved in ATM's stable association with DNA ends and ATM activation (Lavin and Kozlov, 2007). ATM then promotes phosphorylation of H2AX and BRCA1, two proteins also part of the DNA repair machinery. ATM also phosphorylates checkpoint kinase (CHK) 2 and protein 53 (p53), which activates cell cycle arrest during DNA repair, or initiates apoptosis in case of extensive and irreparable damage (Lavin and Kozlov, 2007) (**Fig. 1.5**).

ATM undergoes further PTMs, such as additional phosphorylation events (Kozlov *et al*., 2006), which may also modulate its activity. Human ATM protein has two other serine residues, serine 367 and serine 1893 besides the residue in position 1981, that can also undergo autophosphorylation. In mice these serine residues correspond to positions 367, 1899 and 1987. Curiously, the work done by the Nussenzweig group using ATM transgenic mice, where serine 1987 (human serine residue 1981) or all three serine residues were mutated to alanines and were phosphorylation deficient, has demonstrated that autophosphorylation of these residues upon DNA damage were not necessary for ATM activity (Daniel *et al*., 2008; Pellegrini *et al*., 2006).

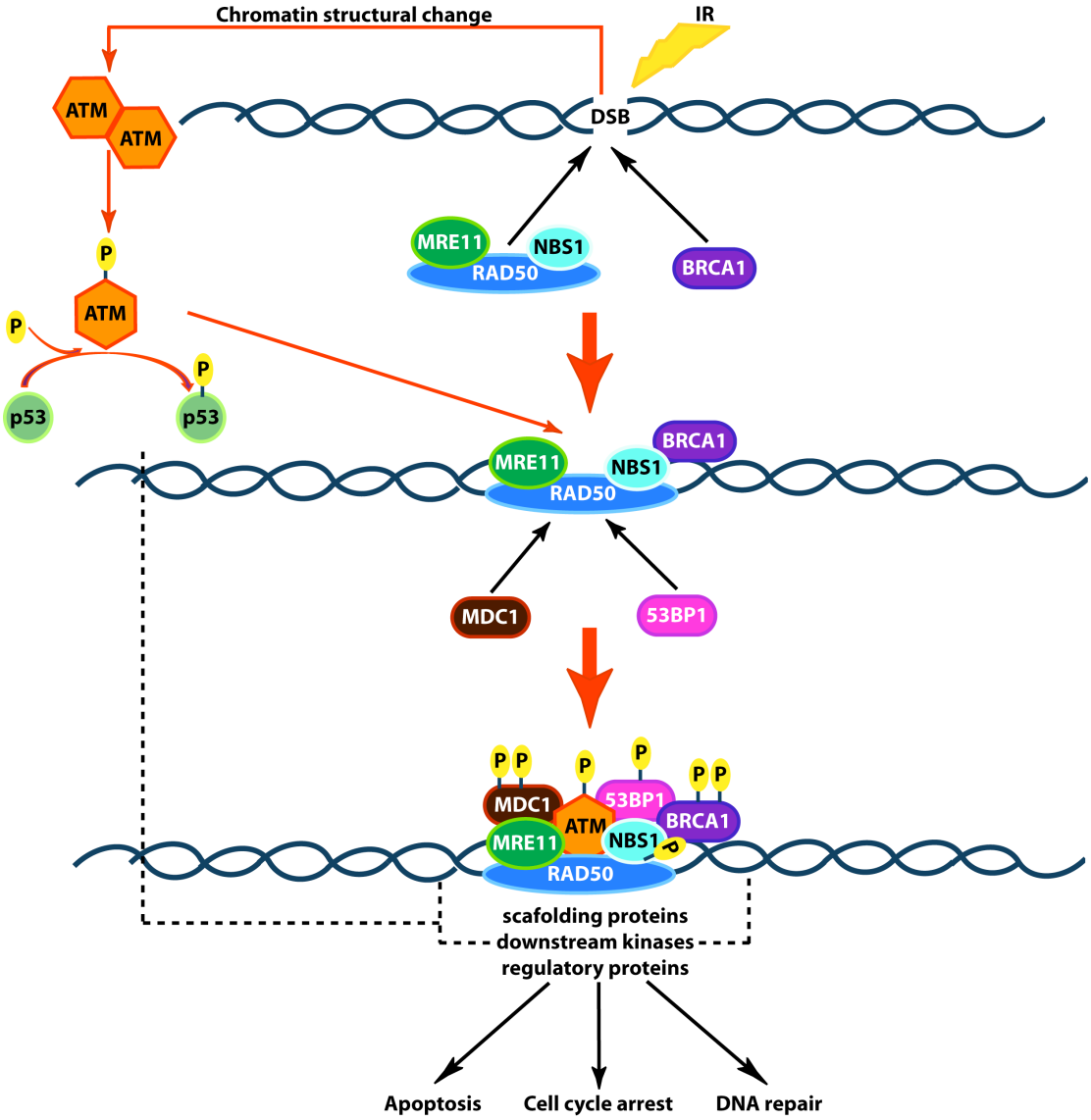


Figure 1.5. Signalling pathways activated by ATM in response to DNA DSBs. ATM is found as a latent dimer in the absence of DNA damage. The occurrence of DSBs, caused by insults such as ionising radiation, is thought to induce topological alterations in the chromatin structure that function as a signal for ATM activation and recruitment to the sites of damage. ATM undergoes auto-phosphorylation, which determines the release of active serine/threonine kinase ATM monomers. The MRN complex formed by MRE11, NBS1 and RAD50 proteins localise at the lesion site together with BRCA1. This complex stabilises the DNA extremities and act as a scaffolding platform for the co-localisation of ATM and its downstream effectors, such as MDC1 and 53BP1. Upon recruitment into this complex, ATM phosphorylates and thus activates MRE11, NBS1, BRCA1, MDC1 and 53BP1. ATM also phosphorylates p53, involved in the DDR. The signalling cascade orchestrated by ATM and facilitated by this multimeric complex is then responsible for coordinating cell cycle arrest and to initiate either repair mechanisms or apoptosis when the damage is to extensive to be properly repaired.

Although dispensable for ATM kinase activity, there is some evidence for serine 1981 phosphorylation interaction with mediator of DNA damage checkpoint protein (MDC) 1 to stabilise ATM localisation at DSBs (So *et al*., 2009). More recently Kozlov and co-workers have identified an additional serine residue at position 2996 that also suffers DNA damage-induced autophosphorylation. Phosphorylation of this residue, or of serine 367 were not important for ATM localisation to the DSBs sites, however, they seemed to be required for promoting ATM-mediated S phase check point arrest. In addition, the authors have also reported that the threonine residue at position 1885 was an ATM phosphorylation site, the function of which is unknown (Kozlov *et al*., 2011).

Following the occurrence of DNA lesions, ATM is also acetylated at lysine residue 3016 within the FATC domain by the TIP60, which, as previously mentioned, determines DNA damage-induced up-regulation of ATM enzymatic activity (Jiang *et al*., 2006; Murr *et al*., 2006; Sun *et al*., 2005b; Sun *et al*., 2007). Additionally, ATM activity may also be dependent on chromatin structure alterations (Lavin and Kozlov, 2007).

Although ATM activation is known to be dependent of DSBs formation, how exactly this takes place is still matter of debate. The ATM homologue in yeast, the kinase Mec1, can be directed to DSBs because of its affinity towards DNA ends, which was suggested to be also the mechanism by which human ATM could sense and be recruited to DSBs. However, another possibility has been proposed, according to which DSBs alter certain topological features of the chromatin structure that function to recruit ATM to the site of DNA lesions (Bakkenist and Kastan, 2003). Besides DSBs, other stimuli are thought to induce such modifications of the chromatin higher organisational order and to activate ATM, even in the absence of DNA lesions (Ju and Muller, 2003). Bakkenist and Kastan reported that both hypotonic stress and inhibition of DNA replication by hydroxyurea treatment were able to induced ATM activation unrelated to DSBs (Bakkenist and Kastan, 2003).

HDAC activity inhibition was shown to induce the expression of genes, such as protein 21 (p21), in an ATM-dependent fashion and in the absence of DSBs (Ju

and Muller, 2003). The authors initially suggested that ATM could act as a regulator of histone acetylation by controlling the activities of both HAT and HDAC. Nonetheless, no differences regarding the histone 3 pattern of acetylation supported this hypothesis, although acetylation of histone 4 was found delayed. As a consequence, the authors assumed that absence of ATM function did not affect chromatin acetylation *per se*, but rather the chromatin-based steps that followed and led to transcription (Ju and Muller, 2003). A more recent study determined that HDAC inhibition with TSA and consequent alteration of the histone acetylation pattern was sufficient to activate ATM and to promote phosphorylation of H2AX and CHK2 in the absence of DNA damage (Lee, 2007). Another study demonstrated that ATM-mediated DDR may be involved in an anti-tumour mechanism activated by hypoxia, a characteristic of the tumour environment. ATM was found phosphorylated and active under conditions of oxygen deprivation. Despite the absence of DNA lesions, ATM stress-induced activity did not require the MRN complex, but recruited MDC1 in order to activate KAP1 (Bencokova *et al*., 2009).

New roles for ATM, apart from DSBs repair, have been reported. For example, ATM was implicated in the regulation of the synthesis of deoxyribonucleoside triphosphates, by affecting the enzyme ribonucleotide reductase and in the control of mitochondrial homeostasis, the disruption of which may contribute to AT pathology (Eaton *et al*., 2007). It was also described that ATM kinase is responsible for mediating full activation of PKB/ AKT by insulin in muscle cells (Halaby *et al*., 2008). These studies provide evidence for the importance of ATM in several cellular functions other than just DSB repair. ATM requirement for DSB repair is not associated with damage complexity, but rather with heterochromatin state of condensation and ATM is thought to be essential for DNA relaxation (Goodarzi *et al*., 2008).

1.5.2. Possible link between ATM chromatin remodelling ability and regulation of IFN-γ responses

The basic repeating unit in DNA structural and functional organisation is the nucleosome and DNA is further packed in a higher order structural fibre organisation (Van Holde *et al*., 1974; Woodcock, 2005). It is now well established that the structural organisation of the chromatin and its condensation status play a role in the regulation of gene transcription and other processes that depend on DNA accessibility (Downey and Durocher, 2006; Felsenfeld, 1992; Morrison and Shen, 2009; Paranjape *et al*., 1994). In fact, chromatin condensation inhibits the processes of transcription and elongation by not allowing the binding of proteins (Laybourn and Kadonaga, 1991), such as transcription regulators and RNA polymerase (Williamson and Felsenfeld, 1978).

Chromatin-mediated repression can be reversed by cellular molecular mechanisms that are capable of altering the nucleosomal structural organisation and consequently, chromatin condensation (Downey and Durocher, 2006; Kingston *et al*., 1996). Cellular processes that require DNA processing rely, therefore, on chromatin modifying complexes. This is the case for transcriptional processes mediated in human cells by the human SWI/SNF and INO80 chromatin-remodelling complexes (Batsche *et al*., 2006; Lee *et al*., 1999a; Reyes *et al*., 1997; Xue *et al*., 2000) .The INO80 chromatin-remodelling complex in *Saccharomyces cerevisiae* can be phosphorylated by ATM and ATR kinases *in vitro* and *in vivo*. These PTMs are thought to regulate the chromatin-remodelling complex so that proper processing of the DNA ends in DSBs repair can occur and thus a role for ATM as a chromatin-modifying factor has started to emerge.

Nonetheless, the existence of ATM-mediated chromatin alterations has only become evident when the ability of ATM to regulate chromatin structural condensation *via* direct modulation of KAP1 was established. KAP1 is known to localise in the nucleus and to bind a range of proteins *via* their KRAB domains. These include HP1 and HDACs. These interactions are thought to be the basis of KAP1-mediated heterochromatin formation and repression of chromatin relaxation [reviewed in (Craig, 2005)]. KAP1 was first identified by Ziv and colleagues (2006)

as a direct downstream target of ATM. More importantly, the authors showed that ATM-induced KAP1 serine 824 phosphorylation provoked temporarily a more open and relaxed structure of the chromatin.

Work done by Goodarzi and co-workers has demonstrated that in the context of DDR and repair of DSBs, the importance of ATM function correlates with regions of heterochromatin, where the DNA structure has increased structural complexity and repression (Goodarzi *et al*., 2008). The transient relaxation of heterochromatin imposed by ATM activation in response to DSBs required the disruption of a transcriptional silencing complex composed of KAP1, HP1 and/or HDAC1/2, which the authors suggested to weaken the interactions between KAP1 and heterochromatin (Goodarzi *et al*., 2008). Silencing of KAP1, HP1 or HDAC1/2 by RNAi was found to revert the blockage in DSB repair in cells where ATM activity had been inhibited, demonstrating that these chromatin-associated factors were responsible for the restrictions that must be overcome by ATM in order for DNA repair to take place (Goodarzi *et al*., 2008).

ATM has been shown to be involved in other cellular functions besides the DDR, such as IFN-γ signalling (Watling *et al*., 2008), and several of these activities are transcription-dependent. Therefore, the ability of ATM to alter the heterochromatin condensation status and thus increase DNA accessibility (Goodarzi *et al*., 2008) may also be required for adequate cytokine-induced gene transcription by permitting the binding of transcription factors to DNA.

In addition, the idea that effectors of the DDR may act as regulators of transcription is further supported by BRCA1, a known downstream effector of ATM, which has also been shown to promote large-scale chromatin decondensation (Ye *et al*., 2001). Several studies support a role for BRCA1 in DNA repair and transcription (Monteiro, 2000; Scully and Livingston, 2000; Zhang *et al*., 1998b). In addition, BRCA1 is also able to interact with RNA polymerase II (Neish *et al*., 1998; Scully *et al*., 1997), several transcription factors (Houvras *et al*., 2000; Somasundaram *et al*., 1997; Zhang *et al*., 1998a), as well as with p300 and HDAC1 and HDAC2 (Neish *et al*., 1998; Yarden and Brody, 1999). It has been suggested that BRCA1 can modulate transcription *via* direct binding to BRG1, a

component of the human chromatin-remodelling complex SWI/SNF (Bochar *et al*., 2000). Moreover, it was shown that BRCA1 acts together with STAT1 to induce expression of a subset of IFN-γ-responsive genes, of which p21 can serve as an example (Ouchi *et al*., 2000). Given the functional relationship between ATM and BRCA1 in the DDR, it would be of interest to assess if the same is true in cytokine-mediated transcription, or even for other effectors of this mechanism, such as the members of the MRN complex.

Besides its key function in orchestrating the damage response upon DNA DSBs, ATM might be involved in other important and clinically relevant cellular functions, possibly by regulating chromatin condensation status and its accessibility at specific promoters. The capability of ATM to alter chromatin accessibility to the transcription machinery may explain its involvement in the regulation of IFN-γ responses, namely, HLA Class II induction (Watling *et al*., 2008). Several functional links can be drawn directly between ATM and STAT1, or involving communal interacting proteins. STAT1 has been shown to participate in ATM responses by mediating ATM-dependent intra-S-phase and G2-M cell cycle checkpoints in response to DNA damage (Townsend *et al*., 2005). Moreover, STAT1 was found to mediate ATM-dependent phosphorylation of CHK2, NBS1 and p53, possibly by controlling 53BP1 and MDC1 expression (Townsend *et al*., 2005). Curiously, ATM silencing in HeLa cells was found to up-regulate the expression of ISGs and this expression pattern was also observed in AT patients. However, these ISGs were vastly type I-inducible genes, such as OAS1 (Chen *et al*., 2004). More recently, the induction of DSBs by treatment with etoposide was reported to cause IFN-α and IFN-λ production, as well as expression of ISGs, such as IRF-1 and IRF-7, therefore, establishing a link between DDR and IFN signalling (Brzostek-Racine *et al*., 2011).

KAP1 is known to be a downstream target of ATM in the context of DSBs repair within heterochromatin, acting as a chromatin-modifying factor (Goodarzi *et al*., 2008; Ziv *et al*., 2006). KAP1 is recognised by its role as a repressor of chromatin relaxation through interaction with HP1, HDACs and methyltransferases (Li *et al*., 2007; Li *et al*., 2010; Zeng *et al*., 2008). As a consequence, KAP1 is also recognised as a repressor of transcription and it is thought that KAP1 does so by

regulating the histone acetylation/methylation pattern of the promoter region (Xu and Price, 2011). In addition, KAP1 has been shown to interact with members of the STAT family, including STAT1 and to act as a negative regulator of STAT3 phosphorylation (Tsuruma *et al*., 2008). Moreover, KAP1 has been identified as a regulator of STAT1-mediated transcription in response to type I IFNs and TNF-α (Kamitani et al., 2008; Kamitani *et al*., 2011).

With regards to the activity of HDACs, HDAC1/2 acted as chromatin repressors in the study conducted by Goodarzy and co-workers and it is usually accepted that these enzymes negatively regulate transcription. HDACs may indeed act as repressors of IFN-γ-induced transcription and biological activities (Kong *et al*., 2009; Murphy *et al*., 2011; Zika *et al*., 2003). However, several studies seem to support that in the context of IFN-γ-induced transcription, HDAC activity may also be required and that this may be related to RNA polymerase II recruitment to the promoters of ISGs, in a similar way to what has been observed for type I IFNs (Chang *et al*., 2004; Guo *et al*., 2006; Hacker *et al*., 2009; Klampfer *et al*., 2004; Nusinzon and Horvath, 2003; Sakamoto *et al*., 2004).

Work from our laboratory has implicated ATM in IFN-γ signalling and a considerable amount of evidence suggest that it may modulate the accessibility of the transcription machinery to ISGs promoters, including of STAT1, the primary transcription factor activated by this cytokine. Several proteins, recognised to functionally interact with both ATM and STAT1, such as KAP1, HDACs and DDR effectors, may be involved in ATM modulation of the IFN-γ-induced JAK/STAT1-mediated biological responses **(Fig. 1.6)**.

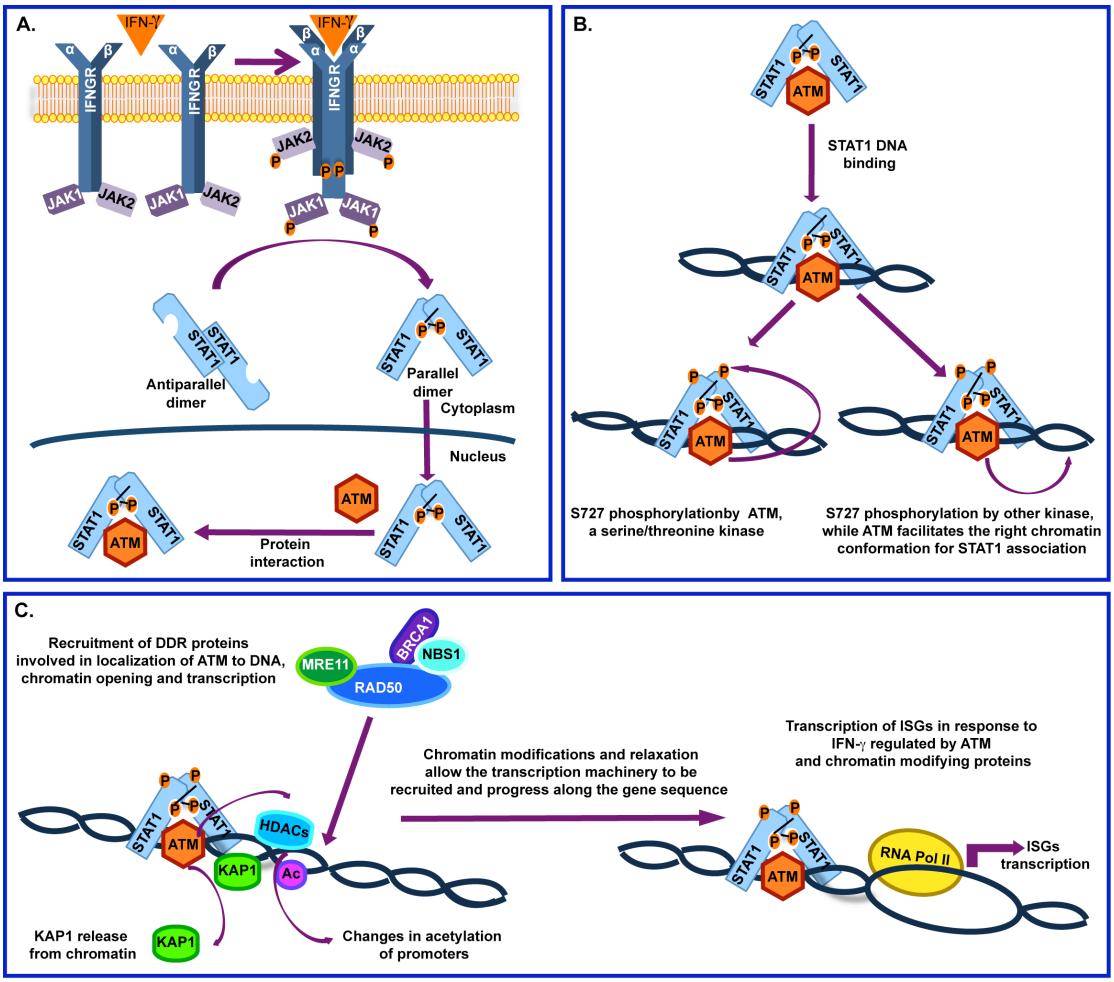


Figure 1.6. Molecular mechanism(s) by which ATM may regulate the transcription of a subset of γ-ISGs. **(A.)** Binding of IFN-γ to the IFNGR determines JAK activation and JAK-mediated STAT1 phosphorylation on tyrosine residue 701. Translocation of the parallel STAT1 tyrosine-phosphorylated dimer into the nucleus may allow its interaction with ATM. **(B.)** This interaction with ATM may be necessary for STAT1 to be able to stably bind DNA, permitting the subsequent serine 727 phosphorylation of STAT1 either by ATM or another serine/threonine kinase. **(C.)** In addition to facilitating STAT1 DNA binding, ATM may interact and use several DDR proteins and chromatin-modifying proteins, such as KAP1 and HDACs, as downstream effectors in order to regulate transcription of a specific subset of ISGs, through chromatin relaxation and recruitment of the transcriptional machinery.

CHAPTER 2

Hypothesis and objectives

Data from Dr Costa-Pereira's laboratory has established an important role for ATM as a modulator of the IFN-γ signalling (Watling *et al*., 2008). The fact that two very distinct responses were severely inhibited, namely the induction of Class II HLA molecules and of an antiviral state, suggests a prominent unrecognised function of ATM in JAK/STAT signalling and warrants a detailed analysis. Moreover, the different degree of impairment caused by ATM silencing on HLA class I expression compared to the one of HLA class II, known to be differently regulated at the transcriptional level, indicates a certain specificity regarding ATM modulation of these two IFN-induced responses. In addition, ATM has been found to control chromatin remodelling during DNA repair by regulation of chromatin-modifying proteins (Goodarzi *et al*., 2008).

Against this background we have hypothesised that ATM plays a key role in IFN-γ-mediated responses, and that it may do so at the transcriptional level, by regulating STAT1 chromatin binding and/or chromatin-modifying proteins.

Accordingly, the main objectives of this project were to:

- Establish the requirement for ATM in IFN-γ-mediated responses by using siRNA-mediated depletion of ATM and chemical inhibition of ATM enzymatic activity;
- Determine the involvement of transcription-induced DSBs in the activation/ recruitment of ATM following IFN-γ treatment;
- Investigate which ISGs are ATM-dependent for IFN-γ-induced transcription and the existence of differences regarding STAT1 binding to the promoters of ATM-dependent and -independent ISGs;
- Determine if STAT1 and ATM interact, and, during the course of this work, we sought to also investigate STAT1 and KAP1 interactions;
- Investigate the role of additional DNA repair molecules, as well as of chromatin modifying proteins, such as KAP1 and HDACs in IFN-γ-mediated signalling.

CHAPTER 3

Materials and Methods

3.1. Materials

3.1.1. Reagents

Ammonium persulphate (APS), bovine serum albumin (BSA), Dulbecco's modified eagle medium (DMEM), 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA) and EDTA 0.02% (w/v), ethylene glycol tetraacetic acid (EGTA), etoposide, formaldehyde 37% (w/v), glycine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), hygromycin B, magnesium chloride ($MgCl_2$), merbarone, 2-β-mercaptoethanol, neomycin (G418), Nonidet P-40 (NP-40), N,N,N,N-tetrametiletilenodiamine (TEMED), para-formaldehyde (PFA), phenol:chloroform:isoamylalcohol (25:24:1), phenylmethylsulfonyl fluoride (PMSF), proteinase K, trypsin 10X solution [2.5% (w/v)], Triton X-100, saponin, sodium acetate (CH_3COONa), sodium azide (NaN_3), sodium chloride (NaCl), sodium deoxycholate ($C_{24}H_{39}O_4Na$), sodium orthovanadate (Na_3VO_4) were all purchased from Sigma-Aldrich (Steinheim, Germany). Foetal calf serum (FCS) was from FirstLink (Birmingham, UK). Agarose, L-glutamine (200 mM), penicillin (5000 units/ml) and streptomycin 5000 μg/ml, iBlot® Gel Transfer Stacks Nitrocellulose, lipofectamine® RNAiMAX reagent, salmon sperm DNA, sodium fluoride (NaF), SYBR® safe DNA gel stain, ProLong® Gold antifade reagent with DAPI and To-PRO®-3-iodide were purchased from Invitrogen (Oregon, USA). High capacity cDNA reverse transcription kit and the Power SYBR® Green PCR master mix were purchased from Applied Biosystems (Warrington, UK). Aprotinin, leupeptin and KU-55933 were purchased from Calbiochem (Darmstadt, Germany). Autoradiography film was purchased from Kodak (Colorado, USA). Protein quantification assay and bromophenol blue were from Bio-Rad (Munich, Germany). Polyvinylidene difluoride (PVDF), nitrocellulose membranes and Immobilon™ western chemiluminescence horseradish peroxidase (HRP) substrate were purchased from Millipore (Bedford, USA). Milk was from Fluka BioChemika (Buchs, Switzerland). Enhanced chemiluminescence (ECL™) western blotting detection reagents and protein A sepharose beads were acquired from GE Healthcare (Buckinghamshire, UK). Protein G sepharose beads for fast flow were obtained from Pharmacia Biotech AB (Uppsala, Sweden).

QIAshredder™ columns, RNeasy® Mini kit and Allstars siRNA negative control were purchased from QIAGEN (Hilden, Germany). Ethanol, methanol, potassium chloride (KCl) and tris(hydroxymethyl)aminomethane (Tris-base) were purchased from BDH Chemicals (Poole, UK). Complete mini EDTA-free protease inhibitor cocktail tablets and glycogen were obtained from Roche diagnostics (Indianapolis, USA). Phosphate buffered saline (PBS) tablets were from Oxoid limited (Hampshire, England) and Phase Lock Gel Heavy tubes were from 5 Prime (Hamburg, Germany). Acrylamide solution [30% (v/v)] was obtained from National Diagnostics (Atlanta, USA). Glycerol, hydrochloric acid (HCl), potassium hydroxide (KOH) and sodium dodecyl sulphate (SDS) were acquired from Fisher Scientific (Loughborough, UK). Page Ruler™ plus prestained protein molecular weight markers, Dharmacon small interfering RNA (siRNA) oligonucleotides (both pools and individual oligonucleotides) against ATM, ATR, STAT1, STAT3, CIITA, KAP1, HDAC1, HDAC2, HDAC3, MRE11, NBS1, RAD50, p53, BRCA1 and H2AX, non-specific control siluciferase and transfection reagent DharmaFECT® II were obtained from ABgene (Surrey, UK). IFN-γ was a generous gift of G. Adolf (Ernst-Boehringer Institut für Arzneimittelforschung, Vienna, Austria) to Dr. Ian M. Kerr.

Table 1. Targeting sequences for each individual Dharmacon siRNA oligonucleotide. (Continued on pages 79 and 80).

Target	Name and accession number	siRNA oligonucleotides target sense sequences
ATM MU-003201-04-0010 NM_138292	D-003201-27 D-003201-28 D-003201-29 D-003201-30	27.GCAAAGCCCUAGUAACAUA 28.GGGCAUUACGGGUGUUGAA 29.UCGCUUAGCAGGAGGUGUA 30.UGAUGAAGAGAGACGGAAU
ATR MU-003202-05-0002 NM_001184	D-003202-05 D-003202-17 D-003202-31 D-003202-32	5.GAACAACACUGCUGGUUUG 17.GCAACUCGCCUAACAGAUA 31.UCUCAGAAGUCAACCGAUU 32.GAAUUGUGUUGCAGAGCUU
BRCA1 MU-003461-01-0002 NM_007294	D-003461-05 D-003461-06 D-003461-07 D-003461-08	5.CAGCUACCCUUCCAUCAUAUU 6.GGGAUACCAUGCAACAUAAUU 7.GAAGGAGCUUUCAUCAUUCUU 8.CUAGAAAUCUGUUGCUAUGUU

Table 1. Targeting sequences for each individual Dharmacon siRNA oligonucleotide. (Continued on page 80).

CIITA MU-011083-01-0002 NM_000246	D-011083-01	1.ACAAGGAGCUCCCGCAGUA
	D-011083-02	2.AGGCUAAGCUUGUACAAUA
	D-011083-03	3.CAGAUGAAGUGAUCGGUGA
	D-011083-04	4.GGCGAUGGCCAAAGGCUUA
HDAC1 MU-003493-02-0002 NM_004964	D-003493-01	1.CUAAUGAGCUUCCAUACAA
	D-003493-02	2.GAAAGUCUGUUACUACUAC
	D-003493-04	4.GGACAUCGCUGUGAAUUGG
	D-003493-09	9.CCGGUCAUGUCCAAAGUAA
HDAC2 MU-003495-02-0002 NM_001527	D-003495-01	1.CCAAUGAGUUGCCAUAUAA
	D-003495-04	4.GCAAAGAAAGCUAGAAUUG
	D-003495-05	5.CGGUAUCAUUCCAUAAAUA
	D-003495-18	18.CCAUAAAGCCACUGCCGAA
HDAC3 MU-003496-02-0002 NM_003883	D-003496-01	1.GGAAAGCGAUGUGGAGAUU
	D-003496-02	2.AAAGCGAUGUGGAGAUUUA
	D-003496-03	3.GCAUUGAUGACCAGAGUUA
	D-003496-04	4.GGAAUGCGUUGAAUAUGUC
TRIM28 (KAP1) MU-005046-01-0002 NM_005762	D-005046-03	3.GACCAAACCUGUGCUUAUG
	D-005046-04	4.GAUGAUCCCUACUCAAGUG
	D-005046-05	5.GCGAUCUGGUUAUGUGCAA
	D-005046-06	6.AGAAUUAUUUCAUGCGUGA
MRE11A MU-009271-00-0002 NM_005590	D-009271-01	1.GAUGAGAACUCUUGGUUUAUU
	D-009271-02	2.GAAAGGCUCUAUCGAAUGUUU
	D-009271-03	3.GCUAAUGACUCUGAUGAUAUU
	D-009271-04	4.GAGUAUAGAUUUAGCAGAAUU
NBS1 MU-009641-01-0002 NM_002485	D-009641-02	1.GGAGGAAGAUGUCAAUGUUUU
	D-009641-03	2.GAAGAAACGUGAACUCAAGUU
	D-009641-04	3.GAAAUGGAUUCAGUCAAUAUU
	D-009641-05	5.GUACGUUGUUGGAAGGAAAUU
RAD50 MU-005232-01-0002 NM_005732	D-005232-01	1.GAAACAAACUGCAGAAUGUUU
	D-005232-02	2.GAACAAGGAUCUGGAUAUUUU
	D-005232-03	3.GCUCAGAGAUUGUGAAAUGUU
	D-005232-05	5.UAACCUCACUGUUGGGAUAUU
STAT1 MU-003543-01-0002 NM_007315	D-003543-01	1.AGAAAGAGCUUGACAGUAAUU
	D-003543-03	3.UAAAGGAACUGGAUAUAUCUU
	D-003543-04	4.GAGCUUCACUCCCUUAGUUUU
	D-003543-05	5.GAACCUGACUUCCAUGCGGUU

Table 1. Targeting sequences for each individual Dharmacon siRNA oligonucleotide. (Continued from page 79).

STAT3 MU-003544-01-0002 NM_003150	D-003544-01 D-003544-02 D-003544-05 D-003543-06	1.CCAACGACCUGCAGCAAUAUU 2.GGAGAAGCAUCGUGAGUGAUU 5.GAGCAGAUGUGGGAAUGUU 6.GGAAACAACCAGUCAGUGAUU
TP53 MU-003329-01-0002 NM_000546	D-003329-05 D-003329-06 D-003329-07 D-003329-08	5.GAGGUUGGCUCUGACUGUAUU 6.CAGUCUACCUCCCGCCAUAUU 7.GCACAGAGGAAGAGAAUCUUU 8.GAAGAAACCACUGGAUGGAUU

3.1.2. Antibodies

Antibodies used in this work are listed in table 2, including their applications and conditions of use. Primary antibodies against ATM (2C1), CIITA (7-1H), IRF-1 (C-20), Lamin B (C-20), polymerase II (N-20), STAT1 p84/p91 (E-23) X, STAT1α p91 (C111), TIF1β (TRI5I093) (also known as TRIM28 and KAP1) and a mouse immunoglobulin G (IgG) control were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). ATM antibody (Ab-3) was purchased from Calbiochem (Darmstadt, Germany). Antibodies against phosphorylated ATM (pATM-S1981) (10H11.E12), phosphorylated p53 (pp53-S15) (16G8), serine 727 phosphorylated STAT1 (pSTAT1-S727), tyrosine 701 phosphorylated STAT1 (pSTAT1-Y701) (58D6) and phosphorylated TIF1β (p-TIF1β-S824) were purchased from Cell Signalling Technology (Hitchin, UK). Primary antibody against STAT1α p91 (NCL-STAT1A) was obtained from Novocastra (Newcastle-upon-Tyne, UK). STAT1α (C-terminal) (KV) antibody was a kind gift from Professor Pavel Kovarik (Max F. Perutz Laboratories, University of Vienna, Austria). STAT1 (N-terminal) antibodies were acquired from BD Transduction Laboratories (Erembodegem, Belgium). Primary antibody used against ATR was bought from Novus Biologicals (Littleton, UK). Primary antibodies against β-actin and α-tubulin and the secondary anti-goat-HRP antibody were acquired from Sigma-Aldrich (Steinhelm, Germany). Primary antibody against heat shock protein (HSP)-90 was obtained from Thermo Fischer Scientific (Cheshire, UK). Primary antibody against p53 was from Bethyl Laboratories (Montgomery, USA). Phycoerithrin (PE)-conjugated anti-Class II HLA

DRα antibody was from BD Pharmingen (Erembodegem, Belgium). Fluorescein isothiocyanate (FITC)-conjugated anti-Class I HLA-A, B, C was from Serotec (Oxford, UK). Secondary antibodies Alexa Fluor® 488 donkey anti-mouse and Alexa Fluor® 555 donkey anti-rabbit were purchased from Invitrogen (Oregon, USA). Secondary polyclonal goat anti-mouse and polyclonal swine anti-rabbit IgGs coupled to HRP and the secondary rabbit-FITC antibody were bought from DAKO (Glostrup, Denmark). Secondary rabbit IgG control ChIP grade was from Abcam (Cambridge, USA).

Table 2. Information regarding the animal source, applications, provider and catalogue numbers of the antibodies used in this work and the molecular weight of the proteins being detected. [goat - Gt, mouse - Ms, rabbit - Rb, Chromatin immunoprecipitation - ChIP, Co-Immunoprecipitation - Co-IP, Flow cytometry - FC, Immunofluorescence - IF, Western blotting - WB, BL - Bethyl Laboratories, BDP - BD Pharmigen, BDTL - BD Transduction Laboratories, CBC - Calbiochem, CS - Cell Signalling, NV - Novus Biologicals, NC - Novocastra, SCB - Santa Cruz Biothecnologoies, TFS - Thermo Fischer Scientific, MW - molecular weight (kDa)]. (Continued on page 82).

Antibody	Host	Applications (dilutions)	Provider	Catalogue numbers	MW
β-Actin	Ms	WB (1:1000)	Sigma	A2228	42
Phospho-ATM (Ser1981) (10H11.E12)	Ms	WB (1:1000)	CS	cs-4526	350
ATM (2C1)	Ms	WB (1:1000), IF (1:50), Co-IP (5 μg per IP)	SCB	sc-23921	350
ATM (Ab-3)	Rb	ChIP (5 μg per IP)	CBC	PC116	350
ATR	Rb	WB (1:1000)	NB	NB100-323	250
CIITA (7-1H)	Rb	WB (1:1000)	SCB	sc-13556	135
Heat Shock Protein 90a/hsp90a (HSP86)	Rb	WB (1:1000)	TFS	RB-119-P0	90
HLA ABC (Class I)	Ms	FC (1:20)	Serotec	MCA81F	46
HLA-DRα (Class II)	Ms	IF (1:10)	BDP	555812	34
IRF-1 (C-20)	Rb	WB (1:1000)	SCB	sc-497	48
Lamin B (C-20)	Gt	WB (1:1000)	SCB	sc-6216	67
Phospho-p53 (Ser15) (16G8)	Ms	WB (1:1000)	CS	cs-9286	53

Table 2. Information regarding the animal source, applications, and provider of the antibodies used in this work and the molecular weight of the proteins being detected. [goat - Gt, mouse - Ms, rabbit - Rb, Chromatin immunoprecipitation - ChIP, Co-Immunoprecipitation - Co-IP, Flow cytometry - FC, Immunofluorescence - IF, Western blotting - WB, BL - Bethyl Laboratories, BDP - BD Pharmigen, BDTL - BD Transduction Laboratories, CBC - Calbiochem, CS - Cell Signalling, NV - Novus Biologicals, NC - Novocastra, SCB - Santa Cruz Biothecnologoies, TFS - Thermo Fischer Scientific, MW - molecular weight (kDa)]. (Continued from page 81).

p53	Rb	WB (1:1000)	BL	A300-247A	53
RNA polymerase II (N-20)	Rb	WB (1:1000), Co-IP (5 μg per IP)	SCB	sc-899	240
Phospho-Stat1 (Ser727)	Rb	WB (1:1000), FC (1:50)	CS	cs-9177	91
Phospho-Stat1α/β (Tyr701) (58D6)	Rb	WB (1:1000), IF (1:50), ChIP (1:100), FC (1:50)	CS	cs-9167	91, 84
Stat1α/β (N-terminus)	Rb	WB (1:1000), IF (1:50)	BDTL	610120	91, 84
Stat1α/β (N-terminus)	Ms	WB (1:1000), IF (1:50)	BDTL	610116	91, 84
Stat1α (NCL-STAT1A)	Ms	Co-IP (5 μg per IP)	NC	NCL-STAT1A	91
Stat1 p84/91 (E-23) X	Ms	ChIP (5 μg per IP)	SCB	sc-246 X	91, 84
STAT1α (C-terminus)	Ms	ChIP (3 μl per IP)	KV	- - - - - - -	91
Stat1α p91 (C-111)	Ms	Co-IP (5 μg per IP)	SCB	sc-417	91
Phospho-TIF1β (Ser824)(pKAP1-S824)	Rb	WB (1:1000)	CS	cs-4127	100
TIF1β (TRI5I093) (KAP1)	Ms	WB (1:1000), Co-IP (5 μg per IP)	SCB	sc-81411	100
α-Tubulin	Ms	WB (1:1000)	Sigma	T5168	55

3.2. Methods

3.2.1. Cell lines and culture procedures

In the present work the human osteosarcoma U2OS cell line transfected with an ecotropic receptor (U2OS/EcoR) has been used. This cell line responds in the same way to the cytokines IFN-α, IFN-β and IFN-γ as the parental U2OS cell line and, for simplicity, will be hereinafter referred to as U2OS cells. These cells were kindly provided by Dr J. Downward (Cancer Research UK, London Research Institute, London, UK). The original U2OS cells were obtained from a human mesenquimal tumour, a moderately differentiated sarcoma of the tibia of a 15-year old Caucasian female (Ponten and Saksela, 1967). The transformed osteoblasts, adherent with an epithelial morphology, express, for instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) receptors (Lisignoli *et al*., 1998) and various cytokines including IFN-γ, upon stimulation (Nelissen *et al*., 2000). This cell line was grown in a humidified atmosphere of 10% CO_2 at 37 °C. The medium used for culturing the cells, DMEM, was supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin.

ATM matched cell lines, GM16666 (ATM negative, ATM^-) and GM16667 (ATM positive, ATM^+) were also analysed. These ATM^+ and ATM^- cell lines were a kind gift of Dr. Ana Gamero (Fels Institute for Cancer Research and Molecular Biology, Temple University, USA). The A-T cell line (AT22IJE-T), obtained by immortalization of skin primary fibroblast cells of a female with A-T (Ziv *et al*., 1997; Ziv *et al*., 1989), has the ATM gene mutated (Gilad *et al*., 1996), which results in a truncated unstable (Lakin *et al*., 1996) and undetectable form of the protein (Ziv *et al*., 1997). The ATM^+ and ATM^- cell lines were generated by transfection of the AT22IJE-T cell line with either a construct expressing ATM or the corresponding empty vector and they ectopically express ATM as a functional protein (majority of which is locates in the nucleus similar to normal cells) or maintain the A-T phenotype, respectively (Ziv *et al*., 1997). The ATM^+ and ATM^- cell lines were cultured as U2OS cells and additionally maintained under selection

with 100 µg/ml of hygromycin B. All cell lines were typically passaged twice a week, at a 1 in 10 dilution and were periodically tested for mycoplasma.

3.2.2. Protein extraction

Whole cell protein extracts were prepared using Schindler's or Laemli lysis buffer. The culture medium was aspirated and cells were washed twice with cold PBS. Protein lysates obtained with Schindler's buffer [50 mM Tris pH 8, 150 mM NaCl, 0.5% (v/v) NP-40, 0.1 mM EDTA pH 8, 10% (v/v) glycerol, 0.1 mM Na_3VO_4, 50 mM NaF, 2 mM DTT, 100 U/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF] (Schindler *et al*., 1992) were kept on ice for 20 minutes and then cleared by centrifugation at 16.2 x*g* for 15 minutes at 4 °C using a refrigerated microcentrifuge (Heraeus Fresco H from Thermo Scientific, Rockford, USA). After lysis with Laemli buffer [62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 50 mM DTT], extracts were sonicated for a few pulses to break chromatin and reduce viscosity of the samples, using a Bioruptor sonicator (Diagenode, Philadelphia, USA).

3.2.3. Cytoplasmic and nuclear protein fractionation

Protein fractionation of the cytoplasmic and nuclear compartments was also performed. Briefly, U2OS cells or ATM^+ and ATM^- cells were plated (5 x 10^6 cells in a 10 cm dish) and the following day were first stimulated for a period of time with IFN-γ (500 IU/ml) and then washed twice with ice-cold PBS. Cells were then resuspended in cytosolic fractionation buffer [10 mM HEPES pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 5% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM NaF, 0.1 mM Na_3VO_4, 1 µg/ml leupeptin, 10 µg/ml aprotinin and 1 mM PMSF] and 'swelled' on ice for a maximum of 10 minutes. The crude cytosolic fraction was separated from the nuclear pellet by centrifugation at 0.6 x*g* for 5 minutes at 4 °C. The supernatant corresponding to the cytosolic fraction was further pre-cleared by centrifugation at 16.2 x*g* for 10 minutes at 4 °C. The nuclear pellet was resuspended and washed by centrifugation at 0.6 x*g* for 5 minutes at 4 °C in fractionation washing buffer (10 mM HEPES pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 1

mM DTT, 2 mM NaF, 0.1 mM Na_3VO_4, 1 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF). Following this step, the nuclear pellet was resuspended in nuclear fractionation extraction buffer [20 mM HEPES pH 7.6, 1.5 mM $MgCl_2$, 420 mM NaCl, 1 mM DTT, 25% (v/v) glycerol, 2 mM NaF, 0.2 mM EDTA, 0.1 mM Na_3VO_4]. The nuclear fraction was obtained upon sonication for 30 minutes (30 seconds pulses at 30 seconds interval at high power) in ice-cold water, using a Bioruptor sonicator. Pre-clearance of this fraction was done by centrifugation for 10 minutes at 17 x*g* at 4 °C.

3.2.4. Protein quantification by the Bradford method

Protein quantification of whole cell extracts prepared with Schindler's lysis buffer or obtained by cytoplasmic and nuclear fractionation was performed by spectrophotometry using either a spectrophotometer DU® series 500 (Beckman Instruments, Fullerton, USA) or a plate reader Sunrise-Basic (Tecan, Grodig, Austria). BioRad protein assay, based on the colorimetric method of Bradford for quantification of solubilized proteins, uses the Coomassie Brilliant Blue G-250 dye in acidic conditions and was performed according to the manufacturer's instructions. Upon protein binding there is an absorbance shift of the dye from 465 to 595 nm, which allows for protein concentration to be calculated by interpolating the absorbance values of the samples read at 595 nm in a BSA standard curve.

3.2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Denaturation of whole cell extracts, as well as of cytoplasmic and nuclear extracts, obtained from U2OS or matched ATM^+ and ATM^- cells, was performed by boiling the samples for 5 minutes in Laemli buffer at 99 °C, prior to separation of the proteins according to their molecular weight on a SDS-PAGE gel. A 5% acrylamide stacking gel was routinely used [5% (w/v) acrylamide, 0.125 M Tris, pH 6.8, 0.1% (w/v) SDS, 0.06% (w/v) APS, 0.07% (v/v) TEMED)], while the percentage of the acrylamide resolving gel varied depending on the molecular weight of the proteins being analysed [6, 7.5, 10 or 12.5% (w/v) acrylamide, 0.375

M Tris, pH 8.8, 0.1% (w/v) SDS, 0.075% (w/v) APS, 0.08% (v/v) TEMED]. Gels were run using a Tris-glycine running buffer [2.5 mM Tris, 0.2 M glycine and 0.1% SDS (w/v)]. A wet transfer system was invariably used to transfer proteins separated on 6% gels onto a methanol-activated PVDF or nitrocellulose membrane. A Tris-glycine buffer was used [2.5 mM Tris, 0.2 mM glycine, 10% methanol (v/v)] at 4 °C with a fixed voltage of 25 volts for 1 hour for the majority of proteins or 1 hour and 30 minutes for high molecular weight proteins, such as ATM, on an electroblotter (Idea Scientific, Minneapolis, USA). For higher percentages of acrylamide, proteins were transferred using the iBlot® dry blotting system (Invitrogen, Oregon, USA). Blocking of the membranes was done in Tris-buffered saline (TBS)/Tween (TBST) [0.01 M Tris, pH 7.4, 75 mM NaCl, 1.25 mM EDTA, pH 8.0, 0.1% (v/v) Tween 20] with 5% (w/v) BSA and 0.02% NaN_3, or with 5% (w/v) milk if detecting pATM-S1981. Incubation with primary antibodies diluted in blocking buffer was done for 1 hour at room temperature or at 4 °C, followed by incubation with the appropriate secondary antibody for 1 hour at room temperature. Binding detection was performed with ECL or Millipore chemiluminescence reagents. Probing of the membranes was done first for the phosphorylated forms of the proteins, followed by probing of the total proteins and finally detection of proteins used as loading controls. For this purpose, HSP90 and β-actin were used or in the case of cytoplasmic and nuclear fractionation extracts α-tubulin and lamin B were used instead. Stripping of the membranes in between each probing was done in a solution of 2 M of glycine, pH 2.5 with 0.1% (w/v) of SDS.

Image J was used to compare the density of each protein band. For that purpose, gray-scale-converted TIFF images of the western blots were used. A similar area was quantified for each band corresponding to either the phosphorylated, or phosphorylated and unphosphorylated forms of the protein, and the background subtracted from each densitometric value.

Densitometric values of loading controls corresponding to each condition (untransfected or siRNA-transfected) were normalised by dividing them by either the value of the corresponding untreated (whole cell or cytoplasmic lysates) or 60 minutes IFN-γ-treated samples (nuclear lysates). The densitometric values for all

other phosphorylated or total proteins (for example, pSTAT1-S727) were divided by the corresponding normalised loading control values. These were then divided by the value of the corresponding untreated (whole cell or cytoplasmic lysates) or 60 minutes IFN-γ-treated (nuclear lysates) samples.

3.2.6. Protein co-immunoprecipitation (Co-IP)

U2OS cells were plated in 15 cm dishes (10 x 10^6 cells) and the following day stimulated with IFN-γ (500 IU/ml) for variable periods of time. Plates were placed on ice, medium was aspirated and cells washed twice with ice-cold PBS. A volume of 1 ml of cold lysis buffer [50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA pH 8, 0.5% (v/v) Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF] was added and cells were scraped of the plate. Protein lysates were transferred to 1.5 ml cold microcentrifuge tube and incubated on ice for 10 minutes. Samples were centrifuged at 13.8 x*g* for 10 minutes at 4 °C. Supernatants were collected and protein concentration was measured using the Bradford method as previously described. Co-immunoprecipitation samples were set up in new microcentrifuge tubes at 1 mg of total protein and final volume was adjusted to 1 ml with lysis buffer. Samples were pre-cleared by rotation for 1 hour at 4 °C with 35 μl of washed protein G beads (50% slurry), centrifuged for 1 minute at 0.4 x*g* and transferred into clean microcentrifuge tubes. For immunoprecipitation of ATM, 1 μg of ATM (2C1) antibody was added to each sample, while for immunoprecipitation of STAT1, 5 μg were added of either STAT1α p91 (NCL-STAT1A) antibody or STAT1 α p91 (C111) antibodies. For KAP1 Co-IP, 5 μg of TIF1β (TRISI093) antibody was used. A control 'beads-only' sample was also set up, together with a non-specific control using a mouse IgG at the same concentration as the specific antibody. Samples were left rotating with the antibodies overnight at 4 °C. Precipitation of the antibody-bound protein was achieved by incubating the samples with 35 μl of washed protein G beads rotating for 2 hours at 4 °C. Samples were centrifuged for 1 minute at 0.4 x*g*, the supernatant aspirated and the beads were washed twice with 500 μl of lysis buffer. Immunoprecipates were diluted with 2X Laemli buffer in a 1:1 ratio, heated at 99 °C for 10 minutes together

with a whole cell extract sample. Samples were separated on a 6% resolving gel, followed by wet transfer and western blotting as previously described.

3.2.7. RNA interference (RNAi)

Depending on the experiment, U2OS cells were plated in 96-well plates (2 x 10^4 cells per well), 6-well plates (2 x 10^5 cells per well), 10 cm dishes (1 x 10^6 cells per dish) or 15 cm dishes (2.5 x 10^6 cells). Briefly, the day after being plated, cells were transfected with 20, 30 or 75 nM of a particular siRNA pool using DharmaFECT® or the transfection reagent Lipofectamine® RNAiMAX. In all experiments, non-specific, non-targeting siRNAs, such as siluciferase and 'Allstars' were also included. Cells were treated 48 hours after transfection with IFN-γ for variable periods of time, as required.

3.2.8. Flow cytometric detection of cell surface antigens and intracellular proteins

3.2.8.1. Cell surface antigens

HLA Class I and II expression was assessed by flow cytometry as a read-out for how different conditions affected IFN-γ-induced biological responses. Depending on the experiment, U2OS were plated on 96- or 6-well plates (2 x 10^4 or 1 x 10^5 cells per well, respectively), while ATM^+ and ATM^- cells were plated on 6-well plates (1 x 10^5 cells per well) and HLA Class I and II expression assessed after a period of 24, 48 and/or 72 hours upon stimulation with IFN-γ. Succinctly, upon stimulation, the procedure included washing the cells with EDTA solution [0.02% (w/v)] and detachment from the plates with a solution of 0.25% (w/v) of trypsin diluted in a 0.02% (w/v) EDTA, followed by inactivation of this enzyme with FCS-supplemented DMEM. Cells were then washed in ice-cold PBS and incubated with a PE-conjugated anti-HLA-DRα Class II or a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody for 45 minutes at 4 °C in the dark. After three washes with ice-cold PBS, cells were finally fixed in 1% (v/v) PFA in PBS and analysed on a flow

cytometer FACSCanto (Becton, Dickinson and Company, Erembodegem Belgium).

3.2.8.2. Intracellular proteins

Forty-eight hours after transfection with RNAi, U2OS cells were treated with IFN-γ for 10 minutes and intracellular detection of STAT1 phosphorylated in serine 727 and tyrosine 701 residues was performed as described. Cells were washed twice with EDTA [0.02% (w/v)] and detached with 0.25% (w/v) of trypsin in 0.02% (w/v) EDTA. Cells were fixed for 10 minutes at 37 °C in 4% (w/v) PFA/PBS solution and then chilled on ice for 1 minute. After removal of the fixative by centrifugation (Sorvall Legend RT centrifuge) (178 x*g*), cells were resuspended an incubated in 90% (v/v) cold methanol in cold PBS for 30 minutes at 4 °C. Cells were subsequently washed three times with cold PBS. The unconjugated primary antibodies directed against pSTAT1-S727 or pSTAT1-Y701 were incubated for 1 hour at room temperature, after which cells were washed 3 times with incubation buffer [0.5% (w/v) BSA solution in PBS]. Incubation with the secondary rabbit-FITC antibody was done in the dark for 30 minutes at room temperature, followed by three washes in incubation buffer. Samples were resuspended in PBS and analysed on a flow cytometer FACSCanto. Data mining and analysis were done using FlowJo (Tree Star Inc., Oregon, USA).

3.2.9. RNA extraction and cDNA preparation

Cells were plated on 6-well plates (2 x 10^5 cells per well) and treated with IFN-γ as required (25 and 500 IU/mL). Isolation of cytoplasmic RNA was performed using the RNeasy kit (Quiagen) as directed by the manufacturer. In brief, subsequent to stimulation, medium was aspirated and cells disrupted by adding buffer RLT supplemented with 2-β-mercaptoethanol. Lysates were transferred into Qiashredder spin columns coupled to collection tubes and centrifuged at 16.1 x*g* for 2 minutes. Lysates were mixed with 70% ethanol in a 1:1 proportion and centrifuged at 9.3 x *g* for 15 seconds through RNeasy spin columns. These columns were treated with buffer RW1 and centrifuged at 9.3 x*g* for 15 seconds.

RPE buffer was then added twice and centrifugation carried at 9.3 x*g*, first for 15 seconds and then for 2 minutes, to ensure that no ethanol would be carried over during RNA elution. Elution was done with 50 μl of diethylpyrocarbonate (DEPC)-treated water and by centrifuging for 1 minute at 9.3 x*g*. All steps of the RNA extraction procedure were done at room temperature. Concentration of RNA samples was measured using a NanoDrop ND1000 spectrophotometer from Labtech International (East Sussex, UK). Reverse transcription of template RNA into first strand cDNA was carried out as recommended by the manufacturer using the high capacity cDNA reverse transcription kit. Briefly, 1 μg of RNA was reverse transcribed in a 20 μl reaction containing 1X RT buffer, 1X dNTP mixture, 1X RT random primers, 1 μl of RNnase inhibitor and 1 μl of Multiscribe™ reverse transcriptase. Thermocycler conditions consisted of an initial incubation step at 25 °C for 10 minutes, followed by reverse transcription at 37 °C for 30 minutes, after which enzyme inactivation was done at 85 °C for 5 minutes. Expression of several different ISGs was analysed using the cDNA samples.

3.2.10. Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription of RNA into cDNA and amplification by PCR were carried out sequentially in the same tube by using a one-step RT-PCR kit. The template RNA (15 ng) was reverse transcribed and cDNA amplified in a 50 μl reaction volume containing 1x RT-PCR buffer, 1x Q-Solution, 400 μM of each dNTP, 0.6 μM of each primer for CIITA and 1.2 μM of each primer for GAPDH and 2 μl of RT-PCR enzyme mix. The RT-PCR amplification conditions were set up according to the manufacturer's guidelines, which included 30 minutes at 50 ºC for the reverse transcription step, followed by 15 minutes at 95 ºC to activate the HotStarTaq polymerase, inactivate the reverse transcriptases and denature the cDNA template. Forty cycles of 45 seconds at 94 ºC, 1 minute at optimum annealing temperature (determined as 52 ºC for ATM primers and 60 ºC for IRF-1, GBP-1 and CIITA pIV) and 1 minute at 72 ºC were performed, with a final extension at 72 ºC for 10 minutes. RT-PCR products together with loading dye [5% (v/v) glycerol, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol) were run on a 2% (w/v) agarose gel containing SYBR green safe dye and the products were checked

based on their size. An extra reaction without RNA template (negative control) was carried out for each RT-PCR mix in order to detect potential contaminations.

Table 3. List of forward (FWR) and reverse (RVS) sequences and final concentrations of primers used in RT-PCR reactions.

Gene	Orientation	Target Sequence (5'3')	[nM]
ATM	FWR	AGGCCAAATGATTTCAGTGC	600
	RVS	ACCTTCAACACCCGTAATGC	600
CIITA pIV	FWR	GCTATGATACTGGCCCCATC	400
	RVS	CCTTAAGCCCTCCCTACACC	400
GBP1	FWR	TGAGAAATCTTTAAACCCTCCC	400
	RVS	TGGCTTCTAGCACTTCTGTGTC	400
IRF-1	FWR	CTTCGCCGCTAGCTCTACAACAG	400
	RVS	GCTCCGGGTGGCCTCGGTTCG	400

3.2.11. Chromatin immunoprecipitation (ChIP)

U2OS cells were plated in 15 cm dishes (2.5 x 10^6 cells), the following day transfected with RNAiMAx and 20 nM of RNAi against ATM or a non-targeting control and 48 hours after transfection cells were stimulated with IFN-γ (500 IU/ml). Upon stimulation for variable periods of time, ChIP was performed. In brief, proteins were crosslinked to DNA with formaldehyde [1% (w/v) final concentration) for 10 minutes at room temperature and with agitation on an orbital shaker. The crosslinking process was finished by quenching excess formaldehyde through incubation with glycine (125 mM final concentration) for 5 minutes at room temperature with agitation on an orbital shaker. Cells were washed twice with cold PBS on ice and lysed with ChIP lysis buffer [0.05 M HEPES, pH 7.4, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, 140 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) NP-40, 0.25% (v/v) Triton-X100], supplemented with 1 mM PMSF and 1/100 of complete mini EDTA-free protease inhibitor cocktail solution (1 tablet per 1 ml of distilled water). Cells were transferred into 15 ml tubes and lysis was carried on for 10 minutes on turning wheel at 4 °C. Supernatant was removed after centrifuging for 5 minutes at 138 x*g* at 4 °C (Sorvall Legend RT centrifuge). Cells were washed on a wheel for 10 minutes at 4 °C with ChIP wash buffer [0.01 M Tris, pH 8.0, 1 mM EDTA, pH

8.0, 0.5 mM EGTA, 200 mM NaCl], supplemented with 1mM PMSF and 1/100 of complete mini EDTA-free protease inhibitor cocktail solution. Washing buffer was removed by centrifuging for 5 minutes at 138 x*g* at 4 °C. After an additional centrifugation for 2 minutes at 138 x*g* at 4 °C to ensure removal of all ChIP washing buffer, cells were resuspended in 1 ml cold Radioimmunoprecipitation assay (RIPA) buffer [0.01 M Tris, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, 140 mM NaCl, 5% (v/v) glycerol, 0.1% (m/v) SDS, 1% (v/v) Triton X-100], complemented with 1 mM PMSF and 1/100 of complete mini EDTA-free protease inhibitor cocktail solution. Cells were transferred into cold 2 ml microcentrifuge tubes and sonication performed for 25 minutes. Samples were sonicated in ice-cold water (Bioruptor sonicator, Diagenode, Philadelphia, USA) and using pulses of 30 seconds at 30 seconds interval at high power. An aliquot of 50 μl was taken as input from the supernatants obtained by centrifugation at 16.2 x*g* at 4 °C.

Pre-clearing of lysates was performed with 50 μl of protein A sepharose beads, for 1 hour on a wheel at 4 °C. Beads were previously prepared by incubating overnight at 4 °C in a wheel with a blocking solution [2 μl of salmon sperm DNA, 10 μl of 10% (w/v) of BSA, 5 μl of 5% (w/v) NaN_3 and 87 μl of 1X TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0)], in a proportion of 104 μl of this solution to each 100 μl of beads. After the pre-clearing step, samples were centrifuged for 3 minutes at 4 °C at 0.4 x*g* and the supernatant equally divided into two 1.5 ml microcentrifuge tubes and incubated overnight at 4 °C on a wheel with either a specific antibody against the protein to be immunoprecipitated or with an IgG-matched isotype control. A volume of 3 μl of STAT1 antibody given by Professor Pavel Kovarik, 5 μg of STAT1 p84/p91 (E-23) X, 5 μl of pSTAT1-Y701 and 5 μg of ATM (Ab-3) were used. DNA/protein immunocomplexes were recovered through binding to 40 μl of protein A sepharose beads, for 2 hours on a wheel at 4 °C, after which supernatant was discarded by centrifugation for 5 minutes at 0.4 x*g* at 4 °C. Beads-bound DNA/protein immunocomplexes where then washed five times with 1 ml of RIPA buffer, by centrifuging for 5 minutes at 0.4 x*g* at 4 °C.

Crosslinks between DNA and proteins were reverted by incubating the beads and the inputs with proteinase K (0.2 μg/μl) in 200 μl or 150 μl of proteinase K buffer

[50 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 100 mM NaCl, 0.5% (w/v) SDS], respectively. This was performed with shaking for 2 hours at 55 °C on a heating block (Thermomixer Compact, Eppendorf, UK), followed by incubation of the cleared supernatant at 65 °C overnight. After reverting the crosslinks, samples were transferred into phase lock gel heavy tubes, 250 μl of phenol:chloroform:isoamylalcohol (25:24:1) added and the contents mixed by inverting the tubes. After 1 minute centrifugation at 13.8 x*g*, supernatants were transferred into clean microcentrifuge tubes and sodium-acetate precipitation of DNA carried out by adding 1 ml of 96% (v/v) of ethanol, 25 μl of 3 M sodium acetate and 1.5 μl of glycogen at 10 mg/ml, followed by incubation at -80 °C for 50 minutes. After centrifugation for 45 minutes at 4 °C at 16.2 x*g*, the supernatant was discarded, 1 ml of 70% (v/v) ethanol added and centrifuged at 16.2 x*g* for 5 minutes at 4 °C. Supernatant was removed, DNA pellets dried out and resuspended with 50 μl of DEPC-treated water.

3.2.12. SYBR green-based real time quantitative-PCR (qPCR)

Both cDNA samples obtained by reverse transcription of RNA and genomic DNA samples ChIP assays were analysed by qPCR. In brief, cDNA samples were diluted 1:10 and a cDNA standard was prepared by pooling equal amounts of each diluted sample. Four sequential 1:4 dilutions were made from this cDNA standard to obtain a standard curve (1, 1/4, 1/16, 1/64, 1/256). This standard curve served the purpose of checking primers efficiency, if the samples were in the linear range of amplification and to obtain quantification values.

Regarding genomic DNA samples from ChIP assays, antibody immunoprecipitated and IgG control samples were diluted 1:2.5, while inputs were diluted 1:10, and used to prepare a DNA standard, which was used in serial 1:4 dilutions to obtain a standard curve (1, 1/4, 1/16, 1/64, 1/256), as described for cDNA samples. A volume of 5 μl of cDNA and DNA samples was used as template in a RT-qPCR reaction of 25 μl total volume, containing 12.5 μl of a 2X Power SYBR Green master mix, 2.5 μl of each forward and reverse primers and 2.5 μl of water. Amplification was carried in a 7900HT Fast Real-Time PCR system (Applied

Biosystems, Warrington, UK), with an initial activation step at 95 °C for 10 minutes and 40 cycles consisting of 15 seconds at 95 °C, followed by 1 minute at 60 °C. All reactions were carried out in triplicates and a non-template control (blank) was included. The house keeping gene hypoxanthine phosphoribosyl-transferase (HPRT) was used as a reference gene to normalise gene expression of cDNA samples and data analysis was performed to determine the mRNA levels of several ISGs using the qbasePLUS 2.1 Academic Premium software (Biogazelle, Zwijnaarde, Belgium).

For ChIP genomic DNA samples, relative quantity values were extrapolated for the Ct values from the standard curve and presented as occupancy (percentage of input) of promoter, or as fold change in promoter binding. Primers were optimised by testing different combinations of the forward and reverse sequences at final concentrations of 50, 300 and 900 nM. The combination of primers that yielded the lowest Ct value for the amplification of standard DNA or cDNA, with less variation among triplicates was chosen. In addition, a melting curve analysis of the products was performed to ensure the quality of the amplification. Sequences and final concentration of forward and reverse primers are listed in **table 4**. All primers were purchased from Invitrogen (Oregon, USA).

Table 4. List of forward (FWR) and reverse (RVS) sequences and final concentrations of primers used in qPCR reactions. (Continued on page 95).

	Gene	Orientation	Target Sequence (5'3')	[nM]
Genomic DNA from ChIP assays	CIITA pIV	FWR	TCACGGTTGGACTGAGTTGG	900
		RVS	CCTGAGTTGCAGGGAGCTTG	300
	HLA-DRα	FWR	TGACCACGCTGGTACCTAAA	900
		RVS	GGAGAGGGCTCTCAAGATCA	300
	Invariant chain	FWR	GTATTTCCAGCCTTTGTAGCTTTCA	900
		RVS	CTGGAGAGGAATCTGATTCGTCC	300
	IRF-1	FWR	CCAGGGCTGGGGAATCC	900
		RVS	TCGGGCGCACGTCTTGC	300
	TAP1	FWR	AACTGGTGCAAGTGGAAAGG	300
		RVS	GCGAGAAGCTCAGCCATTTA	900

Table 4. List of forward (FWR) and reverse (RVS) sequences and final concentrations of primers used in qPCR reactions. (Continued from page 94).

	Gene	Orientation	Target Sequence (5'3')	[nM]
cDNA from RNA samples	CIITA pIV	FWR	TCACGGTTGGACTGAGTTGG	900
		RVS	CCTGAGTTGCAGGGAGCTTG	300
	CIS	FWR	AGCCCAGACAGAGAGTGAGC	900
		RVS	TGACAGCGTGAACAGGTAGC	900
	HLA-DRα	FWR	AGACCACCTTTTCCGCAAGT	900
		RVS	ATAATGATGCCCACCAGACC	300
	HPRT	FWR	TGACACTGGCAAAACAATGCA	900
		RVS	GGTCCTTTTCACCAGCAAGCT	900
	IP10	FWR	GAGCCTCAGCAGAGGAACC	300
		RVS	GAGTCAGAAAGATAAGGCAGC	900
	IP30	FWR	ATCCTCAATGTCACGCTGGT	900
		RVS	TGGCAGACTTCTCTCCATGTC	300
	Invariant chain	FWR	GACCTTATCTCCAACAATGAGCAA	900
		RVS	CAGCAGAGTCACCAGGATGGAA	900
	IFR1	FWR	CAAATCCCGGGGCTCATCTGG	900
		RVS	CTGGCTCCTTTTCCCCTGCTTTGT	300
	PRAME	FWR	CCATGACAAAGAAGCGAAAA	900
		RVS	CATCTGGCCCAGGTAAGGAG	300
	SOCS1	FWR	TGGTAGCACACAACCAGGTG	900
		RVS	GAGGAGGAAGAGGAGGAAGG	900
	SOCS3	FWR	GCCACCTACTGAACCCTCCT	900
		RVS	ACGGTCTTCCGACAGAGATG	900
	TAP1	FWR	CGGAAACCGTGTGTACTTATCC	FWR
		RVS	TCAGGGCTTTCGTACAGGAG	RVS

3.2.13. Immunofluorescence (IF)

3.2.13.1. ATM and STAT1 co-staining

U2OS cells were plated (12.5 x 10^4 cells per well) on top of glass coverslips on 24-well plates. The following day medium was aspirated and cells stimulated with IFN-γ (500 IU/ml), after which medium was removed and cells washed once with PBS. Cells were fixed with 4% PFA for 30 minutes at room temperature. Cells

were washed three times with ice-cold PBS and permeabilized with 100% methanol at -20 °C for 5 minutes, after which cells were washed three times with PBS. All following steps were performed at room temperature. Blocking was done with 2% FCS in PBS for 30 minutes at room temperature, followed by the co-staining of ATM (ATM 2C1) with total STAT1 (STAT1α/β N-terminus). All primary and secondary antibodies were diluted in blocking buffer and incubated for 1 hour at room temperature. Cells were first incubated with primary antibody ATM (2C1) (1:50). Coverslips were washed three times with blocking buffer and incubated with an Alexa fluor® 555 donkey anti-mouse secondary antibody (1:500). Coverslips were washed 3 times with blocking buffer, incubated with a primary antibody against STAT1 (1:50), followed by three washes with blocking buffer and incubation with an Alexa fluor® 488 donkey anti-rabbit secondary antibody (1:500). Coverslips were washed three times with blocking buffer and stained with DAPI (1 μg/ml) and To-PRO®-3-iodide (1:100) diluted in blocking buffer for 15 minutes. Coverslips were washed twice with blocking buffer and one time with PBS, after which were mounted onto glass slides with ProLong® Gold antifade reagent, left overnight and sealed with nail-polish the following day. The immunostaining was visualised and images acquired using a Zeiss LSM 510 Meta Confocal microscopy (Carl Zeiss International, USA).

3.2.13.2. γ-H2AX foci staining

U2OS cells were plated on top of glass coverslips on 24-well plates (12.5 x 10^4 cells per well) or in Optilux™ 96-well plates (BD Bioscience) (2 x 10^4 cells per well). The following day the DMEM medium was aspirated and cells stimulated with either IFN-γ (500 IU/ml) (10, 60 and 240 minutes for the glass coverslips or 1, 5, 10, 30, 60, 240 minutes for the 96-well plates) or with etoposide (100 μM, for 240 minutes) to serve as a positive control for γ-H2AX foci formation. Medium was removed and cells washed once with 1X PBS. The entire procedure was done at room temperature. Cells were fixed with 4% PFA for 30 minutes. Cells were washed three times with PBS and permeabilized with 0.1% of Triton X-100 in PBS for 10 minutes, after which cells were washed three times with PBS. Blocking was done using with a 2% FCS solution in PBS for 30 minutes. Staining was performed

using a pH2AX-S139 uncoupled primary antibody, diluted in blocking buffer (1:50) for 1 hour. Cells were washed three times with blocking buffer and then incubated for 1 hour with an Alexa fluor® 488 donkey anti-rabbit secondary antibody (1:500). After washing the cells three times with blocking buffer, cells plated on coverslips were counterstained with DAPI (1 μg/ml) and To-PRO®-3-iodide (1:100) and the cells nuclei in 96-well plates were counterstained with DAPI (1 μg/ml) diluted in blocking buffer, for 15 minutes. Cells were washed two times with blocking buffer and one time with PBS and the incubated with PFA 4% for 10 minutes, after which their were washed again three times with PBS. Coverslips were mounted onto glass slides with ProLong® Gold antifade reagent, left overnight and sealed with nail polish the following day. The immunostaining was visualised and images acquired using a Zeiss LSM 510 Meta Confocal microscopy (Carl Zeiss International, USA). Cells plated in 96-well plates were left in PBS at 4 °C until the moment of image acquisition. Forty-two fields of view were acquired per condition using the high-throughput microscope ImageXpress Micro (Molecular Devices Inc) and then analysed using the analytical pluggins available in the software, Metamorph (Molecular Devices Inc) for the number and area per cell of the γ-H2AX foci.

3.2.14. Statistical analysis

Mean and standard deviation (SD) and/or standard error of the mean (SEM) of either three independent experiments or duplicates/triplicates of one representative experiment were calculated. A two-tail Student's t-test was performed for statistical comparison of different conditions and p values superior to 0.05 were considered non-significant [non-significant (ns) - $p>0.05$], while those inferior to 0.05 were considered statistically significant [* - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$].

CHAPTER 4

Results

Requirement for ATM in early and late phase events of the IFN-γ signalling pathway: a structural or an enzymatic role?

4.1. Introduction

IFN-γ is an important cytokine that mediates several biological processes through the regulation of innate and acquired immunity, from antiviral and antibacterial protection, to inflammation and tumour immune surveillance, specifically in the elimination phase (Agnello *et al*., 2003; Dunn *et al*., 2006; Hu *et al*., 2008; Kunz *et al*., 1999; Mukai *et al*., 1999; Schroder *et al*., 2004; Seliger *et al*., 2008; Weber and Rosenberg, 1988). This cytokine's prominent role in anti-tumoural immunity involves direct anti-proliferative effects on the tumour cells, while increasing tumour immunogenicity through regulation of the mediated adaptive immune response (Bui *et al*., 2006; Gattoni *et al*., 2006; Kunz *et al*., 1999; Mukai *et al*., 1999; Nishikawa *et al*., 2003; Seliger *et al*., 2008; Tannenbaum and Hamilton, 2000; Whitmire *et al*., 2005). An example of how IFN-γ is able to increase anti-tumoural immunity is by mediating upregulation of HLA class I molecules. This leads to augmented presentation of tumour antigens by antigen presenting cells (APCs) (Seliger *et al*., 2008) to cytotoxic T lymphocytes (CTL), which can additionally be recruited to the tumour environment upon IFN-γ-induced chemokine release and, thus, consequently improve elimination of tumour cells (Kunz *et al*., 1999; Mukai *et al*., 1999).

IFN-γ biological activity is mediated through activation of the JAK/STAT signalling pathway (**Fig. 1.3**). Binding of the cytokine to the extracellular part of the IFN-γ receptor determines the formation of the IFN-γ-receptor complex (Bach *et al*., 1996). This event allows JAK1 and JAK2, which are associated with the intracellular portion of the IFN-γ receptor chains 1 and 2, respectively, to become activated. The JAKs then recruit STAT1, the primary transcription factor of the pathway, into the IFN-γ-receptor complex *via* its SH2 domain and phosphorylate STAT1 on a tyrosine on position 701 (Darnell, 1997; Pestka *et al*., 2004; Platanias, 1995; Stark *et al*., 1998). This specific phosphorylation is considered an activation marker since it is responsible for an alteration in the STAT1 dimers conformation. This conformational change allows these dimers to translocate into the nucleus and bind specific DNA consensus sites in order to activate transcription of IFN-γ-responsive genes (Wenta *et al*., 2008; Zhong *et al*., 2005).

Expression of ISGs occurs in a timely organised sequence and can be divided in primary, secondary and tertiary responses. IRF-1 is also a transcription factor and the first ISG to be transcribed upon STAT1 binding to its promoter after IFN-γ signalling. This primary response is followed by a secondary response that is dependent on *de novo* synthesis of IRF-1 protein and which comprises the transcription of genes that are dependent on IRF-1 alone or on the combined action of IRF-1 and STAT1. HLA class I expression is constitutive on every nucleated human cell, but it can be further enhanced by IFN-γ and can be used as an example of a secondary phase of IFN-γ-induced transcription. Transcription of CIITA gene also qualifies as such a secondary response, since it depends on both STAT1 and IRF-1 binding to its IFN-γ-responsive promoter IV (Muhlethaler-Mottet *et al*., 1998). The transactivator CIITA is responsible for controlling both constitutive (in professional APCs) and inducible HLA class II expression (in different non-immune cell types), through the use of different promoters of this gene (Croce *et al*., 2003; Muhlethaler-Mottet *et al*., 1997). IFN-γ-induced expression of HLA class II is mediated by the activation of CIITA promoter IV in response to this cytokine. Transcription of HLA class II and invariant chain (Ii) molecules is dependent on CIITA and, therefore, constitutes a tertiary event of transcription, which occurs much later in time upon cytokine signalling. Loading of HLA class II molecules with antigen peptides and their assembly at cell surface, a process that involves Ii, can already be considered as a quaternary event following IFN-γ-induced transcription of ISGs.

STAT1 also possesses a serine residue on position 727 at the C-terminal, located within a MAPK consensus site. Phosphorylation of this residue has been associated with increased STAT1 transcriptional activity of a subset of ISGs. ATM is known to be a serine-threonine kinase that phosphorylates its downstream targets in SQ/TQ motives (Kim *et al*., 1999; Shiloh, 2003), although the possibility of phosphorylating serine residues located in other consensus sequences cannot be excluded. The sequence of STAT1 includes serine and threonine residues within the SQ/TQ motif recognized by ATM. Additionally, ATM has been shown to modulate phosphorylation of both STAT3 and STAT1 on serine 727 in stress-induced responses caused by ultraviolet A rays (Yang *et al*., 2003). Despite being

generally accepted that transcriptional dependence of STAT1 on serine 727 phosphorylation varies among ISGs, the genes showed to be dependent on it, and the degree of dependence on this post-translational modification, diverges between different *in vitro* and *in vivo* studies (Kovarik *et al*., 2001; Varinou *et al*., 2003). Nonetheless, variability of different ISGs' requirement for STAT1 serine 727 phosphorylation in our *in vitro* system could account for the persistent impairment of HLA class II, but not class I expression, and explain the differential effects on distinct IFN-γ-induced responses when ATM is silenced. Therefore, we investigated if ATM could regulate the phosphorylation of STAT1 on serine 727 residue and its activation in the context of cytokine signalling.

In order to establish the importance of ATM as a modulator of the IFN-γ pathway we further analysed events subsequent to STAT1 phosphorylation and activation upon ATM knockdown by RNAi. For that purpose, the expression of HLA class I and II proteins at cell surface was determined. In addition, the transcription of several different ISGs, including IRF-1 and CIITA, was analysed in order to establish if the differences observed regarding HLA class I and II expression at cell surface were the result of changes at the transcriptional level. IRF-1 and CIITA protein levels were also assessed to establish how differences in the mRNA amount would affect protein production. ATM enzymatic activity was chemically inhibited in an attempt to distinguish between the relevance of structural and kinase functions regarding the modulation of IFN-γ signalling by ATM. In addition, negative and fully matched ATM-complemented cell lines were compared regarding STAT1 phosphorylation and activation, HLA class I and II expression and ISGs mRNA production following IFN-γ stimulation.

4.2. ATM played a role in IFN-γ signalling, from STAT1 phosphorylation and activation to ISG transcription and protein expression in U2OS cells

4.2.1. ATM silencing delayed STAT1 phosphorylation and activation

In order to determine the effect of ATM knockdown in the activation status and phosphorylation kinetics of STAT1 upon IFN-γ stimulation, U2OS cells were

transfected with siRNA oligonucleotide pools against ATM. Since ATR is closely related to ATM and did not have a major phenotype in the original siRNA screen (Watling *et al*., 2008), this protein was also silenced as a control for siRNA transfection effects *per se* together with a non-specific, non-targeting, siRNA (siNS). Silencing of STAT1 was used as a positive control for the IFN-γ response as it is an essential transcription factor. Activation of the JAK/STAT signalling pathway in response to IFN-γ treatment, as well as the efficiency of protein knockdown and the siRNA pools specificity, were assessed by western blotting. Accordingly, the phosphorylation of STAT1 on tyrosine 701 and serine 727 residues was analysed during a time-course response ranging from 5 to 30 minutes of IFN-γ stimulation in whole cell extracts (**Fig. 4.1**).

IFN-γ-induced phosphorylation of STAT1 serine 727 residue could be detected after 10 minutes of treatment with the cytokine (orange arrow) and this increased after 30 minutes of stimulation in both untransfected, siNS and siATR conditions. Phosphorylation of STAT1 on this residue was diminished/delayed at the 10 minutes time point when ATM was silenced, even though it could be detected after 30 minutes of IFN-γ stimulation. Western blot analysis showed that ATM knockdown impaired STAT1 serine 727 phosphorylation induced by IFN-γ, when comparing to untransfected cells and cells transfected with siNS or siATR, but this was not attributable to a decrease in the amount of total STAT1 protein.

Curiously, phosphorylation of STAT1 tyrosine 701 residue also seemed to be decreased as a consequence of ATM knockdown by RNAi. As expected, phosphorylation was highly decreased in siSTAT1-transfected cells as a consequence of reduced STAT1 total protein levels. The efficiency of STAT1, ATM and ATR protein knockdown and the siRNA pools specificity were confirmed (**Fig. 4.1**) validating the observations attributed to specific protein silencing.

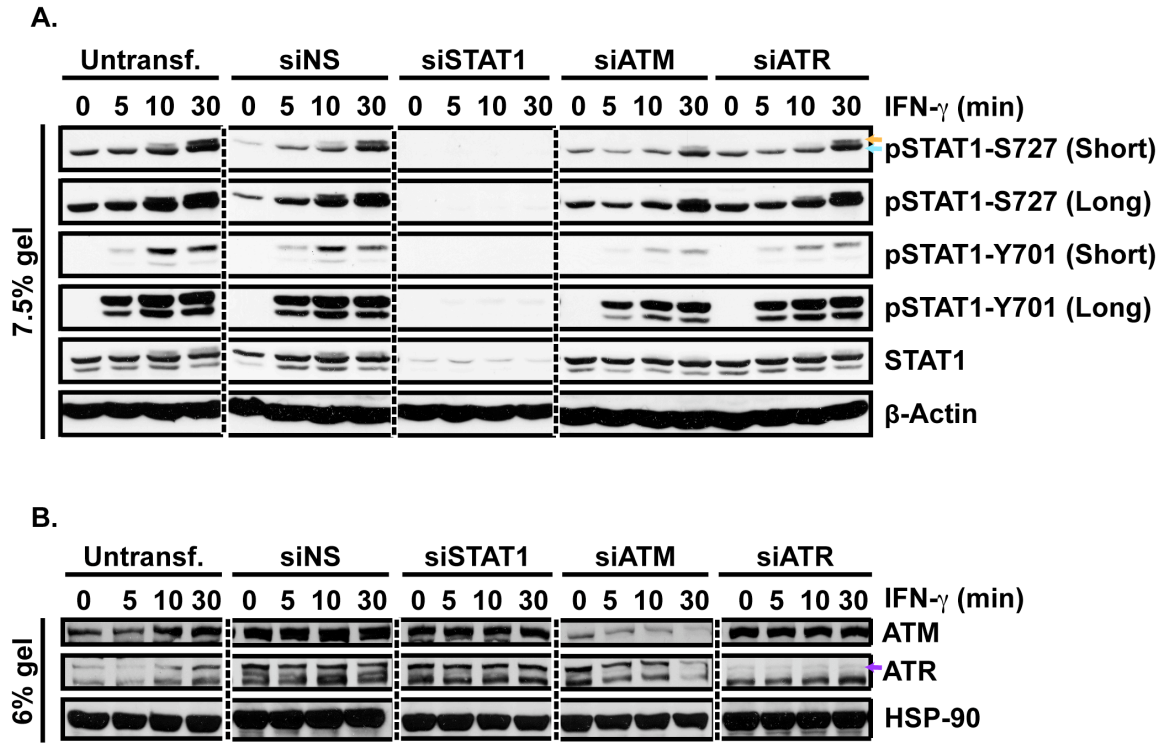


Figure 4.1. ATM knockdown by RNAi delayed STAT1 serine 727 phosphorylation in response to IFN-γ. U2OS cells were transfected with 20 nM of the indicated siRNAs, using RNAiMAX or left untransfected. After forty-eight hours cells were stimulated with IFN-γ (500 IU/ml) for the time points shown in the figure (min - minutes). Total cell lysates were prepared using Schindler's buffer. **A.** Proteins were resolved using 7.5% SDS-PAGE gels and western blotting analysis was performed using antibodies against pSTAT1-S727 and pSTAT1-Y701 to assess phosphorylation levels in response to IFN-γ (long - long exposure, short - short exposure). An antibody against total STAT1α/β was used to determine the total amount of this protein and efficiency of its knockdown by RNAi. β-actin was used as a loading control. The orange arrow indicates IFN-γ-induced STAT1 serine 727 phosphorylation, while the blue arrow shows basal phosphorylation detected with the same antibody. These results are representative of three independent experiments. **B.** Proteins were additionally separated on a 6% SDS-PAGE gel and efficiency of ATM and ATR (purple band) knockdown by RNAi was confirmed by western blotting analysis, using antibodies against these proteins. HSP-90 was used as a loading control.

4.2.2. ATM knockdown led to significantly decreased levels of activated STAT1 in the nucleus

As mentioned previously, both STAT1 tyrosine 701 phosphorylation and nuclear translocation are indispensable for IFN-γ-induced STAT1 serine 727 phosphorylation to occur (Kovarik *et al*., 2001). In order to become serine 727

phosphorylated and achieve maximum transcriptional activity in response to IFNs, this transcription factor needs to be assembled into chromatin-associated transcriptional complexes (Sadzak *et al*., 2008). The delay observed in serine residue phosphorylation when ATM was silenced could be the result, therefore, of the DNA being less accessible to tyrosine 701-phosphorylated STAT1. In an attempt to clarify the mechanism by which ATM regulated STAT1 serine 727 phosphorylation, both phosphorylation and distribution of this transcription factor were assessed in the cytoplasm and in the nucleus upon IFN-γ stimulation following knockdown of ATM and other appropriate control molecules, namely ATR and STAT1 (**Fig. 4.2**).

STAT1 serine 727 phosphorylation was detected in the nucleus after 10 minutes of stimulation with IFN-γ and also in the cytoplasm (upper lighter band indicated by the orange arrow) of untransfected and siATR-transfected cells (**Fig. 4.2.A**), which would be consistent with nuclear phosphorylation followed by shuttling of STAT1 to the cytoplasm. ATM knockdown impaired serine 727 phosphorylation at this time point, but did not block it, being visible, albeit at lower levels, for longer periods of stimulation of 20, 30 and 60 minutes in both cytoplasmic and nuclear compartments.

A decrease in STAT1 tyrosine 701 residue phosphorylation in the nucleus was also observed for the 10 minutes time point of cytokine stimulation, but no clear increase in its cytoplasmic level upon silencing of ATM in comparison to untransfected and siATR conditions was seen.

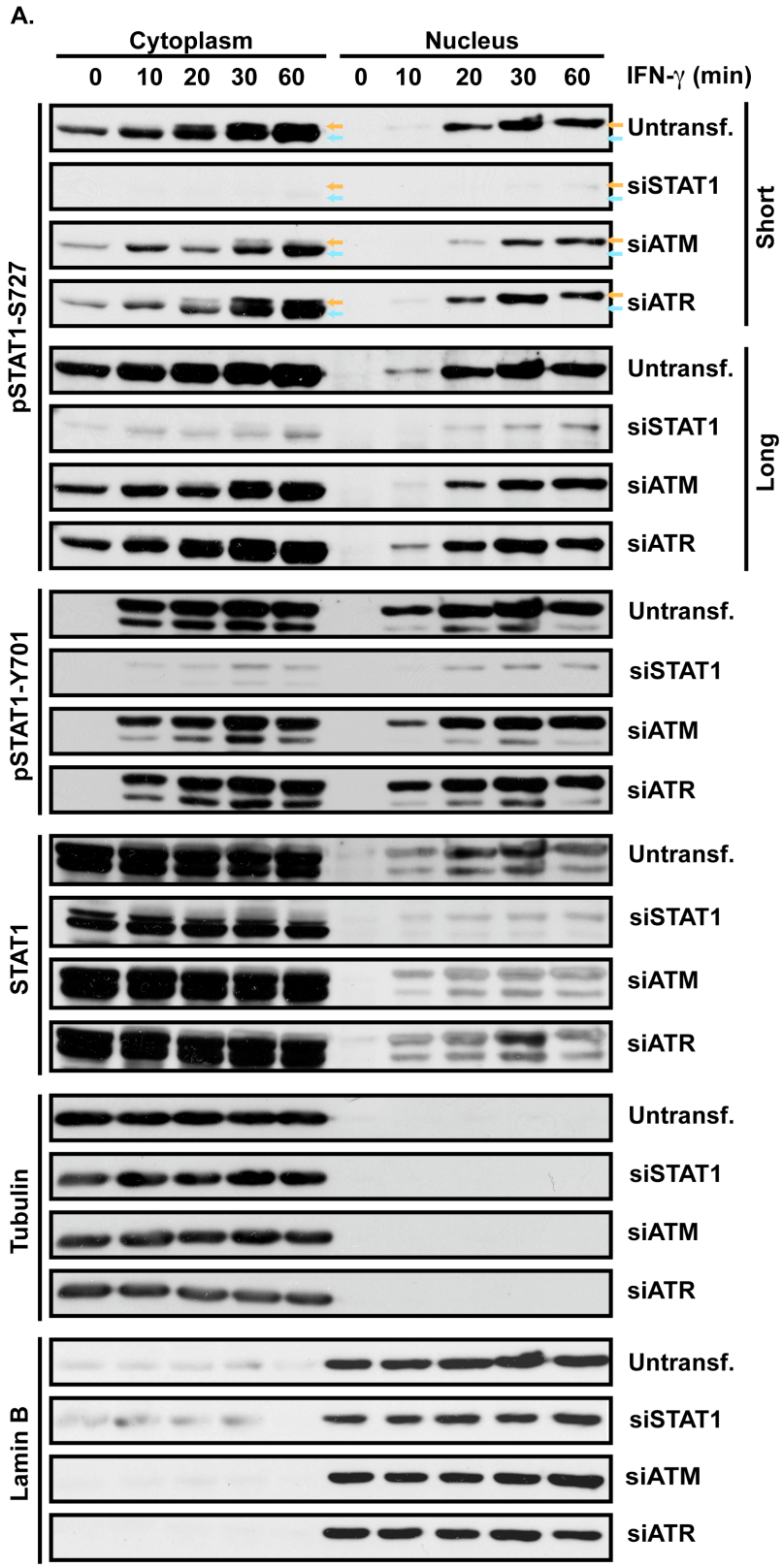


Figure 4.2. ATM knockdown by RNAi significantly decreased/delayed STAT1 serine 727 phosphorylation in the nucleus after stimulation with IFN-γ for up to 30 minutes, while this effect was only significant in the cytoplasm after this time point. (Continued on pages 106 to 108).

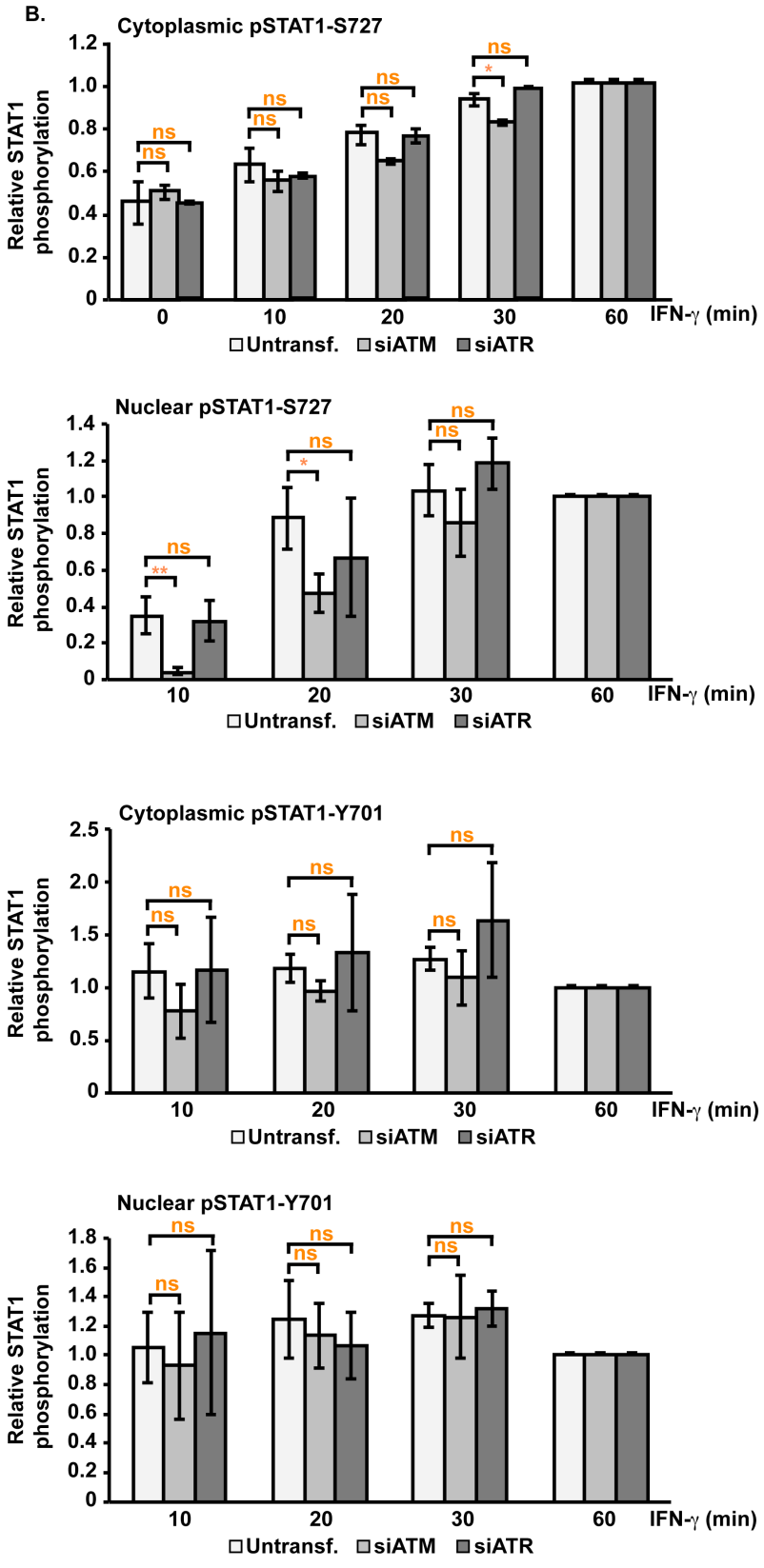


Figure 4.2. ATM knockdown by RNAi significantly decreased/delayed STAT1 serine 727 phosphorylation in the nucleus after stimulation with IFN-γ for up to 30 minutes, while this effect was only significant in the cytoplasm after this time point. (Continued on pages 107 and 108).

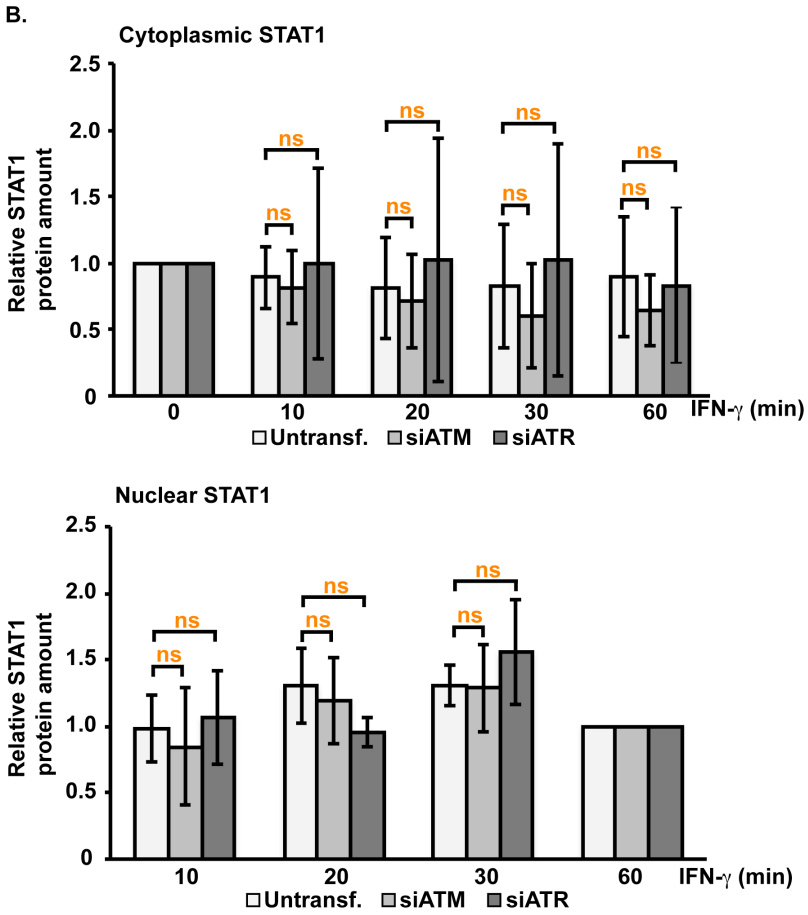


Figure 4.2. ATM knockdown by RNAi significantly decreased/delayed STAT1 serine 727 phosphorylation in the nucleus after stimulation with IFN-γ for up to 30 minutes, while this effect was only significant in the cytoplasm after this time point. U2OS cells were transfected with 75 nM of the indicated targeting siRNAs or left untransfected. Cells were stimulated 48 hours after transfection with IFN-γ (1000 IU/ml) for the time points shown (minutes - min). Cytoplasmic and nuclear proteins were fractionated. **A.** Cytoplasmic and nuclear fractions were separated using a 7.5% SDS-PAGE gel and proteins detected by western blotting. Membranes were probed with antibodies against pSTAT1-S727, pSTAT1-Y701 and total STAT1α/β (long - long exposure, short - short exposure). Lamin B and α-tubulin were used as nuclear or cytoplasmic loading controls, respectively. These results are representative of three independent experiments yielding identical results. **B.** Levels of STAT1 total protein, STAT1 serine 727 and tyrosine 701 phosphorylation were quantified using Image J. Mean ± SD of densitometric values from two or three experiments are expressed as fold change over the 0 or 60 minutes time point. Statistical analysis was performed using Student's t-test relative [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. **C.** Three time points (0, 10 and 60 minutes) of each condition were run in the same 7.5% resolving gel, for two of the experiments performed in (**A.**). This was done to guarantee that the effect of ATM knockdown on STAT1 serine 727 and tyrosine 701 phosphorylation in response to IFN-γ was not an artefact from SDS-PAGE and/or transfer processes. Western blotting analysis was performed as in (**A.**).

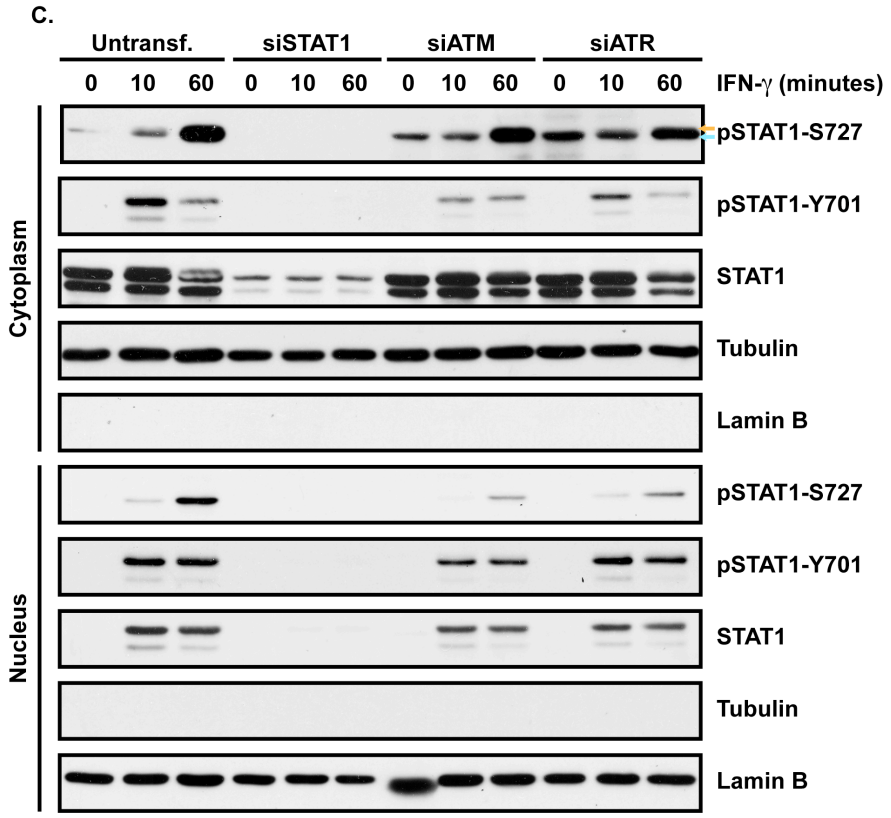


Figure 4.2. ATM knockdown by RNAi significantly decreased/delayed STAT1 serine 727 phosphorylation in the nucleus after stimulation with IFN-γ for up to 30 minutes, while this effect was only significant in the cytoplasm after this time point. (Continued from page 107).

In order to determine how significant the impairment caused by ATM silencing on STAT1 serine and tyrosine phosphorylation was, in both cellular compartments, densitometric analyses of the western blots for two or three experiments was performed using Image J software (**Fig. 4.2.B**). The extent of nuclear and cytoplasmic serine 727 and tyrosine 701 phosphorylation are expressed as mean ± SD values normalised to the 60 minutes time point of stimulation for each transfection condition. ATM silencing was found to significantly decrease IFN-γ-induced STAT1 serine 727 phosphorylation in the nuclear compartment after 10 and 20 minutes of stimulation with the cytokine ($p<0.01$ and $p<0.05$, respectively). Analysis of the western blots revealed a similar trend for STAT1 serine 727 phosphorylation in the cytoplasmic where ATM had been knocked down by RNAi (**Fig. 4.2.A**). However, only the 30 minutes time point statistically significant ($p<0.05$). siATM-induced impairment of STAT1 tyrosine phosphorylation in the nuclear compartment for the 10 minutes time point of stimulation was not

statistically significant as assessed by densitometric analysis possibly due to variation between experiments. No difference was seen between conditions regarding STAT1 tyrosine 701 phosphorylation in the cytoplasm.

STAT1 protein levels in the cytoplasmic and nuclear compartments were also quantified across three identical western blot experiments using the Image J software (**Fig. 4.2.B**). Cytoplasmic and nuclear quantifications were expressed as mean ± SD values of the STAT1 protein amount normalised to the value of the 0 minutes or 60 minutes time point of stimulation, respectively, for each condition. This was done to address if the delay in nuclear accumulation of serine 727 phosphorylated-STAT1 occurred as the result of a reduction in the total amount of STAT1 accumulated in the nuclear compartment. The quantification of the western blots did not show any significant impairment in STAT1 nuclear localization when ATM was silenced.

The cytoplasmic extracts obtained from untransfected and siSTAT1-transfected cells were slightly contaminated with nuclear proteins in the particular experiment shown here (**Fig. 4.2.A**). This contamination was small considering that the amount of STAT1 present in the cytoplasm largely exceeds that in the nucleus. Thus, this level of contamination is unlikely to interfere with the assessment of STAT1 phosphorylation events in the cytoplasm. Furthermore no contamination of the cytoplasmic extracts for siATM and in the control siATR-transfected conditions was observed. Moreover, no contamination from the cytoplasm was detected on the nuclear extracts of either untransfected or transfected cells, which legitimates our observations and conclusions.

To confirm that the differences observed regarding STAT1 phosphorylation (**Fig. 4.2.A**) were not an artefact of SDS-PAGE and transfer processes, samples corresponding to three time points (0, 10 and 60 minutes) for each condition, from two experiments, were run in the same 7.5% resolving gel (**Fig. 4.2.C**). The decrease in IFN-γ-induced STAT1 serine 727 and tyrosine 701 phosphorylation was evident in the nuclear compartment upon ATM silencing (**Fig. 4.2.C**) and confirmed that this observation was *bona fide*.

Given the limitations of the western blot technique and of western blot-based densitometry quantification, we further assessed STAT1 serine 727 and tyrosine 701 phosphorylation by intracellular staining using flow cytometry (**Fig. 4.3**). U2OS cells were transfected with siRNA targeting a non-specific sequence, ATM or ATR or left untransfected. Forty-eight hours after transfection cells were stimulated with IFN-γ for a period of 10 minutes, time point at which the impairment in STAT1 phosphorylation caused by ATM silencing seemed to be more pronounced as seen by western blot (**Fig. 4.2**).

STAT1 phosphorylation at serine 727 and tyrosine 701 was assessed in individual samples and the results analysed using FlowJo are represented as geometric mean values in the top right end corner of each graph (**Fig. 4.3.A** and **4.3.B**, respectively). The inducible effect of IFN-γ on STAT1 phosphorylation was calculated for each condition by subtracting the geometric mean of the untreated sample to the geometric mean of the treated sample. After this subtraction, normalisation was done by dividing all values by the value of the siNS non-targeting control and statistical significance of the fold change relative to the non-specific control was determined applying Student's t-test (**Fig. 4.3.C** and **4.3.D**).

Flow cytometric analysis showed an increase in IFN-γ-induced STAT1 serine phosphorylation in the untransfected and siNS or siATR transfected cells, which was above the basal phosphorylation level (**Fig. 4.3.C**). A significant impairment ($p<0.001$) was observed in STAT1 serine 727 phosphorylation upon IFN-γ treatment, which was caused by ATM silencing, as compared to the siNS control (**Fig. 4.3.C**). This impairment was particularly evident when observing the overlap between the basal and the induced STAT1 serine 727 phosphorylation graphs (**Fig. 4.3.A,** 3rd horizontal panel). STAT1 tyrosine phosphorylation was only detected after IFN-γ stimulation in all conditions, but was decreased as a consequence of ATM knockdown (**Fig. 4.3.B**). Although the level of reduction in the fold change of STAT1 tyrosine 701 phosphorylation relative to the siNS control was not as prominent as for serine 727 phosphorylation, this was still significant ($p<0.01$) (**Fig. 4.3.D**).

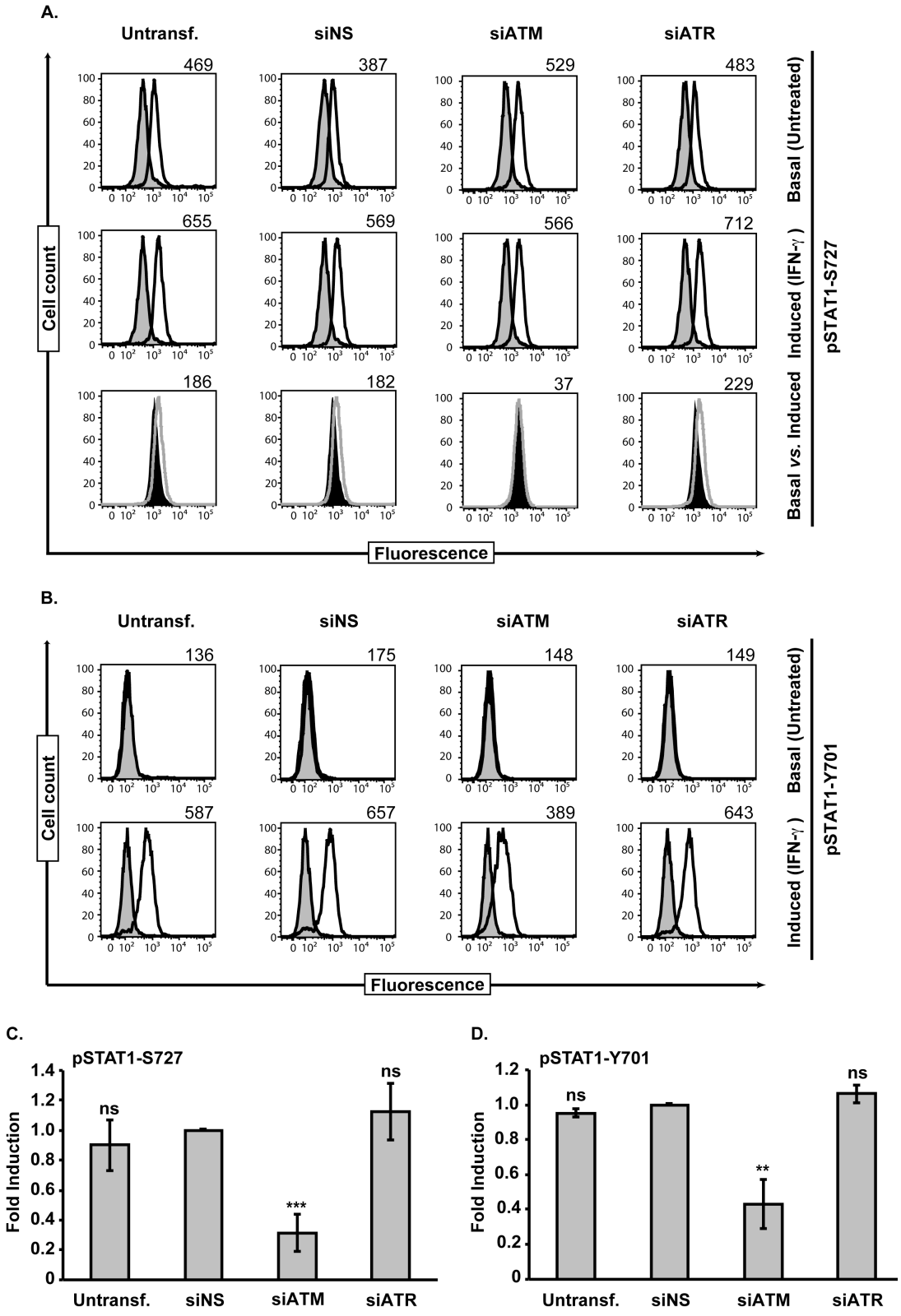


Figure 4.3. ATM knockdown by RNAi reduced both STAT1 serine 727 and tyrosine 701 phosphorylation after 10 minutes of treatment with IFN-γ. U2OS cells were left untransfected or transfected with 75 nM of targeting siRNAs against ATM or ATR or with a non-targeting sequence, using Dharmafect 2. Forty-eight hours after transfection cells were stimulated with IFN-γ

(500 IU/ml) for 10 minutes. Cells were then intracellularly stained with specific antibodies against STAT1 phosphorylated in the serine 727 or tyrosine 701 residues. Fluorescence was detected using a FACSCanto and results were analysed using FlowJo. **A.** and **B.** Results are presented as the geometric mean values of stained samples (numbers on the top right hand corner) for STAT1 serine 727 (**A.**) and tyrosine 701 (**B.**) phosphorylation. The matched IgG controls and stained samples correspond to the shadowed and transparent peaks, respectively. The black filled peak corresponds to the basal phosphorylation of STAT1 and the grey outlined transparent peak (3rd row of graphs in A.) represents the induced STAT1 serine 727 phosphorylation. One representative experiment of three independent experiments yielding similar results is shown. **C.** and **D.** For each condition (untransfected, siNS, siATM or siATR) the value of the geometric mean of the stained untreated sample was subtracted from the value of the stained IFN-γ treated sample and then normalised by dividing by the value obtained for the siNS non-targeting control. Values are expressed as fold change relative to the siNS control. Mean ± SD values obtained using three independent experiments after normalisation are shown for STAT1 serine 727 (**C.**) and tyrosine 701 (**D.**) phosphorylation. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. Untransf. - untransfected, NS - non-specific.

The impairment observed in IFN-γ-induced STAT1 serine 727 and tyrosine 701 phosphorylation caused by ATM silencing in western blot analysis (**Fig.4.2**) was corroborated by flow cytometric analysis (**Fig.4.3**). Results showed that both STAT1 serine 727 and tyrosine 701 phosphorylation were significantly reduced (**Fig.4.3.C** and **4.3.D**) and probably the effect caused by ATM knockdown on these post-translation modifications was more pronounced in the nucleus (**Fig.4.2.B** and **4.2.C**).

4.2.3. ATM knockdown resulted in a severe reduction of HLA class II but not of HLA class I expression in response to IFN-γ

Since expression of both HLA class I and II molecules can be induced by IFN-γ, these responses were frequently used as an experimental read-out to assess the importance of ATM and other molecules for adequate IFN-γ signalling. Additionally, because HLA class I and II expression is differently regulated at the transcriptional level, we thought this would provide further insight into to the mechanisms underlying ATM's function. U2OS cells were left untransfected or transfected with a non-targeting siRNA sequence, or transfected with sequences targeting ATM or

ATR. Stimulation was initiated 48 hours after transfection for a period of 24 hours after which IFN-γ induction of HLA class I and II was assessed by flow cytometry (**Fig. 4.4**).

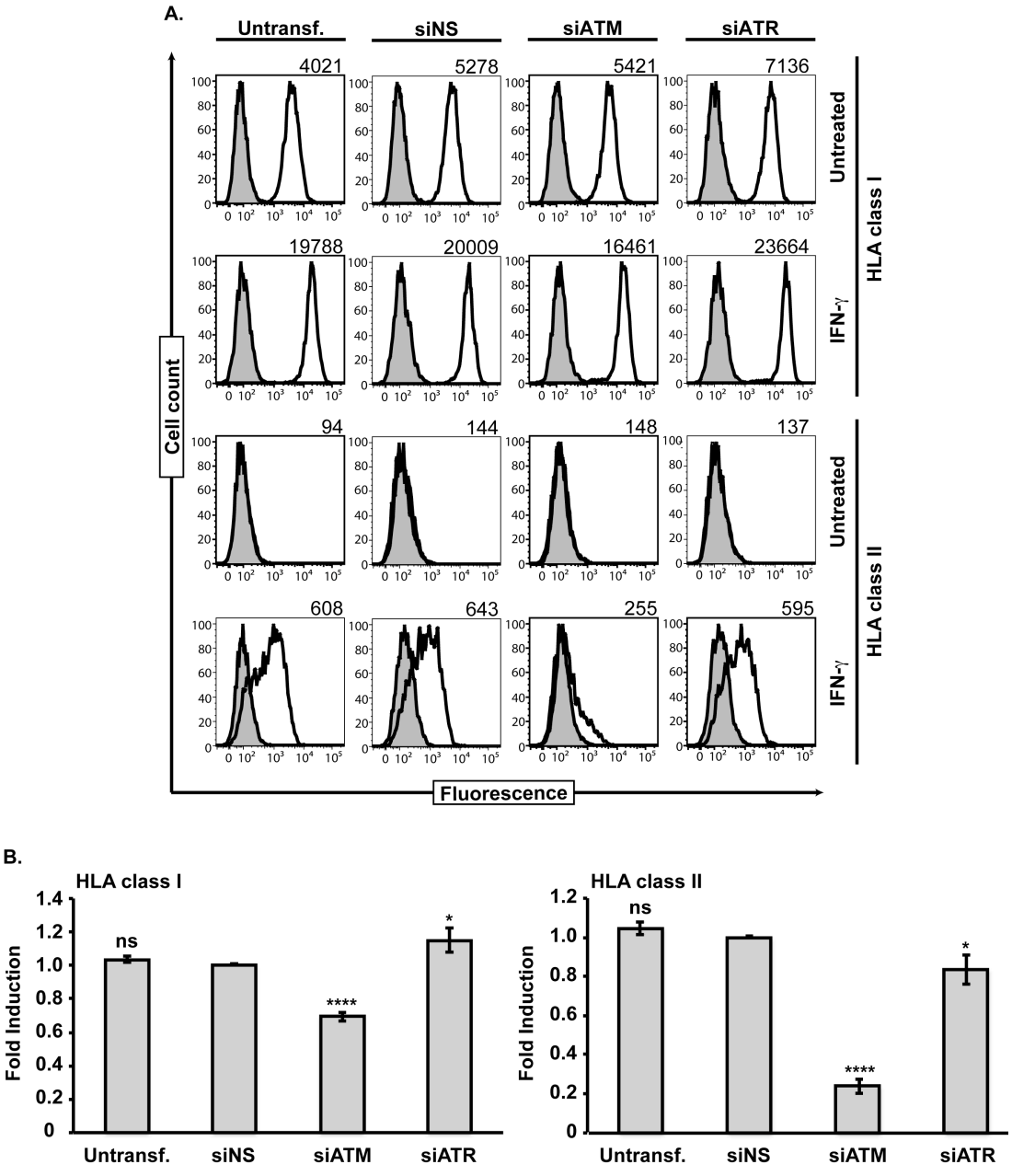


Figure 4.4. ATM knockdown by RNAi reduces cell surface expression of HLA Class I and II molecules induced after 24 hours treatment with IFN-γ. U2OS cells were left untransfected or transfected with 20 nM of targeting siRNAs against ATM or ATR or with a non-specific sequence, using RNAiMAX. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for 24 hours. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody or with a PE-conjugated anti-HLA-DRα Class II antibody and cell surface expression of these

molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-Class I (1st and 2nd horizontal panels) and HLA-DRα Class II (3rd and 4th horizontal panels) expression are presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **B.** For each condition (untransfected, siNS, siATM or siATR) the value of the geometric mean of the untreated sample was subtracted from the value of the IFN-γ treated sample and then normalised by dividing the obtained value by the siNS non-targeting control. Mean ± SD obtained using three independent experiments after normalisation are shown for HLA class I (upper graph) and class II (lower graph). Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. Untransf. - untransfected, NS - non-specific.

All siRNA transfected conditions led to a small increase in the basal expression of HLA class I and II (**Fig. 4.4.A**). Therefore, in order to calculate the inducible effect of IFN-γ, this was subtracted from the geometric mean of each treated condition and then normalised by dividing it by the value of the siNS non-targeting control. Statistical significance of the fold change relative to the non-specific control was determined using three independent experiments after normalisation (**Fig. 4.4.B**).

In agreement to what has been previously described, HLA class I was detected at the cell surface of unstimulated U2OS cells and this could be further induced by IFN-γ treatment regardless of transfections, which was reflected by the increase in the geometric mean values shown on the top right of each graph (**Fig. 4.4.A**). No significant difference was detected regarding HLA class I induction by IFN-γ when comparing the siNS control and untransfected conditions, which suggested that the transfection *per se* was without effect. Knockdown of ATM was responsible for impairing IFN-γ-inducible expression of HLA class I at 24 hours and this was statistically significant ($p<0.0001$) (**Fig. 4.4.B**). ATR knockdown led to a small, but consistently observed, significant increase ($p<0.05$) in IFN-γ-induced expression of HLA class I molecules, showing a contrary phenotype to siATM. The sensitivity of HLA class I to ATM silencing was evident 24 hours after stimulation with a saturating concentration of IFN-γ (500 IU/ml).

In these non-immune cells HLA class II molecules were not expressed at the cell surface of unstimulated U2OS cells and could only be detected after IFN-γ treatment (**Fig. 4.4.A**). Expression upon stimulation was similar in both siNS and untransfected conditions. ATM knockdown consistently resulted in a highly significant impairment of the induction of HLA class II molecules ($p<0.0001$) after 24 hours of treatment with IFN-γ (**Fig. 4.4.B**), much more severe than the effect obtained for HLA class I. Only a small reduction was observed upon ATR knockdown (**Fig. 4.4.A**), which was nevertheless significant ($p<0.05$) (**Fig. 4.4.B**).

The effect of ATM knockdown by RNAi was also assessed in U2OS cells treated with IFN-γ for a longer period of 72 hours (**Fig. 4.5**), using siNS and siATR as controls and the same experimental and analysis layout as for the 24 hours treatment (**Fig. 4.4**). HLA class I was again constitutively expressed at the cell surface of unstimulated U2OS cells and, upon cytokine treatment, expression was further enhanced for all conditions at 72 hours to a degree greater than that seen for 24 hours of IFN-γ stimulation as seen by the increased geometric mean values (**Fig. 4.5.A**). Nonetheless, contrarily to what was observed after 24 hours of IFN-γ stimulation, ATM knockdown did not impair the inducible expression of HLA class I comparatively to siNS condition (**Fig. 4.4.A** and **4.4.B**). ATR knockdown still led to a small increase in IFN-γ-induced expression of HLA class I molecules at 72 hours.

HLA class II expression was detected after IFN-γ treatment and was higher at 72 hours (**Fig.4.5.A**) than after 24 hours of cytokine treatment (**Fig.4.4.A**) as noticed by the geometric mean values. The significant impairment on IFN-γ-induced expression of HLA class II caused by ATM silencing after 24 hours of stimulation was still consistently observed after a longer 72 hours period ($p<0.0001$) of cytokine treatment (**Fig.4.5.B**). The magnitude of HLA class II reduction regarding ATM silencing was similar on both time points and without any recovery noticed by prolonged stimulation (around 0.2% fold when compared with the siNS condition). The small reduction observed in the expression of these molecules at cell surface upon 24 hours of cytokine treatment, when ATR was silenced, was thus reverted after a longer period of stimulation and no difference was seen between siATR and siNS conditions (**Fig.4.5**).

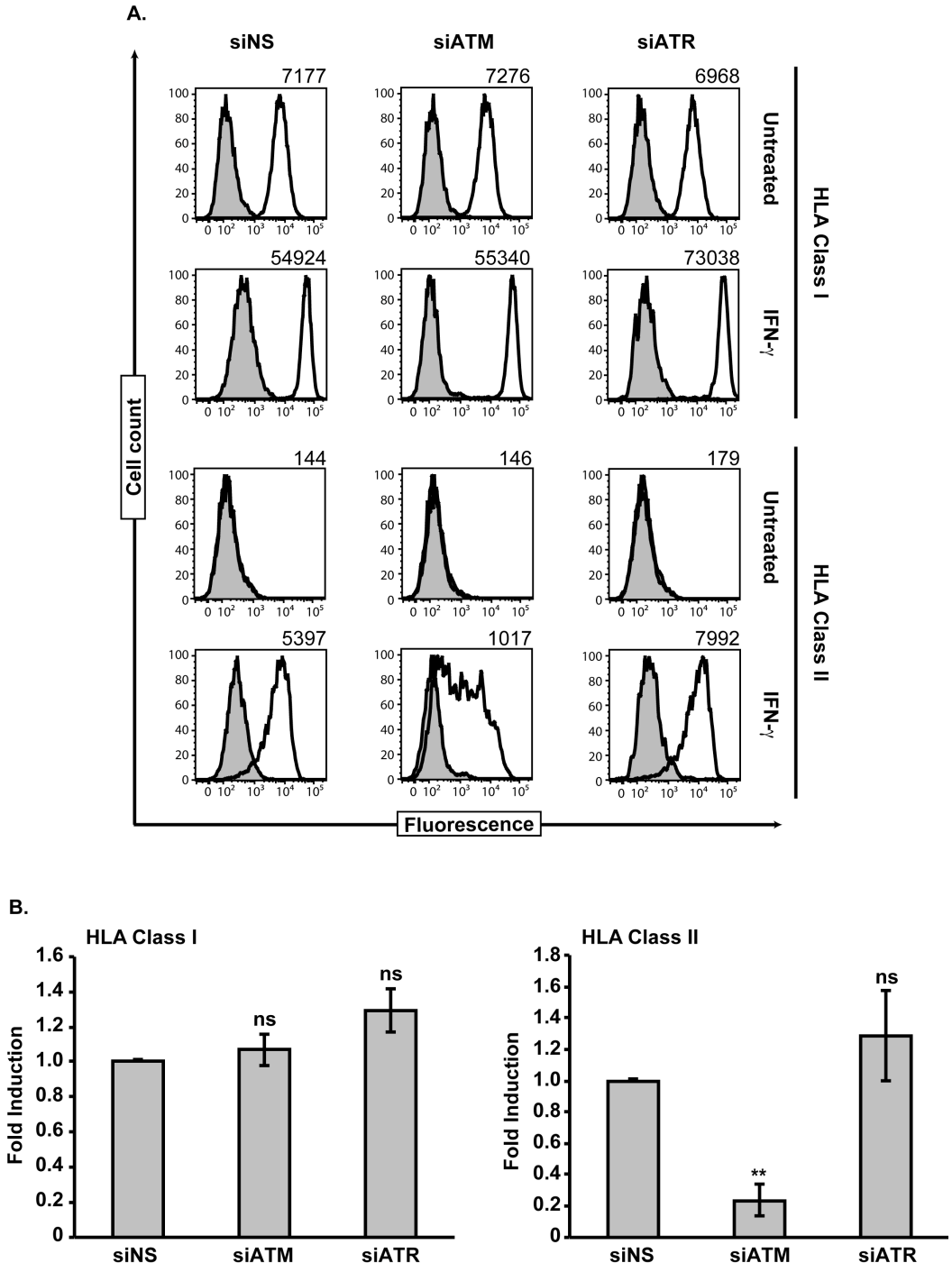


Figure 4.5. Impairment of HLA Class II expression, but not of HLA class I, persisted after 72 hours of treatment with IFN-γ in cells with silenced ATM. U2OS cells were transfected with 20 nM of targeting siRNAs against ATM or ATR or with a non-targeting sequence, using RNAiMAX. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for 72 hours. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody or with a PE-conjugated anti-HLA-DRα Class II antibody and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-Class I (1st and 2nd horizontal panels) and HLA-DRα Class II (3rd and 4th horizontal panels)

expression are presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **B.** For each condition (untransfected, siNS, siATM or siATR) the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample and then normalised by dividing the obtained value by the siNS non-targeting control. These values represent the fold induction of HLA class I and II expression induced by IFN-γ treatment relative to the non-specific control, which is defined as 1. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class I (left graph) and class II (right graph). Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. NS - non-specific.

These data showed a clear difference in the magnitude of the inhibition of HLA class I and II induction by IFN-γ stimulation upon ATM silencing. There appeared to be a temporary impairment of HLA class I expression and a persistent, more profound, inhibition of HLA class II. Taking into consideration the differences known to occur at the transcriptional level between both classes of HLA, we next sought to determine if ATM differently regulated their transcription.

4.2.4. ATM silencing impaired transcription and translation of a specific subset of ISGs

IFN-γ-mediated biological responses are sustained by the immense number of proteins induced by this cytokine. In order to determine if ATM is a general regulator of ISG transcription or if it is only involved in the regulation of a specific subset of genes responsive to IFN-γ, we assessed using qPCR the mRNA levels of a panel of ISGs upon ATM silencing. The panel of ISGs selected included: IRF-1, CIITA, Ii, HLA-DRα, TAP1, SOCS1, SOCS3, CIS, IP10 and PRAME.

Transcription of some of these ISGs is solely dependent on the activity of the main transcription factor of the pathway, STAT1, while others also need newly synthesised transcription factors and transactivators. The genes included in the panel have, therefore, different levels of transcriptional regulation, allowing us to gain a better insight into the underlying mechanism of ATM regulation of IFN-γ-

mediated signalling. IRF-1 and CIITA are classical ISGs. For instance, IRF-1 transcription is directly and exclusively dependent of STAT1 activation upon IFN-γ treatment. IRF-1 is subsequently necessary for the expression of HLA Class I and II molecules. In tissues where HLA class I expression is dependent on IRF1 activity, this involves binding of this transcription factor to a single enhancer on the HLA class I promoter upon IFN-γ stimulation (Jarosinski and Massa, 2002). IRF-1 is necessary for HLA class II expression through the induction of CIITA (Hobart *et al*., 1996; Hobart *et al*., 1997). This involves IRF-1 binding to CIITA promoter IV in association with STAT1 and chromatin modifications by acetylation of histones 3 and 4 (Morris *et al*., 2002). CIITA is the master regulator of HLA class II expression, which is considerably more complex than HLA class I induction, and, as the previously presented data suggests, critically dependent on ATM.

U2OS cells were transfected with a non-targeting siRNA, siSTAT1 as a positive control, siATR as a closely related molecule to ATM, but without phenotype, and siATM. Following 48 hours after transfection, protein levels of these proteins were determined by western blotting analysis (**Fig. 4.6**). Appropriated reduction of ATM, ATR and STAT1 protein levels was achieved by siRNA when compared with the siNS control.

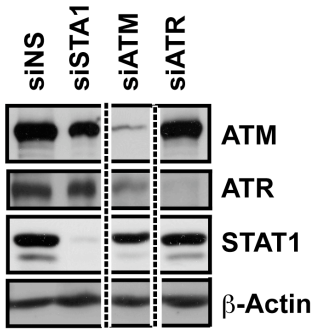


Figure 4.6. Efficient silencing of STAT1, ATM and ATR proteins in samples used for the qPCR assays where ISGs mRNA induction was determined in response to IFN-γ. U2OS cells were transfected with 20 nM of a non-specific, non-targeting, sequence or with sequences targeting STAT1, ATM and ATR. Forty-eight hours after transfection cells were lysed with Schindler's buffer at the same that cells were stimulated in parallel for qPCR analysis of ISG mRNA induction. Proteins were resolved on a 6% SDS-PAGE gel. Western blotting analysis was performed using antibodies against ATM, ATR and STAT1α/β to assess the knockdown efficiency of these proteins by RNAi. β-actin was used as a loading control.

Cells were stimulated with IFN-γ for 1 and 6 hours. The period of stimulation of 1 hour is a reasonably short one to allow detection of early-transcribed genes like IRF-1, but not of ISGs that are highly regulated and dependent on newly synthesised proteins, such as HLA-DRα, for which a longer period like 6 hours was deemed adequate for assessing mRNA levels. RNA was subsequently extracted, reverse transcribed into cDNA and the induction of mRNA levels of several ISGs was analysed using SYBR Green-based qPCR (**Fig. 4.7**). The mRNA levels were expressed as fold change relative to siNS control upon IFN-γ stimulation and statistical analysis was done using Student's t-test. Each transfection condition for any given time of stimulation was first compared to the untreated siNS control (black symbols in the graphs) to determine the effect of the different knockdowns on IFN-γ-induced expression for each ISG. In order to more closely assess variations in the mRNA levels between different transfection conditions, at a specific time point of stimulation, a comparison was established between the different knockdowns and the siNS untreated control for each period of time of cytokine treatment (orange symbols in the graphs).

IRF-1 transcription following IFN-γ treatment (black symbols) is solely dependent on STAT1-activated dimers binding to the IRF-1 promoter. Transcription was clearly detectable and statistically significant ($p<0.01$) after 1 hour of stimulation in siNS-transfected cells (**Fig. 4.7.1.A**). mRNA transcription must occur very rapidly, way before 1 hour, since at this time point IRF-1 protein was already detectable (**Fig. 4.7.1.B**). Significant levels of IFN-γ-induced IRF-1 mRNA in siNS-transfected cells of were detectable after 1 hour, compared to the control unstimulated cells (siNS at 0h) and also when ATM ($p<0.001$) and ATR ($p<0.0001$) were silenced (**Fig. 4.7.1.A**). However, there was a decrease in the magnitude of induction of IRF-1 mRNA upon siATM compared to the induction seen in the siNS condition, but this was not statistically significant. STAT1 silencing was efficient (**Fig. 4.6**), but not complete and, as a consequence, a significant amount of IRF-1 mRNA was still detected upon STAT1 knockdown ($p<0.05$). After 6 hours of treatment, IRF-1 mRNA production was still evident and significant for siNS and siATR ($p<0.001$) (**Fig. 4.7.1.A**). The increase of IRF-1 mRNA levels upon siSTAT1 was no longer significant, while silencing of ATM still allowed a significant induction of

this ISG mRNA ($p<0.0001$) (black symbols). However, IRF-1 mRNA production was considerably reduced upon siSTAT1 and siATM when compared to the siNS induction ($p<0.001$) (orange symbols) (**Fig. 4.7.1.A**).

CIITA transcription is complex. It has been reported that CIITA transcripts need more than 2 hours to be upregulated (Morris *et al*., 2002) and, as a consequence, protein production will take longer than that. Indeed, CIITA mRNA could not be detected 1 hour after IFN-stimulation, but was visible after 6 hours of IFN-γ treatment when the increase in CIITA expression was statistically significant (black symbols) in siNS ($p<0.01$) and siATR-transfected cells ($p<0.05$) (**Fig. 4.7.2.A**). STAT1 and ATM silencing significantly abrogated CIITA mRNA expression induction ($p<0.01$ and $p<0.05$, respectively) when compared to the siNS control (orange symbols) almost to basal levels (black symbols). Newly synthesised CIITA protein (indicated by the orange arrow) could only be detected after 4 hours in control cells and, although, it could not be detected for STAT1 knockdown, there was still a small amount of CIITA produced upon ATM silencing (**Fig. 4.7.2.B**).

Ii transcripts were massively elevated after 6 hours of stimulation with IFN-γ (black symbols) in siNS ($p<0.01$) and siATR transfected cells ($p<0.001$) (**Fig. 4.7.3**). Since Ii transcription is dependent on CIITA we hypothesised that its mRNA levels would also be affected upon STAT1 and ATM knockdown. A significant reduction on Ii transcript was in fact observed as a consequence of STAT1 ($p<0.01$) and ATM ($p<0.01$) silencing when compared to the siNS control (orange symbols) or siATR controls. While the impairment by siSTAT1 and siATM was considerable, the induced levels of mRNA were still significantly above basal levels ($p<0.001$ for both proteins) given the high induction of Ii and the fact that the knockdown is never complete (black symbols). ATR knockdown resulted in a significantly higher induction of Ii transcription in comparison to the siNS control ($p<0.01$) (symbols in orange).

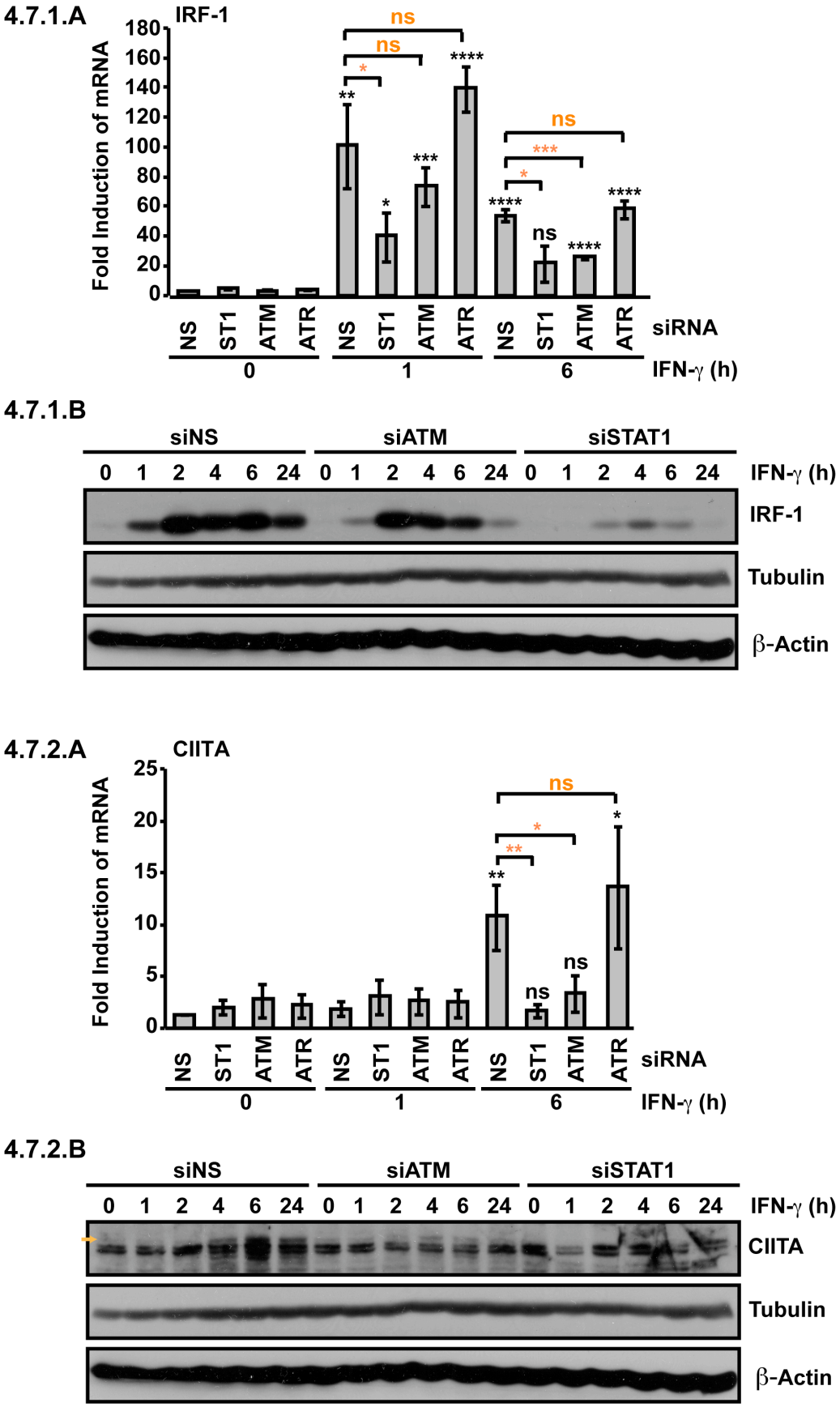


Figure 4.7. ATM was required for the expression of a subset of ISGs. (Continued on pages 122 to 124).

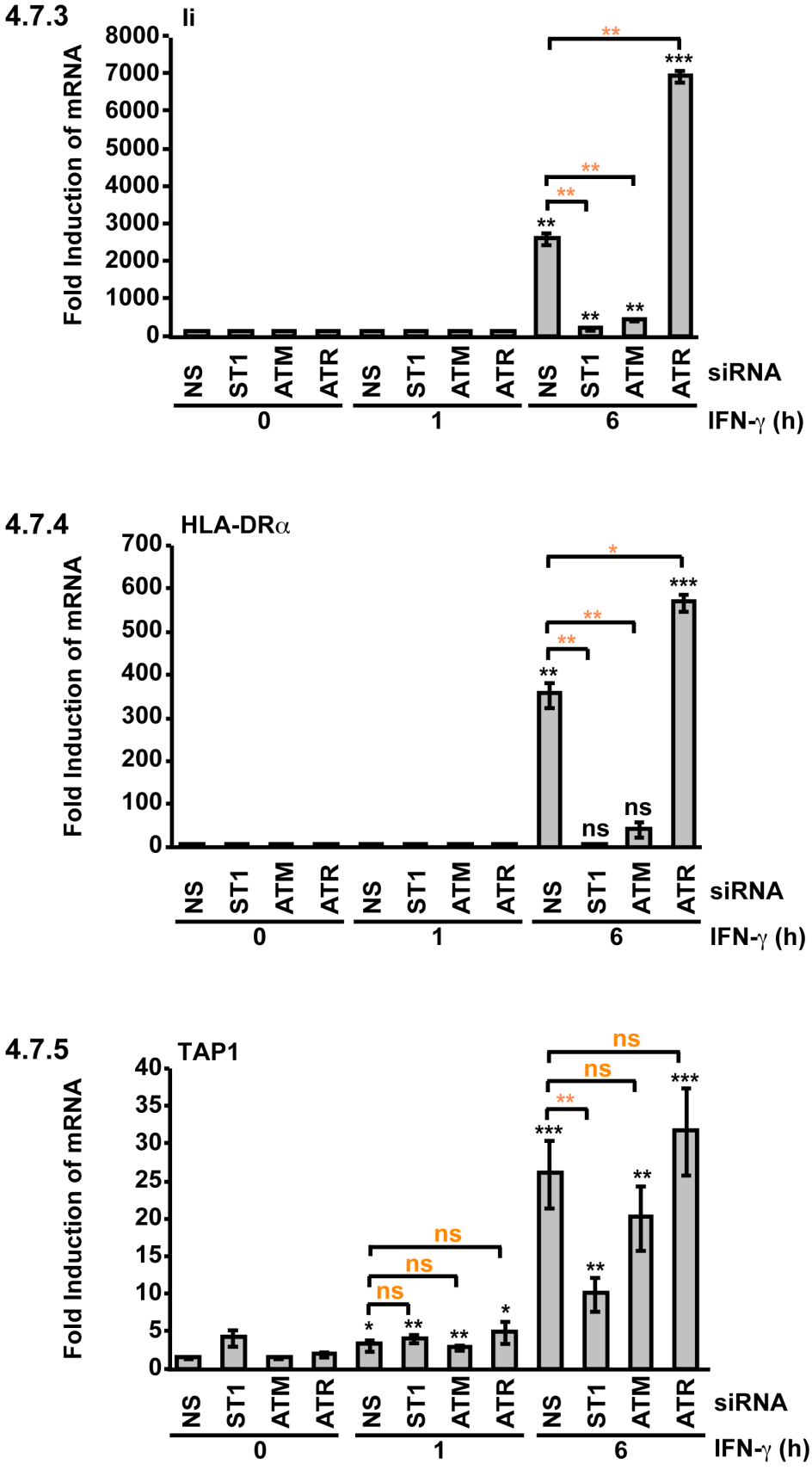


Figure 4.7. ATM was required for the expression of a subset of ISGs. (Continued on pages 123 and 124).

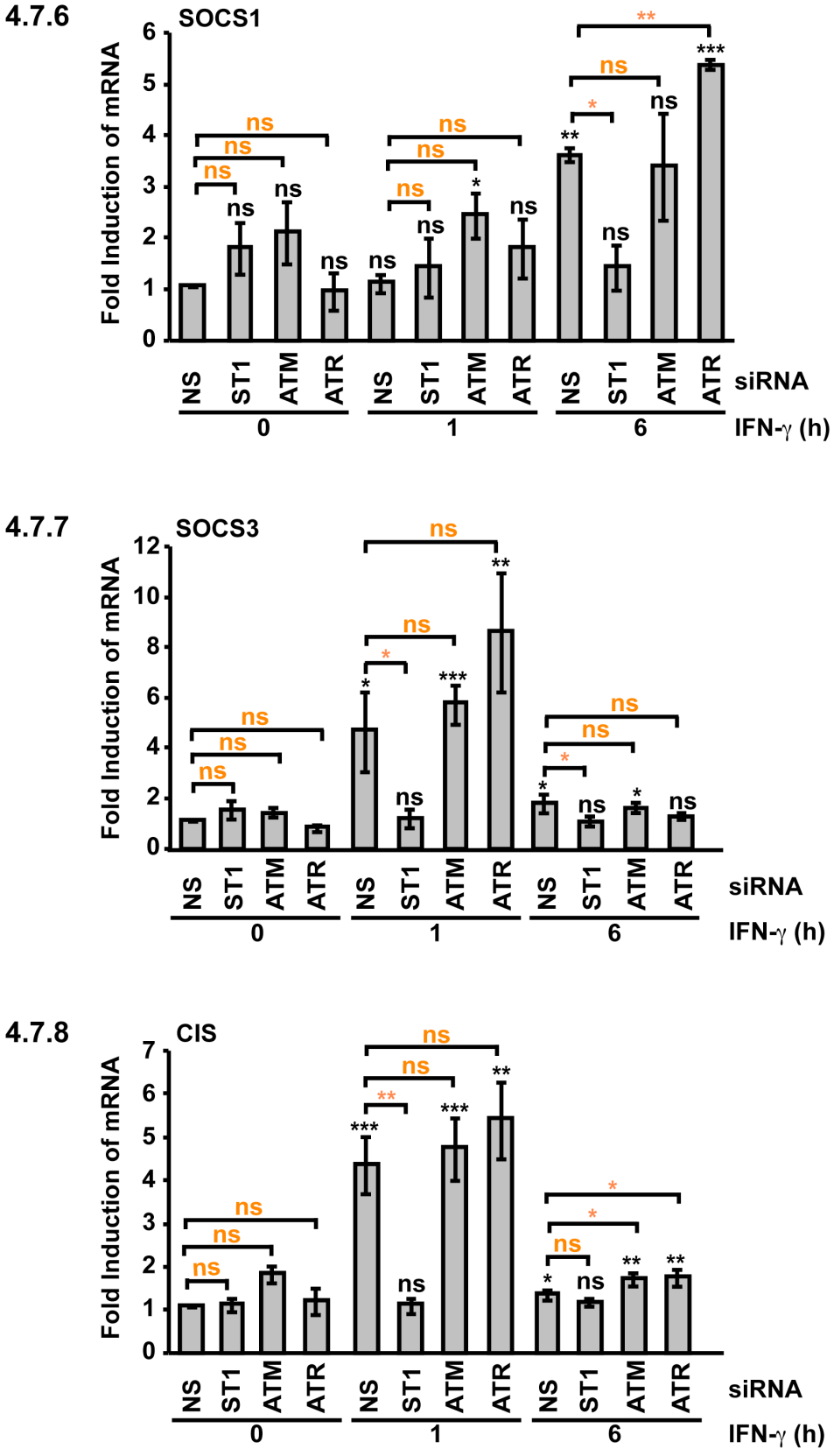


Figure 4.7. ATM was required for the expression of a subset of ISGs. (Continued on page 124).

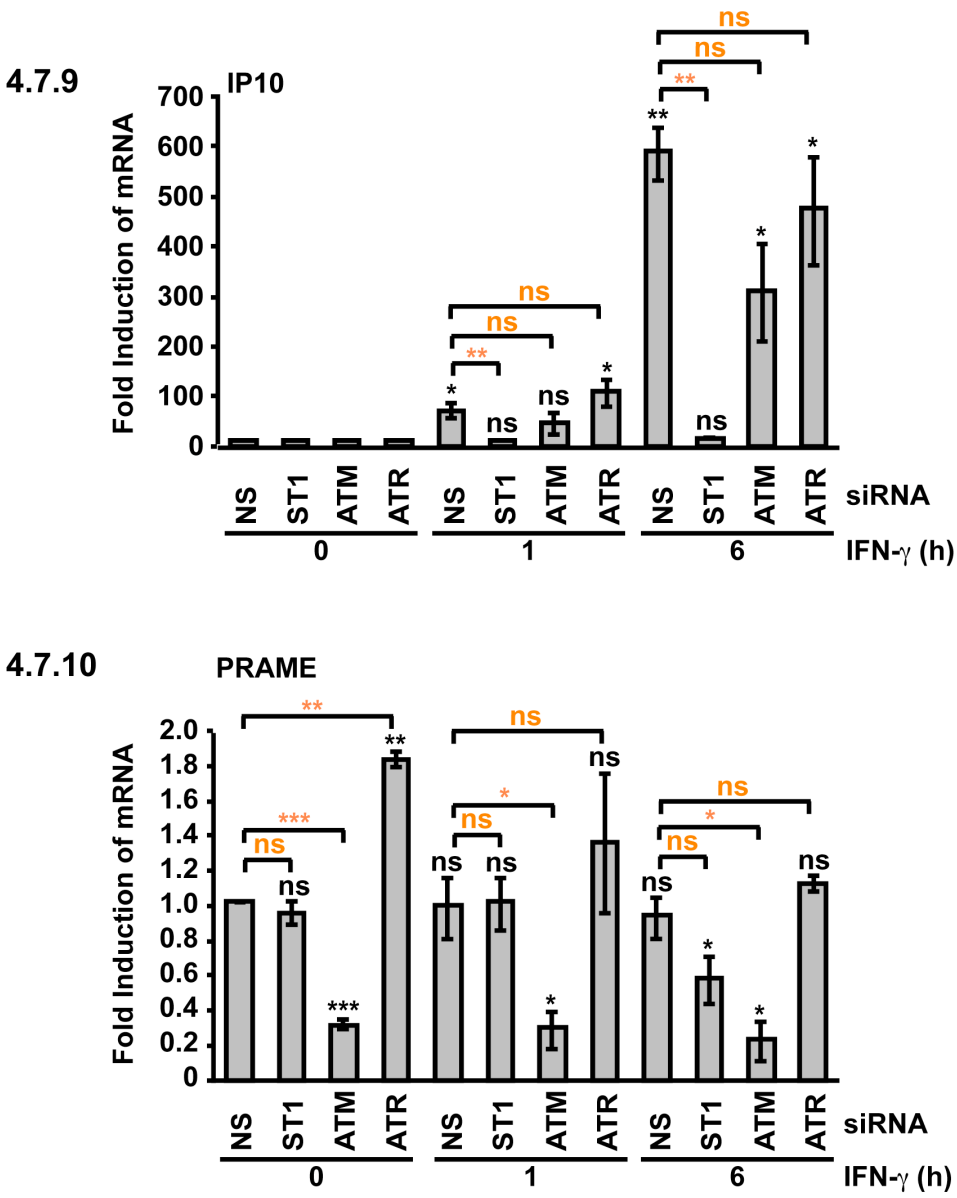


Figure 4.7. ATM was required for the expression of a subset of ISGs. U2OS cells were transfected with 20 nM of targeting siRNAs against STAT1 (ST1), ATM and ATR or with a non-specific siRNA control (NS) with RNAiMAX. Forty-eight hours after transfection, cells were stimulated with IFN-γ (500 IU/ml) for the indicated time points (hours, h). RNA extraction and reverse transcription was performed and the obtained cDNA was amplified with primers specific to detect mRNA of known ISGs, namely IRF1, CIITA, Ii, HLA-DRα, TAP1, SOCS1, SOCS3, CIS, IP10 and PRAME. Detection of amplified products was done using SYBR Green-based qPCR. Each reaction was performed in triplicate. Data was analysed using qBase plus program and normalised by dividing by the untreated value of the NS condition for each individual experiment. Mean ± SD values were obtained using at least two or three independent experiments done in triplicates and yielding identical results. Values are expressed as fold change relative to NS control upon treatment. Statistical analysis was done using Student's t-test and comparing each condition relative to the untreated non-specific control in order to determine the effect of the different

knockdowns on the induction of each ISG with stimulation (symbols in black). [non-significant (ns) - p>0.05, *- p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001]. This test was also used to compare between the different knockdowns relative to the NS control for each time point of stimulation were significant mRNA expression was detected to determine differences (symbols in orange) [non-significant - (ns) - p>0.05, *- p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001]. **1.B** and **2.B.** After IFN-γ treatment ATM knockdown severely decreased CIITA-induced protein expression but it only affected IRF-1 slightly. U2OS cells were transfected and treated as above. Whole cell lysates were prepared with Schindler's buffer and proteins were separated on a 7.5% SDS-PAGE gel. Western blotting analysis was performed using antibodies against CIITA and IRF-1. The orange arrow indicates CIITA protein. Tubulin was used as a loading control. These results are representative of three independent experiments yielding identical results.

STAT1 silencing down-regulated IFN-γ-induced levels of HLA-DRα after 6 hours of treatment to non-significant basal levels (black symbols) when compared to the siNS control (p<0.01) (orange symbols) (**Fig. 4.7.4**). ATM silencing resulted in a similar phenotype to siSTAT1, significantly reducing HLA-DRα mRNA transcripts (p<0.01) (orange symbols). Interestingly, after IFN-γ treatment, ATR knockdown resulted in a significantly higher induction of HLA-DRα mRNA levels (**Fig. 4.7.4**) as it did for Ii (**Fig. 4.7.3**), contrarily to siATM phenotype.

TAP1 induction was noticed as early as after 1 hour of stimulation with IFN-γ, after transfection with siNS (p<0.05), siATM (p<0.01), siATR (p<0.05) and even when STAT1 was silenced (p<0.01) (black symbols) (**Fig. 4.7.5**). No significant difference could be seen between each transfected condition and the siNS control, upon 1 hour of treatment (orange symbols), possibly because at such an early time point there was only a modest increase in mRNA levels. TAP1 induction increased significantly after 6 hours (p<0.001) for the siNS-transfected cells (black symbols). Significant induction was also observed for this time point upon silencing STAT1 (p<0.01), ATM (p<0.01) and ATR (p<0.001) (symbols in black). When STAT1 was silenced, however, mRNA levels of TAP1 were considerably reduced comparatively to the siNS control (p<0.01) (orange symbols), as expected, since STAT1, together with IRF-2, is responsible for its transcription. Similarly to what was observed for IRF-1, there seemed to be a slight repression on TAP1 induction by ATM silencing. However, this was not statistically significant (orange symbols). On the contrary, although also not significant, there was a tendency for ATR

knockdown to increase TAP1 induction (orange symbols) (**Fig. 4.7.5**), as was also seen for IRF-1 (**Fig. 4.7.1.A**).

After 6 hours of treatment with IFN-γ, SOCS1 mRNA levels were upregulated upon siNS ($p<0.01$), siATM or siATR ($p<0.001$) transfection (black symbols) (**Fig. 4.7.6**). As anticipated, STAT1 knockdown abolished SOCS1 transcription (black symbols) and this was significantly reduced ($p<0.05$) when compared to the induction in siNS-transfected cells (orange symbols). Increase of SOCS1 transcripts was visible in all experiments when silencing ATM and no significant difference was apparent when compared to siNS control. However, due to variability among experiments, this increase was not statistically significant. ATM knockdown did not decrease SOCS1 expression directly or indirectly.

SOCS3 transcription was induced early upon 1 hour of stimulation of siNS ($p<0.05$), siATM ($p<0.001$) and siATR-transfected cells ($p<0.01$). It decreased with time to almost basal levels by 6 hours of treatment (black symbols) (**Fig. 4.7.7.**). Induction of SOCS3 upon siATM and siATR did not differ from the siNS control after 1 hour of stimulation with IFN-γ (orange symbols). SOCS3 transcription was absolutely dependent on STAT1. Knockdown of STAT1 abrogated SOCS3 transcription to basal level (black symbols) in strict contrast to the siNS induction (orange symbols).

CIS belongs, together with SOCS1 and SOCS3, to the SOCS family and is induced by IFN-γ (Yamasaki *et al*., 2003). CIS expression followed a similar trend to the one of SOCS3. CIS mRNA levels were upregulated within 1 hour of treatment of siNS ($p<0.001$), siATM ($p<0.001$) and siATR-transfected cells ($p<0.01$) (black symbols) (**Fig. 4.7.8**). Expression of this early-induced gene decreased with time and was only detected slightly above basal levels at the 6 hours time point. No differences were observed between siATM and siATR and the control condition upon 1 hour of stimulation (orange symbols). Silencing of STAT1 completely abrogated CIS1 transcription as expected (black symbols) and this difference was statistically significant in comparison to the siNS condition ($p<0.01$) (orange symbols).

Transcription of the chemokine IP10 was detected upon 1 hour of treatment with IFN-γ when cells were transfected with siNS ($p<0.05$) and siATR ($p<0.05$) (black symbols) (**Fig. 4.7.9**). Although IP10 induction was also detected after this short stimulation in cells transfected with siATM, the variation between experiments meant this was not statistically significant (black symbols). IP10 expression increased with a longer IFN-γ stimulation (6 hours) of siNS ($p<0.01$), siATM ($p<0.05$) and siATR-transfected cells ($p<0.05$) (black symbols). While in some experiments siATM seemed to slightly decrease this chemokine mRNA levels, this was not significant as it was for siSTAT1 transfection, which completely abolished IP10 expression for both 1 and 6 hours of cytokine treatment ($p<0.01$) relatively to the siNS control (orange symbols).

PRAME is expressed in several osteosarcomas and has been reported as an ISG. This gene was constitutively expressed in U2OS cells, but was not inducible by IFN-γ for either 1 or 6 hours of treatment in siNS, siSTAT1 and siATR conditions (symbols in black) (**Fig. 4.7.10**). Curiously, ATM knockdown was responsible for significantly down-regulating PRAME constitutive expression for all treatment time points ($p<0.001$ for 0 hours and $p<0.05$ for 1 and 6 hours of stimulation). ATR silencing increased the levels of PRAME mRNA transcript only in the absence of stimulation ($p<0.01$).

ATM importance in the regulation of IFN-γ signalling was thus confirmed by studying transcription of several relevant ISGs. As predicted, ATM silencing did not result in a similar phenotype to STAT1 knockdown and it was important only for transcription of a subset of ISGs, namely CIITA, Ii and HLA-DRα.

4.3. ATM kinase activity was not necessary for IFN-γ-induced responses in U2OS cells

The accepted role for ATM as a central coordinator of the DSB repair machinery relies on its serine/threonine enzymatic activity. This is necessary for the activation by phosphorylation of a number of downstream effectors, such as p53 (Bakkenist and Kastan, 2003; Banin *et al*., 1998; Canman *et al*., 1998; Lavin and Kozlov, 2007). Even the more recently recognised function of ATM, as a regulator of

chromatin condensation regarding the repair of DSBs in heterochromatin (Goodarzi *et al*., 2008), has also been associated with ATM's enzymatic activity. We, therefore, asked if this could also be the case in the regulation of IFN-γ-responses.

4.3.1. ATM enzymatic activity was not required for IFN-γ-induced phosphorylation of STAT1

In order to determine if ATM plays an enzymatic or structural role in IFN-γ-mediated responses, an ATM-specific chemical inhibitor was used. The commercial inhibitor KU-55933 (2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one) binds competitively to the ATP-binding pocket of ATM and was shown to inhibit efficiently the phosphorylation of several ATM downstream targets upon activation by ionizing radiation (Hickson *et al*., 2004). ATM activation was promoted by treating U2OS cells with 100 μM of the DSB-inducing agent etoposide for 4 hours. A dose response was initially performed by assessing the effect of 1 hour of pre-treatment with KU-55933 on ATM serine 1981 phosphorylation, which is associated with its kinase activation, and on phosphorylation on the ATM downstream target p53 at serine 15, which is known to be strongly induced in U2OS cells upon DNA damage (Allan and Fried, 1999; Banin *et al*., 1998; Canman *et al*., 1998) (**Fig. 4.8**).

Western blot analysis showed that a 10 μM concentration of KU-55933 was sufficient to completely block phosphorylation of ATM serine 1981 residue (**Fig. 4.8**). This concentration was also able to block p53 phosphorylation on its serine 15 residue. In addition, this concentration is commonly used in U2OS cells as attested by a detailed literature search (Hickson *et al*., 2004). Consequently, the inhibitor was used at a concentration of 10 μM to evaluate the relevance of ATM enzymatic activity on IFN-γ responses.

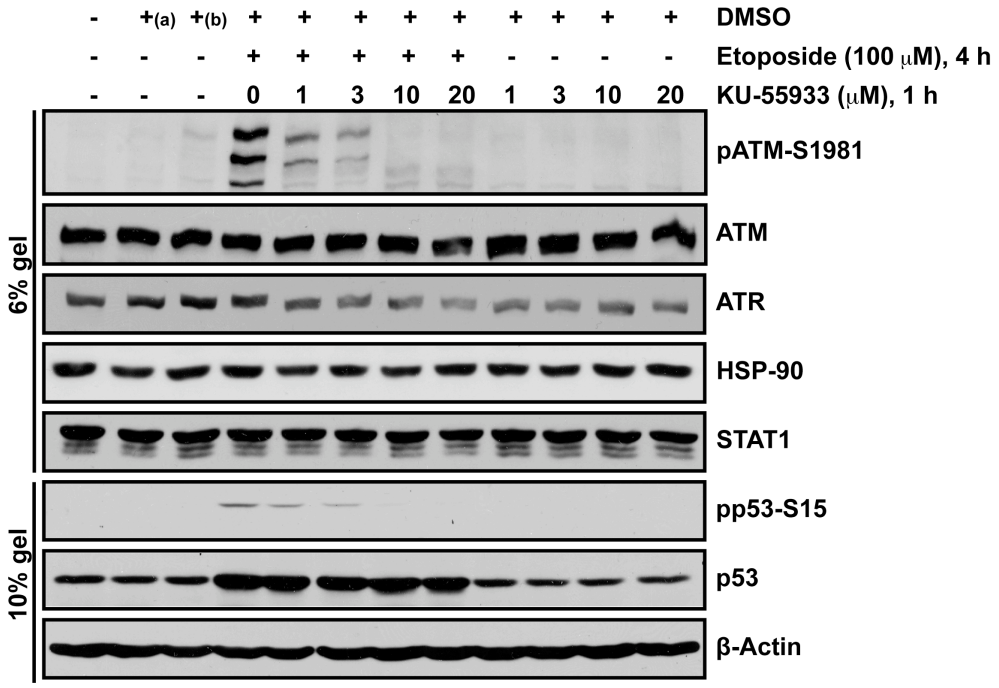


Figure 4.8. KU-55933 efficiently inhibited ATM enzymatic activity. Cells were pre-treated for 1 hour (h) with 0, 1, 3, 10 and 20 μM of KU-55933, followed by treatment with etoposide for 4 hours at 100 μM. Whole cell lysates were prepared using Schindler's buffer and proteins were resolved in 10 or 6% SDS-PAGE gels. Western blotting analysis was performed using antibodies against pATM-S1981 and pp53-S15, as well as against ATM, p53 and STAT1α/β. β-actin and HSP-90 antibodies were used as loading controls for the 10% and 6% resolving gels, respectively. This experiment was only done once. [(a)] and [(b)] correspond to controls with a DMSO concentration similar to the one used in the conditions where both drugs were used, with 10 and 20 μM of KU-55933, respectively.

After pre-treatment for 1 hour with 10 μM of the inhibitor cells were stimulated with IFN-γ for 0, 5, 10, 20, 30 and 60 minutes. STAT1 serine 727 and tyrosine 701 phosphorylation were assessed by western blot **(Fig. 4.9)** in order to verify if blocking ATM enzymatic activity would recapitulate the results obtained using RNAi to silence ATM. The results obtained here indicate that inhibition of ATM enzymatic function had no effect on either STAT1 serine 727 or tyrosine 701 phosphorylation induced by IFN-γ. This suggested that ATM's role in the regulation of IFN-γ biological responses did not depend on its kinase activity but is instead structural.

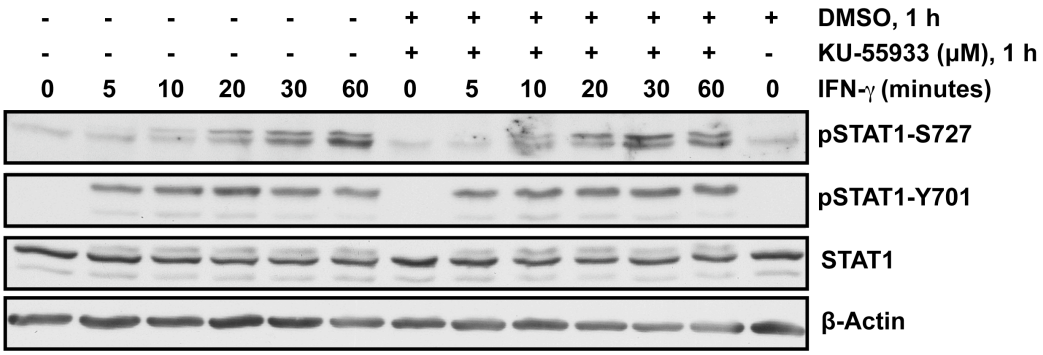


Figure 4.9. Inhibition of ATM enzymatic activity did not affect STAT1 serine 727 or tyrosine 701 phosphorylation in response to IFN-γ. Cells were pre-treated for 1 hour (h) with 10 μM of KU-55933 and then stimulated with 500 IU/ml of IFN-γ for the shown time points. Total cell lysates were prepared with Schindler's buffer and proteins were separated in a 7.5% SDS-PAGE gel. Western blotting analysis was performed using antibodies against pSTAT1-S727 and pSTAT1-Y701 to assess phosphorylation levels in response to IFN-γ and also against STAT1α/β. β-actin was selected as a loading control. These results are representative of three experiments giving similar results.

4.3.2. ATM enzymatic activity was dispensable for HLA Class I and II induction by IFN-γ

The lack of requirement for ATM enzymatic function was further confirmed by assessing the expression of HLA Class I and II in response to IFN-γ. In order to do so, the stability of the inhibitor in culture was first confirmed for the minimum and maximum periods of time that these assays may take. After incubation for 1, 24 and 72 hours with 10 μM of KU-55933, U2OS cells were subjected to 4 hours of treatment with etoposide at 100 μM. As a read-out for the efficiency of the inhibitor, cell extracts were prepared using Schindler's buffer and phosphorylation of ATM on serine 1981 and of p53 on serine 15 assessed by western blot **(Fig. 4.10)**.

Results showed that after a period of 24 or even 72 hours in cell culture, the inhibitor activity was not lost and was comparable to that seen after only 1 hour in culture, which was used for the shorter time experiments. Indeed, ATM phosphorylation was still prevented, as was the phosphorylation of its target p53 upon etoposide treatment (**Fig. 4.10**).

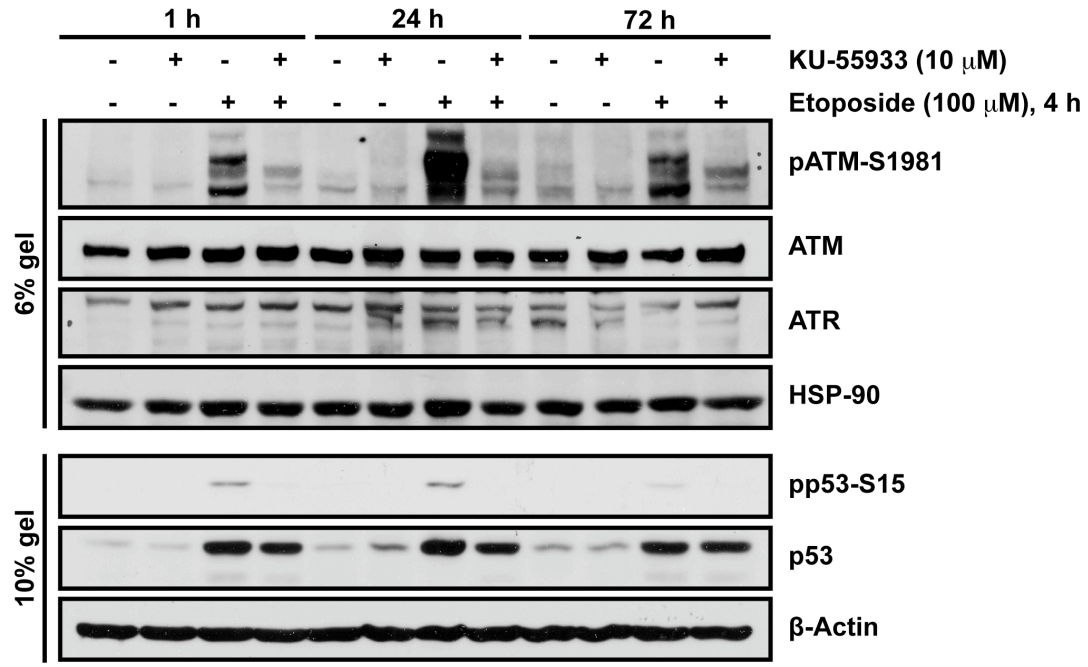


Figure 4.10. KU-55933 inhibitor was stable and active even after being incubated for prolonged periods of time in culture. Cells were pre-treated with 10 μM of KU-55933 for 1, 24 or 72 hours (h) and then ATM activation induced by treating cells with a DSBs causing agent (etoposide for 4 hours at 100 μM). Western blotting analysis was performed using antibodies against pATM-S1981 and pp53-S15, as well as against ATM and p53. β-actin and HSP-90 antibodies were used as loading controls for the 10% and 6% resolving gels, respectively.

The induction of Class I and II HLA was subsequently analysed by flow cytometry in U2OS cells pre-treated for 1 hour with 10 μM of KU-55933 to ensure that ATM enzymatic activity was inhibited from the moment stimulation was started. Cells were then cultured for 24, 48 and 72 hours in the presence of IFN-γ with or without KU-55933 (**Fig. 4.11**).

Treatment with IFN-γ clearly induced the expression of HLA Class I above the basal levels detected in the DMSO control. The induction of HLA class I was statistically significant for all the three time points assessed (24 hours, $p<0.0001$; 48 hours, $p<0.01$; 72 hours, $p<0.001$) (**Fig. 4.11.B**). Induced expression of HLA class I increased with time of stimulation, which was confirmed by the increase in the geometric mean values shown on the top right hand corner of each graph (**Fig. 4.11.A**). ATM inhibition, however, had no effect on this response not even for the 24 hours time point (**Fig. 4.11.A** and **B**) when siATM impaired on the expression of HLA class I (**Fig. 4.4.A** and **B**).

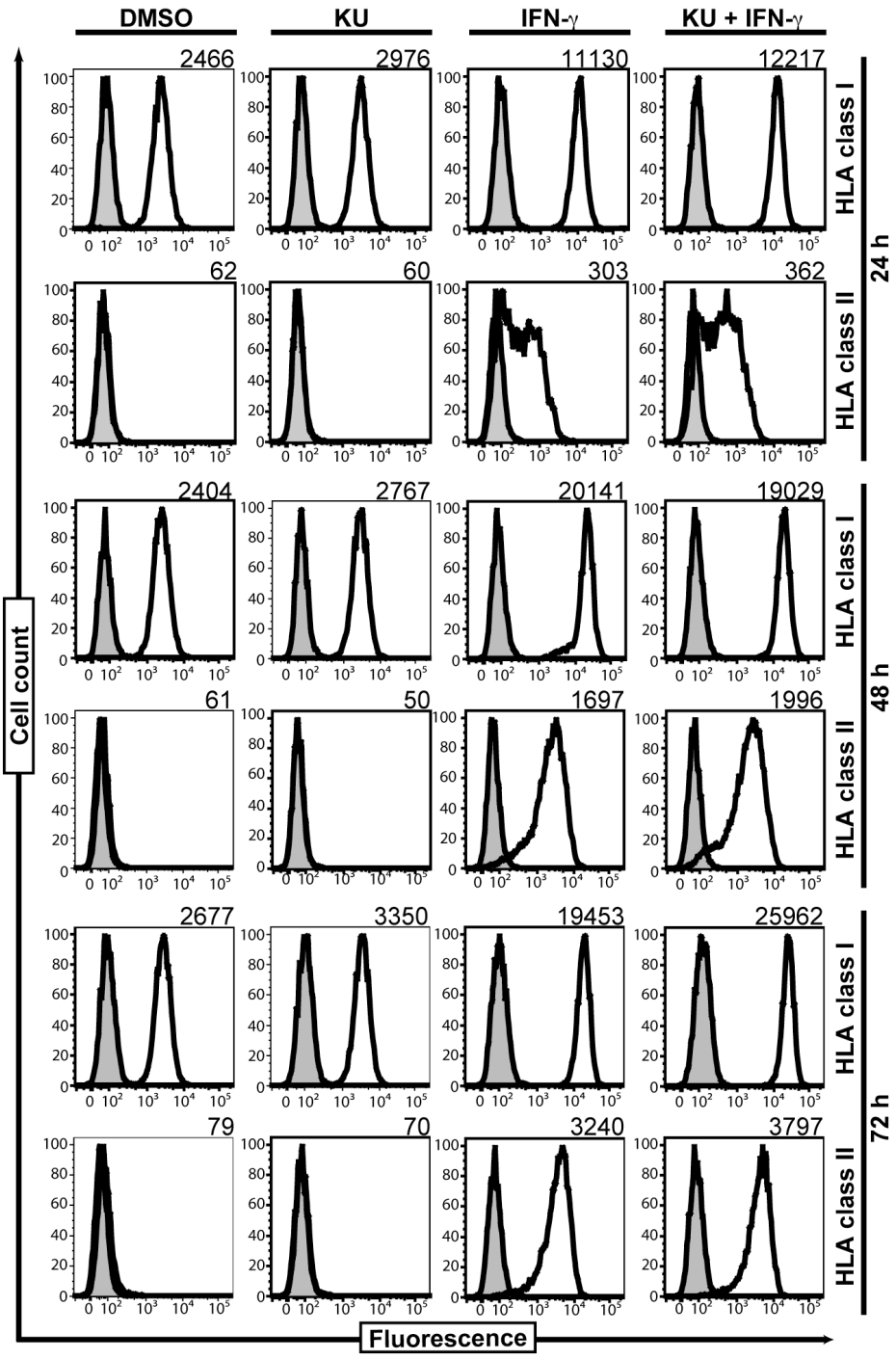


Figure 4.11. Inhibition of ATM enzymatic activity did not affect induction of HLA Class I or II by IFN-γ treatment. (Continued on page 133).

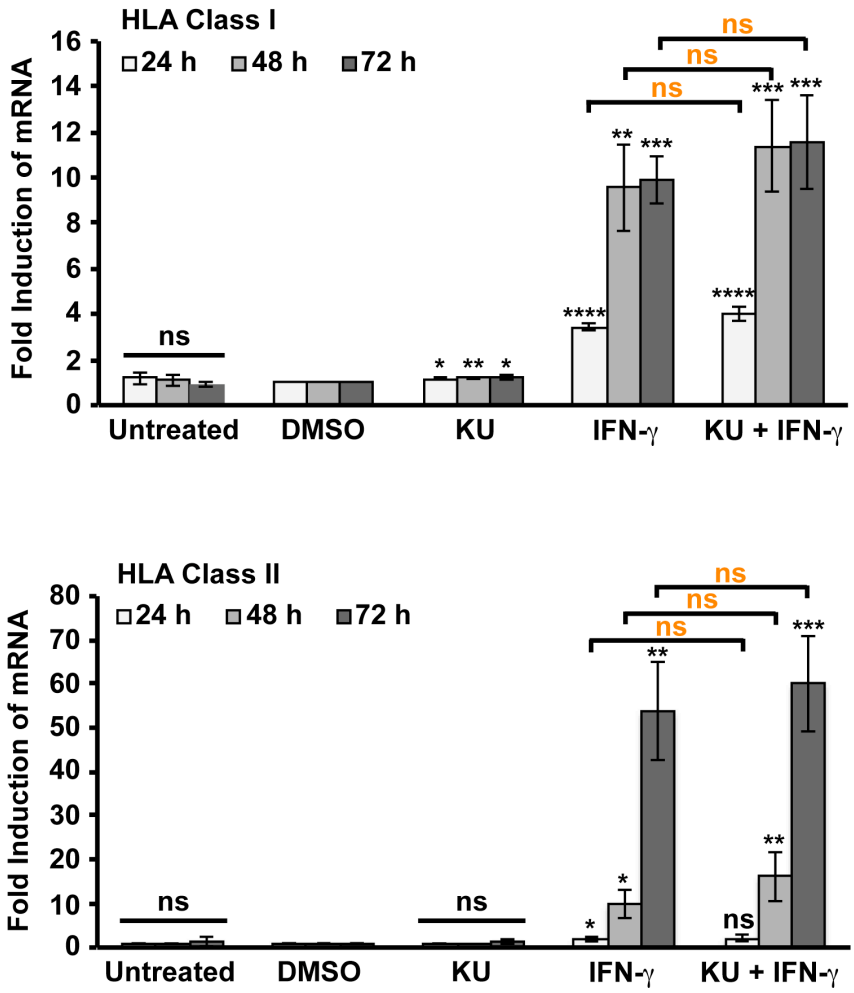


Figure 4.11. Inhibition of ATM enzymatic activity did not affect induction of HLA Class I or II by IFN-γ treatment. Cells were pre-treated for 1 hour (h) with KU-55933 (10 μM), followed by stimulation with IFN-γ (500 IU/ml) for 24, 48 and 72 hours in the presence or absence of the inhibitor. Cells were stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody (1st, 3rd and 5th horizontal panels) or with a PE-conjugated anti-HLA-DRα Class II antibody (2nd, 4th and 6th horizontal panels). Cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. **A.** Results were analysed using FlowJo and are presented as the geometric means of fluorescence of stained samples (numbers on the top right hand corner of each graph). The unstained controls and the stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **B.** Each condition was normalised by dividing the value of the geometric mean of the stained sample by the value of the DMSO control. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class I (upper graph) and class II (lower graph). Values are expressed as fold change. Statistical significance was calculated using Student's t-test relative to the DMSO control and no significant difference was observed between untreated and DMSO control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$].

IFN-γ-induced expression of HLA class II expression was clearly observed when compared with unstimulated cells on the DMSO control (**Fig. 4.11.A**). This significant induction (24 and 48 hours, $p<0.05$ and 72 hours, $p<0.01$) also increased with prolonged stimulation (**Fig. 4.11.B**). However, the chemical inhibitor had no effect on the magnitude of this induction (**Fig. 4.11.A** and **B**), in contrast to the clear reduction of HLA class II expression upon ATM silencing (**Fig. 4.4** and **Fig. 4.5**). These results suggested that ATM enzymatic function was not only dispensable for the effect observed over STAT1 phosphorylation upon IFN-γ stimulation, but also for IFN-γ-induced expression of HLA class II. It seemed thus likely that ATM had a structural rather than an enzymatic role in the regulation of IFN-γ responses.

4.3.3. ATM enzymatic activity was dispensable for transcription and translation of the subset of ISGs that was dependent on ATM protein expression

In order to further confirm that ATM has a structural function, we inhibited this protein's enzymatic activity with KU-55933 and analysed the induction of mRNA levels of the same panel of ISGs that was assessed upon ATM silencing. U2OS cells were pre-treated for 1 hour with 10 μM of KU-55933 so that ATM enzymatic activity was inhibited when stimulation was initiated. Cells, either pre-treated or not, were stimulated with IFN-γ for 1, 2, 4, 6 and 24 hours, in the presence or absence of KU-55933, respectively. As described before, RNA was extracted and reverse transcribed into cDNA. mRNA levels of IRF-1, CIITA, Ii, HLA-DRα, TAP1, SOCS1, SOCS3, CIS, IP10, IP30 and PRAME were once again determined using SYBR Green-based qPCR (**Fig. 4.12**).

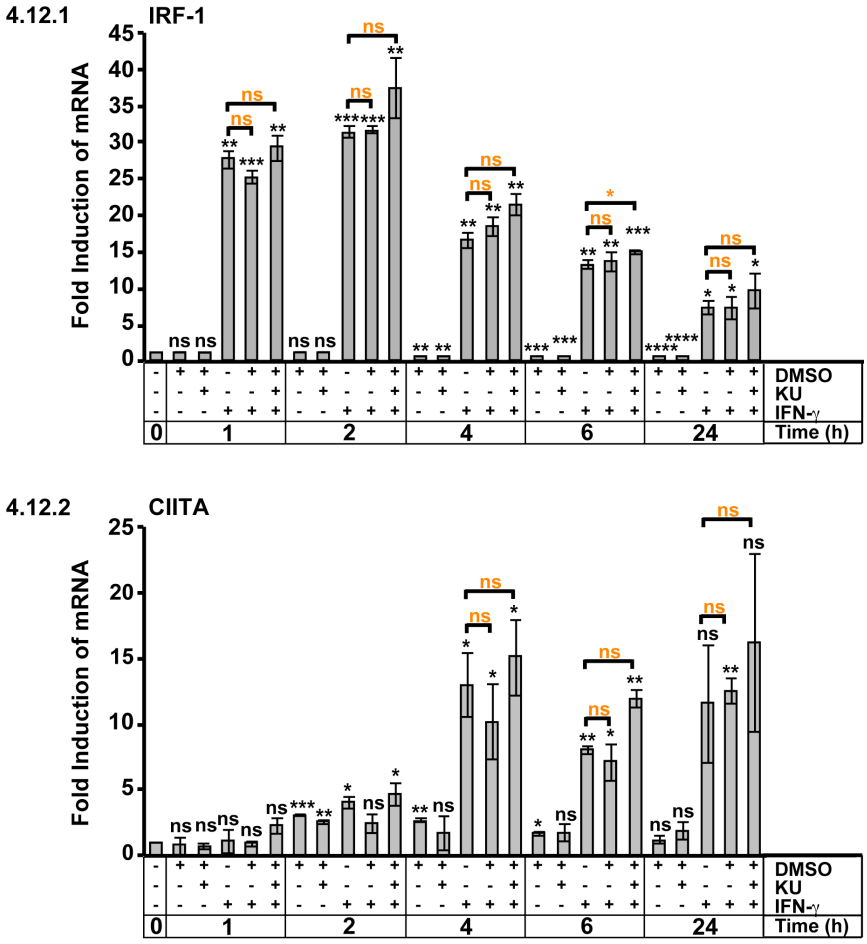


Figure 4.12. Inhibition of ATM's enzymatic activity did not impair the expression of several ISGs in response to IFN-γ. (Continued on pages 136 to 138). Cells were pre-treated for 1 hour (h) with either KU-55933 (10 μM) or with the corresponding amount of solvent (DMSO) or left untreated. Cells were then stimulated with IFN-γ (500 IU/ml) for the indicated periods of time in hours (h). RNA extraction and reverse transcription was performed and the obtained cDNA was amplified with primers specific to detect mRNA of known ISGs namely, IRF-1, CIITA, Ii, HLA-DRα, TAP1, SOCS1, SOCS3, CIS1, IP10 and PRAME. Detection of amplified products was done using SYBR green-based real time qPCR. Each reaction was performed in triplicate. Data was analysed using qBase plus program and normalised dividing by the value of the untreated condition (0 h) of each individual experiment. Mean ± SD values were obtained using two independent experiments done in triplicates and yielding identical results, after normalisation untreated condition and values are therefore, expressed as fold change upon treatment. Statistical analysis was done using Student's t-test relative to the untreated control in order to determine the profile of induction of each ISG with stimulation (black symbols). This test was also used to compare between the condition treated only with IFN-γ and the conditions treated with DMSO or KU-55933 previous to stimulation with this cytokine for each time point were significant mRNA expression was detected (orange symbols) [non-significant (ns) - p>0.05, *- p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001].

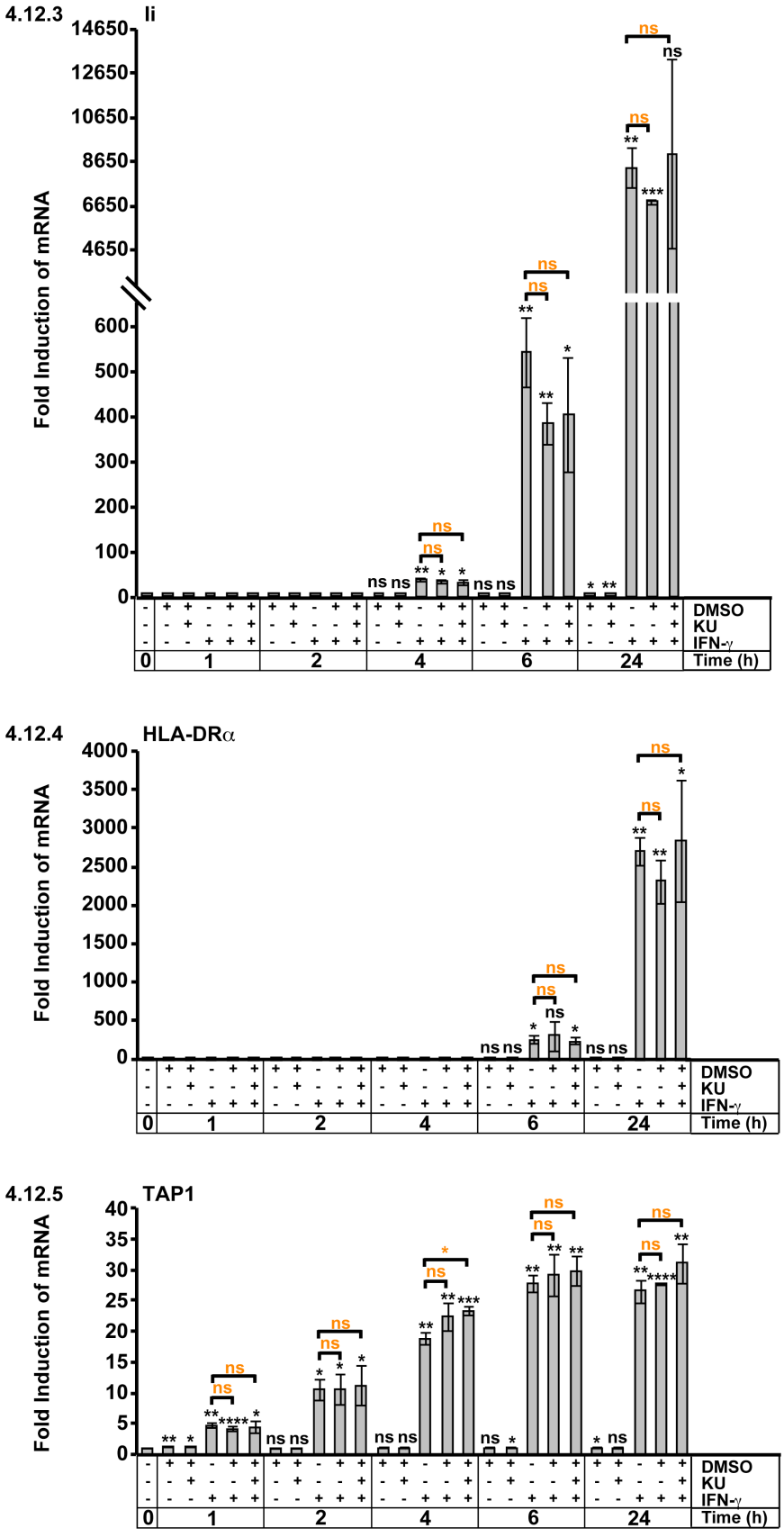


Figure 4.12. Inhibition of ATM's enzymatic activity did not impair the expression of several ISGs in response to IFN-γ. (Continued on pages 137 and 138).

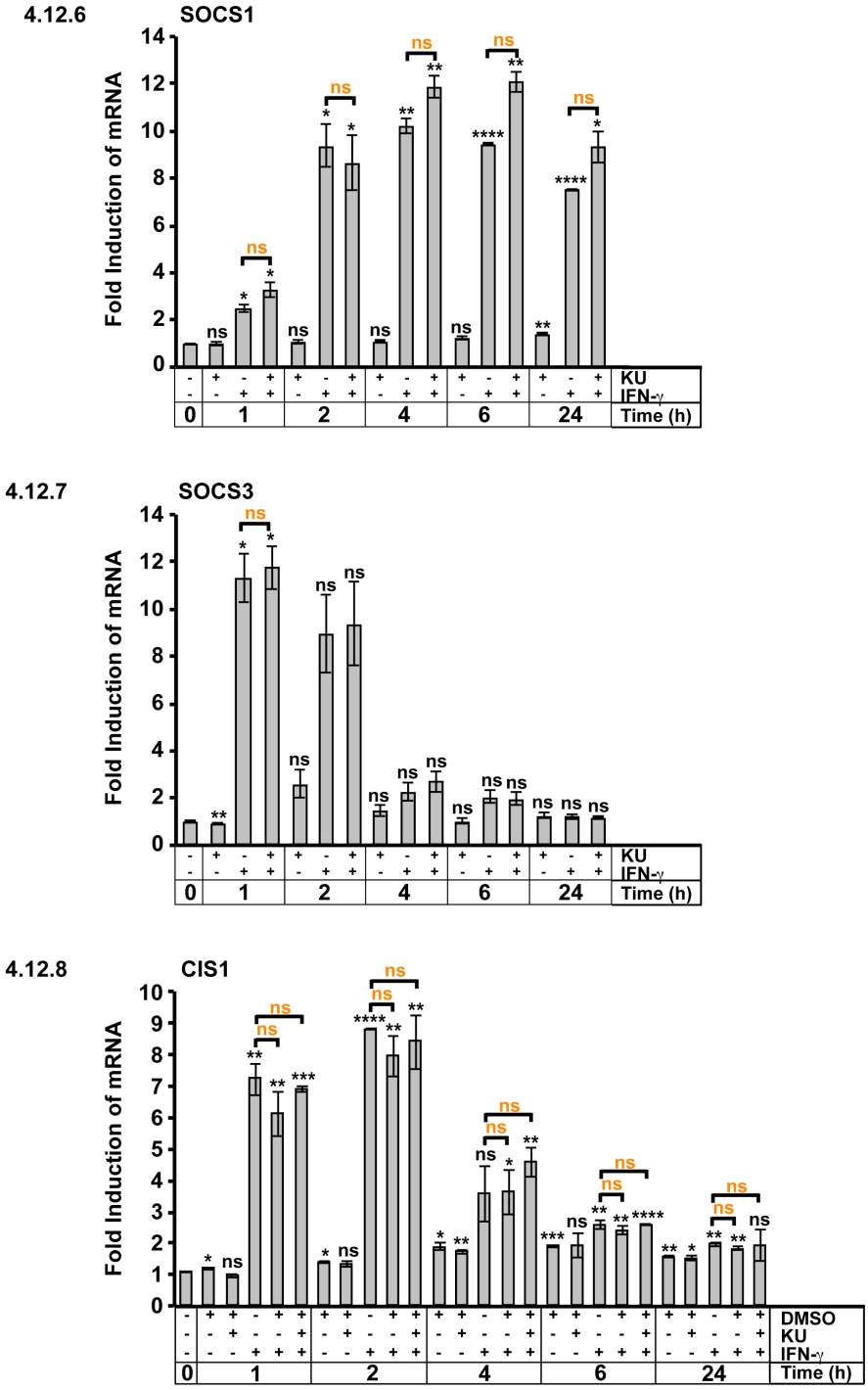


Figure 4.12. Inhibition of ATM's enzymatic activity did not impair the expression of several ISGs in response to IFN-γ. (Continued on page 138).

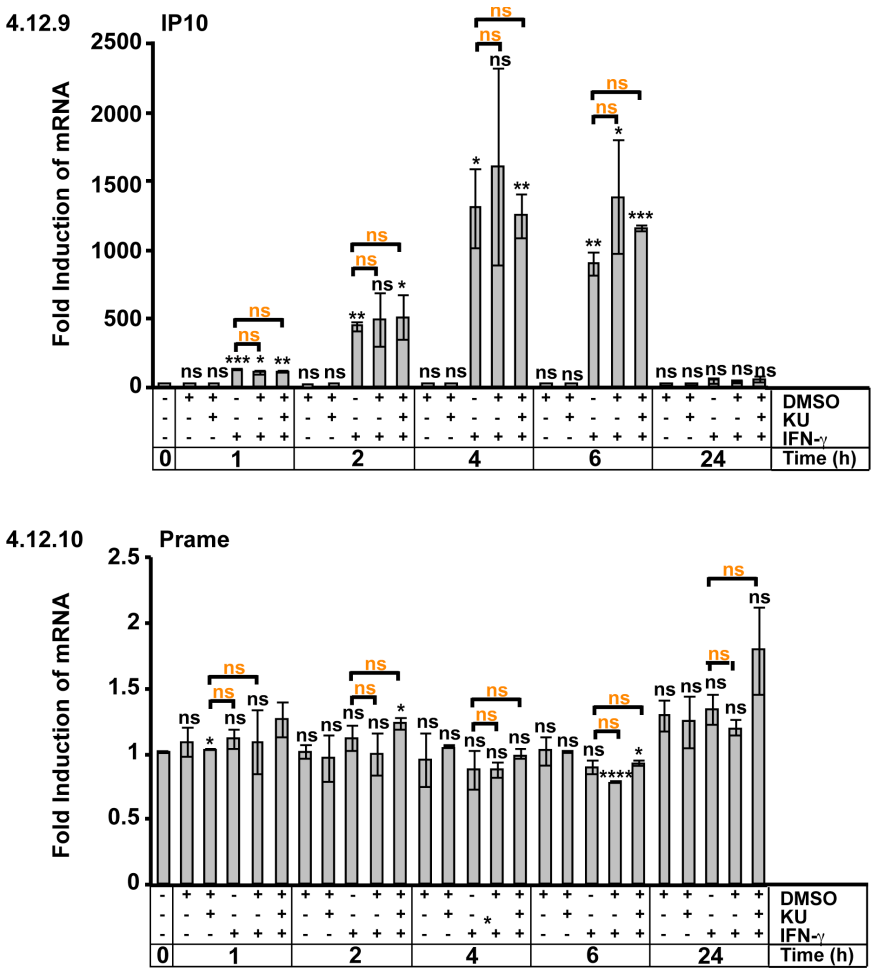


Figure 4.12. Inhibition of ATM's enzymatic activity did not impair the expression of several ISGs in response to IFN-γ. (Continued from page 137).

The early-transcribed ISG IRF-1 was strongly detected after 1 hour of stimulation with IFN-γ ($p<0.01$), similarly induced at the 2 hours time point ($p<0.001$), and this induction decreased slowly along time, albeit it remained statistically significant above basal levels (4 hours, $p<0.01$; 6 hours, $p<0.01$; 24 hours, $p<0.05$) (**Fig.4.12.1**). When silencing ATM, mRNA levels were only assessed after 1 and 6 hours of stimulation and although IRF-1 expression was not impaired for the first time point, we could see a significant impairment for the later one (**Fig. 4.7.1.A**). No significant difference on IRF-1 expression was detected when ATM enzymatic function was inhibited by treatment with KU-55933 for any of the time points of stimulation or for the DMSO control (**Fig. 4.12**).

ATM silencing efficiently impaired CIITA transcription (**Fig. 4.7.2.A**) and, therefore, a similar effect would be expected when using KU-55933 if the enzymatic function of this protein would be required for this ISG transcription. CIITA transcript started to increase after two hours of stimulation, but was only clearly detected for the later time points (**Fig. 4.12.2**). Despite the variation in between experiments, there was never any decrease on the mRNA levels of this transcription factor as a consequence of ATM inhibition. Impairment of Ii and HLA-DRα transcription observed upon ATM silencing (**Fig. 4.7.3** and **Fig. 4.12.3**) was also not recapitulated with the use of the ATM inhibitor KU-55933 (**Fig. 4.12.3** and **Fig. 4.12.4**, respectively) for any of the time points for which significant mRNA induction was observed (4 to 24 hours time points for Ii and for 6 and 24 hours of stimulation for HLA-DRα). These results, together with the lack of impairment on STAT1 serine 727 residue phosphorylation and on HLA class II expression, strongly support the notion that ATM has a structural role, independent of its kinase activity, regarding the regulation of IFN-γ responses.

ATM silencing did not affect significantly TAP1 mRNA transcript levels (**Fig.4.7.5**). Hence, no difference was also expected upon ATM enzymatic inhibition, which was indeed confirmed (**Fig. 4.12.5**). Induction of TAP1 was noticed as early as after 1 hour of IFN-γ treatment ($p<0.01$) and increased steadily through out the experiment (2 hours, $p<0.05$; 4 hours, $p<0.01$). TAP1 transcription stabilised at its maximum fold induction for the 6 and 24 hours time points ($p<0.01$) Inhibition of ATM activity had no effect over any of the time points of stimulation analysed (**Fig. 4.12.5**).

SOCS1 mRNA was detected after 1 hour of IFN-γ stimulation (**Fig.4.12.6**). The levels of SOCS1 mRNA increased with time of stimulation, peaked after 4 hours of treatment and after 24 hours started to decrease. SOCS3 transcription was at its maximum after 1 hour of IFN-γ stimulation and decreased from there onwards, returning to basal levels by 24 hours (**Fig.4.12.7**). Neither SOCS1 nor SOCS3 expression were affected either upon ATM chemical inhibition (**Fig.4.12.6.** and **Fig.4.12.7**, respectively) or by ATM silencing (**Fig.4.7.6.** and **Fig.4.7.7**, respectively). CIS mRNA levels were detected after 1 hour of treatment with the

cytokine ($p<0.01$), they slightly increased at 2 hours ($p<0.0001$) and decreased from there onwards, returning to almost basal levels by the 6 hours of treatment (**Fig. 4.12.8**). At no time of this time course did ATM inhibition affect CIS transcription. This was expected since ATM knockdown also had no effect (**Fig. 4.7.8**).

IP10 mRNA was detected after 2 hours of stimulation, its levels were maximal for 4 hours of stimulation and were undetectable after 24 hours (**Fig. 4.7.9**). IP10 transcription was not affected in the absence of ATM (**Fig. 4.12.9**) and the same was consistently observed when inhibiting this protein's enzymatic activity (**Fig. 4.7.9**). As far as PRAME is concerned, no induction of its mRNA was observed after IFN-γ treatment. However ATM silencing caused a reduction in its basal level of expression (**Fig. 4.7.10**), but, reassuringly, the same did not happen when affecting ATM's enzymatic activity (**Fig. 4.12.10**).

Overall, the data indicate that ATM enzymatic activity was not critical for the role(s) that this protein exerts in specific IFN-γ-induced responses.

4.4. Comparison of matched ATM negative and positive cell lines regarding the role of ATM in IFN-γ pathways

The use of an ATM negative cell line together with a fully matched ATM-complemented one could represent an easier experimental approach to evaluate the role and importance of this protein in IFN-γ signalling. Utilising such cell lines could also mean a reduction in the number of controls necessary to use in each assay compared to silencing ATM using RNAi. It would be, in addition, a way of validating the results obtained with U2OS cells. The immortalised fibroblast cell line GM16667 (ATM^-) was obtained from an A-T donor. It was reported that the absence of ATM protein in this cell line derived from a homozygous frameshift mutation in the ATM gene (Gilad *et al*., 1996), which resulted in a truncated unstable form of ATM protein (Lakin *et al*., 1996). This cell line was stably complemented with an episomal recombinant vector expressing a functional ATM

protein and termed GM16667 (ATM^+), which reversed the A-T phenotype of the parental cell line (Ziv *et al*., 1997).

4.4.1. ATM protein and mRNA levels differed in ATM^+ and ATM^- cells

The ATM^+ and ATM^- cell lines selected had been previously used in our laboratory and were known to be responsive to IFN-γ. We first analysed ATM protein levels, as well as its mRNA expression, to guarantee that these differed between the ATM positive and negative cell lines and established a level of comparison with U2OS cells, thus used here as a positive control for ATM expression (**Fig. 4.13**).

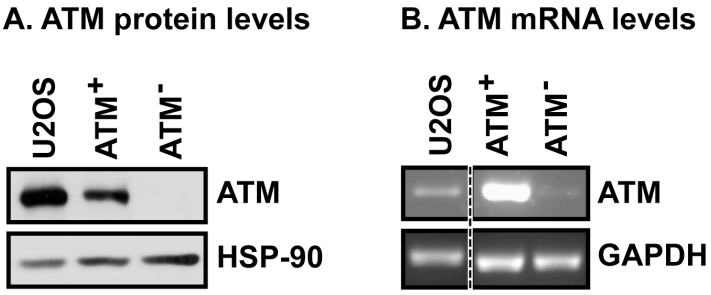


Figure 4.13. The GM16667 (ATM^+) cell line expressed ATM mRNA and protein in contrast to the parental GM16666 (ATM^-) cell line. A. Whole cell extracts were prepared from ATM^+, ATM^- and U2OS cells using Schindler's buffer and proteins were resolved in a 6% SDS-PAGE gel. Western blotting analysis was performed using antibodies against ATM and against HSP-90, which was used as a loading control. **B.** RNA was extracted from ATM^+, ATM^- and U2OS and used in a RT-PCR reaction (40 cycles) to detect mRNA levels of ATM. GAPDH levels were assessed as a loading control.

ATM protein was detectable in the ATM^+ cell line, although the level of expression was significantly lower than in U2OS cells, but no protein was detectable in the ATM^- cells (**Fig. 4.13.A**). ATM mRNA levels of all three cell lines were determined in the same agarose gel, but for clarity, the image was cut and presented in the same loading order of the protein samples. Expression of ATM mRNA was obvious in both ATM^+ and U2OS cells, but it was much higher in the first cell line (**Fig. 4.13.B**). This is possibly due to the protein expression being under control of recombinant vector in the ATM^+ cells. Curiously, this did not translate into a higher amount of ATM protein in the ATM^+ cells, which was in fact superior in U2OS cells

(**Fig. 4.13.A**). A faint level of mRNA was seen in the ATM$^-$ cell line (**Fig. 4.13.B**), which could be attributed to the expression of the endogenous ATM mutated gene, however, no ATM protein was observed in these cells (**Fig. 4.13.A**), in agreement with what has been described in the literature. The differences in mRNA and protein levels of ATM in the ATM$^+$ and ATM$^-$ cell lines served as an indication that this cells could indeed be suitable for investigating the mechanistic aspects of the roles of ATM in IFN-γ signalling in assays such as ChIP. We started to analyse these cells in terms of STAT1 phosphorylation patterns, STAT1 activation/localisation and HLA class I and II expression at the cell surface in response to IFN-γ, in order to draw a parallel with the observations made in U2OS cells upon ATM silencing.

4.4.2. STAT1 phosphorylation and activation differed in ATM$^+$ and ATM$^-$ cells

ATM$^+$ and ATM$^-$ cells were stimulated in time-course experiments with a saturating concentration of IFN-γ of 500 IU/ml. Whole cell lysates were prepared with Schindler's buffer and STAT1 phosphorylation/activation kinetics in response to the cytokine was assessed by western blotting analysis. (**Fig. 4.14**).

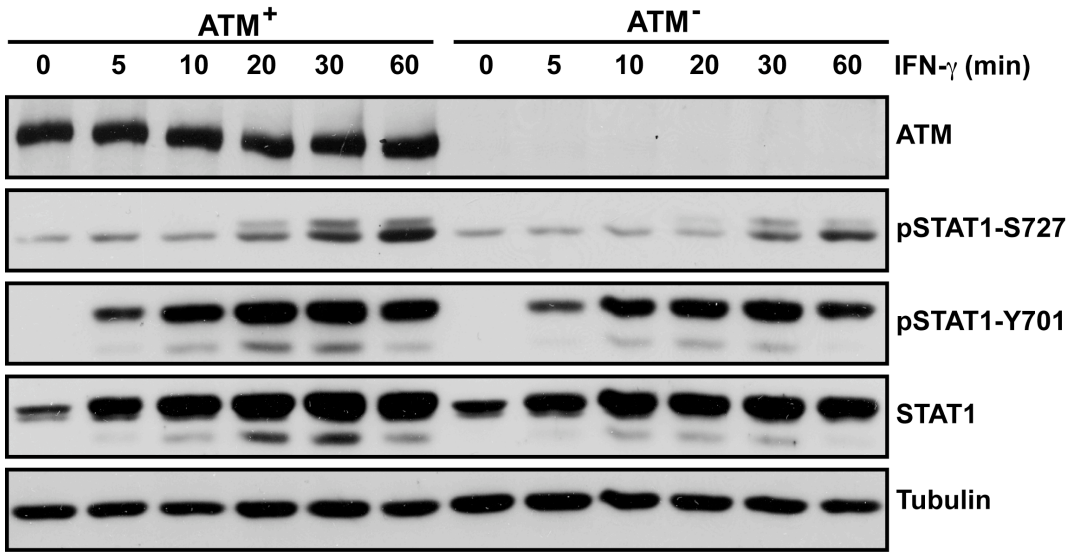


Figure 4.14. STAT1 serine 727 and tyrosine 701 phosphorylation was impaired in ATM deficient cells in response to IFN-γ. ATM$^+$ and ATM$^-$ cells were stimulated with IFN-γ (500 IU/ml) for the indicated time points (minutes - min). Whole cell lysates were prepared using Schindler's buffer and proteins were separated on a 6 % SDS-PAGE gel. Western blotting analysis was

performed using antibodies against pSTAT1-S727 and pSTAT1-Y701 and against ATM and STAT1α/β. Tubulin was used as a loading control. These results are representative of at least three independent experiments yielding identical results.

STAT1 tyrosine phosphorylation was clearly visible in both ATM^+ and ATM^- cells after 5 minutes of stimulation, peaking at 20 and slightly decreasing by 1 hour (**Fig. 4.14**). STAT1 serine 727 phosphorylation induced by IFN-γ could only be clearly detected in ATM^+ cells at 20 minutes, while for ATM^- this only happened at 30 minutes. These phosphorylation kinetics were consistent with what is known about STAT1 tyrosine phosphorylation occurring first in the cytoplasm and serine phosphorylation only occurring later on, upon translocation into the nucleus and binding to DNA. STAT1 tyrosine phosphorylation was consistently diminished after 5 minutes of IFN-γ treatment in ATM^- cells, although it recovered in intensity with increased time of stimulation. Also for the 60 minutes time point, STAT1 tyrosine phosphorylation seemed to be more reduced in these cells, compared to ATM^+ cells. The levels of serine phosphorylation also seemed reduced in ATM^- cells. ATM protein was expressed in ATM^+ cells but was not detectable in the parental cell line. These results appeared comparable to the ones reported using RNAi to silence ATM.

4.4.3. ATM deficiency seemed to result in decreased levels of activated STAT1 both in the nucleus and cytoplasm and this was not attributable to altered localisation of STAT1

Fractionation of cytoplasmic and nuclear proteins of ATM^+ and ATM^- cells was performed to determine if differences in the activation and compartmental distribution of STAT1 dependent on ATM expression could be detected. Cells were stimulated with IFN-γ for 10, 20 and 60 minutes, to focus on a time frame more adequate to assess STAT1 serine phosphorylation (**Fig. 4.15**).

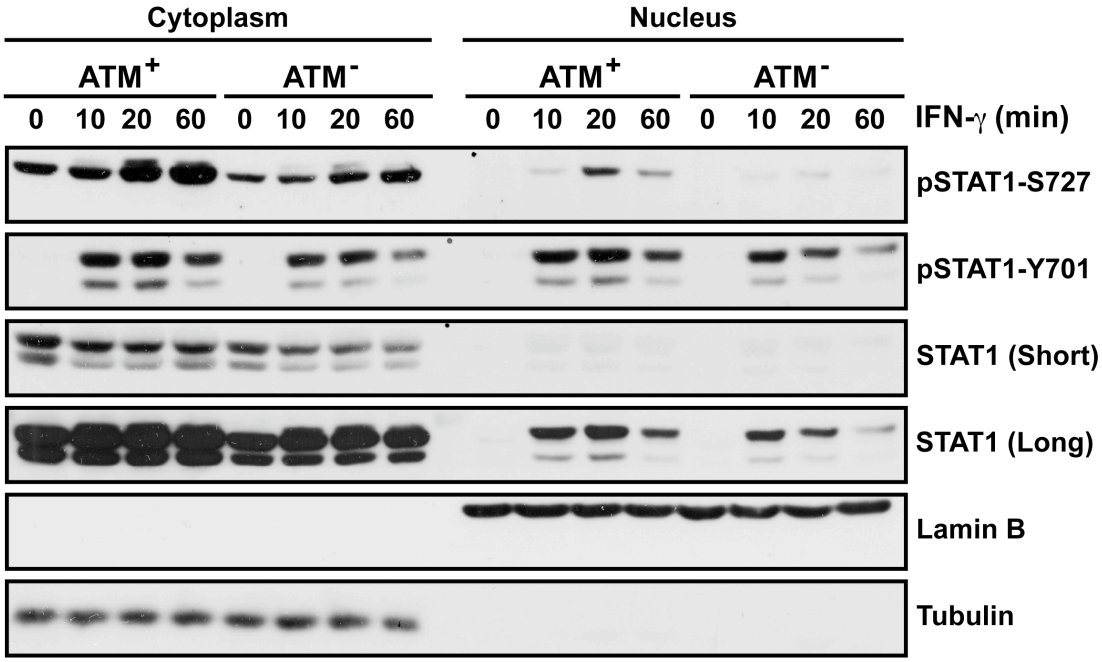


Figure 4.15. STAT1 serine 727 and tyrosine 701 phosphorylation were impaired in ATM deficient cells in response to IFN-γ both in the cytoplasm and nucleus. ATM^+ and ATM^- cells were stimulated with IFN-γ (500 IU/ml) for the indicated time points in minutes (min). Cytoplasmic and nuclear protein fractionation was performed and proteins were resolved on a 7.5 % SDS-PAGE gel. Western blotting analysis was performed using antibodies against pSTAT1-S727 and pSTAT1-Y701 to assess phosphorylation levels in response to IFN-γ and also against STAT1α/β to determine total amounts of this protein. Lamin B and α-tubulin were used as nuclear and cytoplasmic loading controls, respectively. These results are representative of three independent experiments with similar results.

STAT1 serine 727 phosphorylation was noticeable after 20 minutes of treatment, as well as for the longer time points of stimulation, in both ATM^+ and ATM^- cells in the cytoplasm and at 10 minutes in the nuclear compartment (**Fig.4.15**). Nonetheless, the levels of phosphorylation of this specific residue were reduced in ATM^- cells in both compartments, indicating that is not distribution that is impaired, but that are either phosphorylation or dephosphorylation events that are affected. STAT1 tyrosine 701 phosphorylation also seemed to be slightly reduced in ATM^- cells in both compartments, which was particularly visible for the 60 minutes time.

IFN-γ-induced phosphorylation of STAT1 seemed altered in the absence of ATM when comparing the ATM^+ and ATM^- cell lines. This observation prompted us to

further analyse the expression of HLA class I and II and we hypothesised that HLA class II would be impaired in ATM^- cells.

4.4.4. ATM^+ cells presented a distinct profile of HLA class II expression, but do not significantly differ from ATM^- cells in response to IFN-γ

ATM^+ and ATM^- cells were treated with different concentrations of IFN-γ for 72 hours and HLA class I and II expression was analysed as before. Regarding detection of HLA class I at cell surface, we did not detect any significant difference between the cell lines in analysis (**Fig. 4.16.A**). This did not come as a surprise, since all our data so far indicated that ATM is crucial for HLA class II transcription/translation and assembly at the surface of cells, but not for class I. We consistently observed in each individual experiment a higher expression of HLA class II in ATM^+ cells for low concentrations of IFN-γ in the range of 25-50 IU/ml, in comparison with ATM^- cells.

Unexpectedly, however, with increasing concentrations of the cytokine this difference subsided and for the highest concentrations, HLA class II expression in cells not expressing ATM increased and overcame the expression in the complemented cells. Nonetheless, given the minimal differences and the variation in between experiments, upon normalisation, no statistically significant differences were upheld between the ATM^+ and ATM^- cell lines regarding HLA class II expression inducible by IFN-γ (**Fig. 4.16.B**).

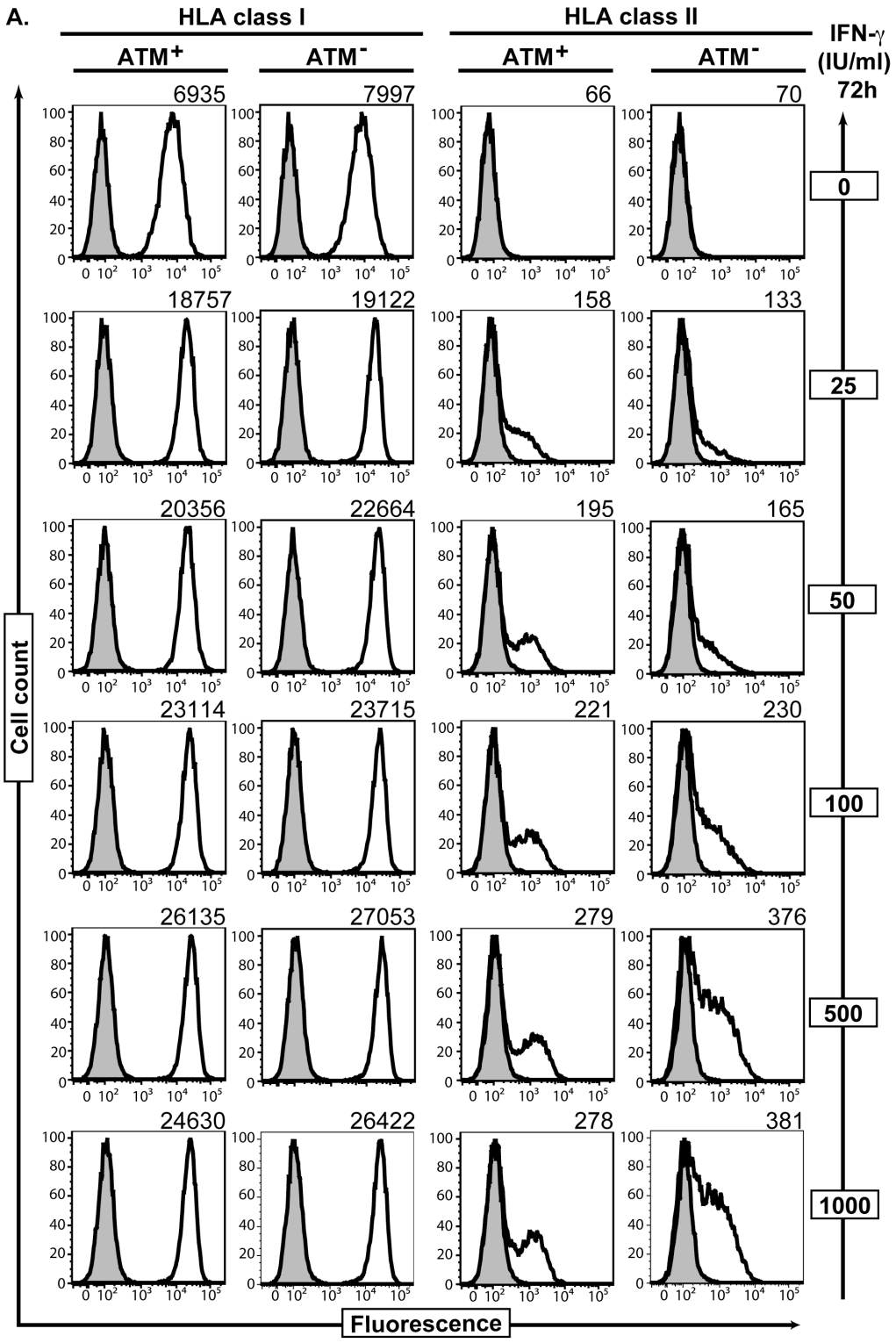


Figure 4.16. Expression of Class I and II HLA in ATM$^+$ and ATM$^-$ cells treated with IFN-γ. (Continued on page 147).

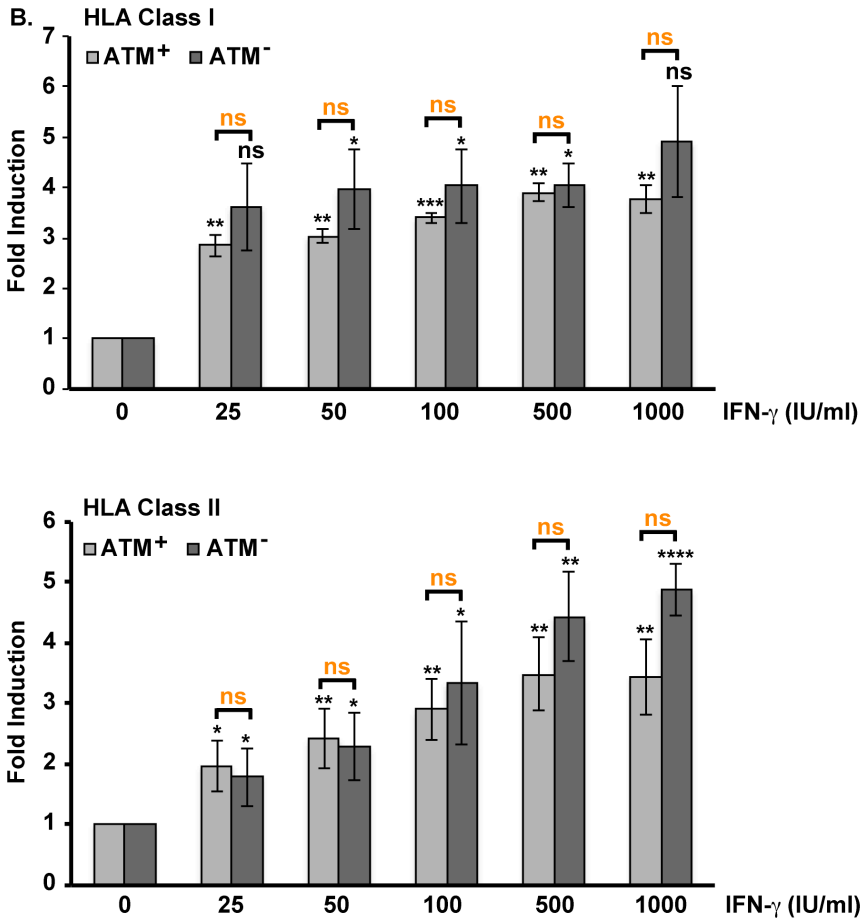


Figure 4.16. Expression of Class I and II HLA in ATM$^+$ and ATM$^-$ cells treated with IFN-γ. ATM$^+$ and ATM$^-$ cells were treated with IFN-γ (25, 50, 100, 500 and 1000 IU/ml) for 72 hours. Cells were then stained using a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody (1st and 2nd vertical panels) or a PE-conjugated anti-HLA-DRα Class II antibody (3rd and 4th vertical panels) and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. **A.** Results were analysed using FlowJo and are presented as the geometric means of fluorescence of stained samples (numbers on the top right hand corner of each graph). One representative experiment of three independent experiments yielding similar results is shown. The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. **B.** Normalisation was done dividing the value of the geometric mean of the treated stained sample by the untreated stained one, for each cell line. Mean ± SD values obtained using two independent experiments after normalisation are shown for HLA class I (upper graph) and class II (lower graph) for both cell lines. Values are expressed as fold change relative to stimulation. Statistical analysis was done using Student's t-test relative to the untreated control of each individual cell line in order to determine the profile of induction of HLA class I and II in each one of them upon IFN-γ treatment (symbols in black). This test was also used to compare between both cell lines for each time point of stimulation (symbols in orange) [non-significant - (ns), $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$].

ATM^+ and ATM^- cells were also treated with low (25 IU/ml) and saturating (500 IU/ml) concentrations of IFN-γ, with or without pre-treatment with the ATM kinase inhibitor, KU-55933 (**Fig. 4.17**).

HLA class I and II expression was analysed as previously. We had anticipated that differences would be observed regarding class II, between ATM^+ and ATM^- cells with the latter presenting a lower expression level, at least for the non-saturating concentration of IFN-γ. We also expected that no difference would be seen upon usage of KU-558933 on ATM^+ cells, therefore, gathering further evidence that, not only HLA class II expression was dependent of ATM in the context of IFN-γ signalling, but also that it was independent of this protein's enzymatic activity. (**Fig.4.17.A** and **Fig.4.17.B**).

Consistently with the data in previous figures (**Fig.4.16.A** and **Fig.4.16.B**), no significant difference was observed in HLA class I expression between both cell lines with or without inhibition of ATM kinase activity (**Fig.4.17.A** and **Fig.4.17.B**). Despite the fact we observed a decrease in STAT1 phosphorylation/activation in the ATM^- cell line compared to the ATM complemented cell line this did not correlate with a difference in terms of final response, indicating that these cell lines were not suitable for the purpose of this study. Consequently all further experiments were performed resorting to ATM silencing in U2OS cells.

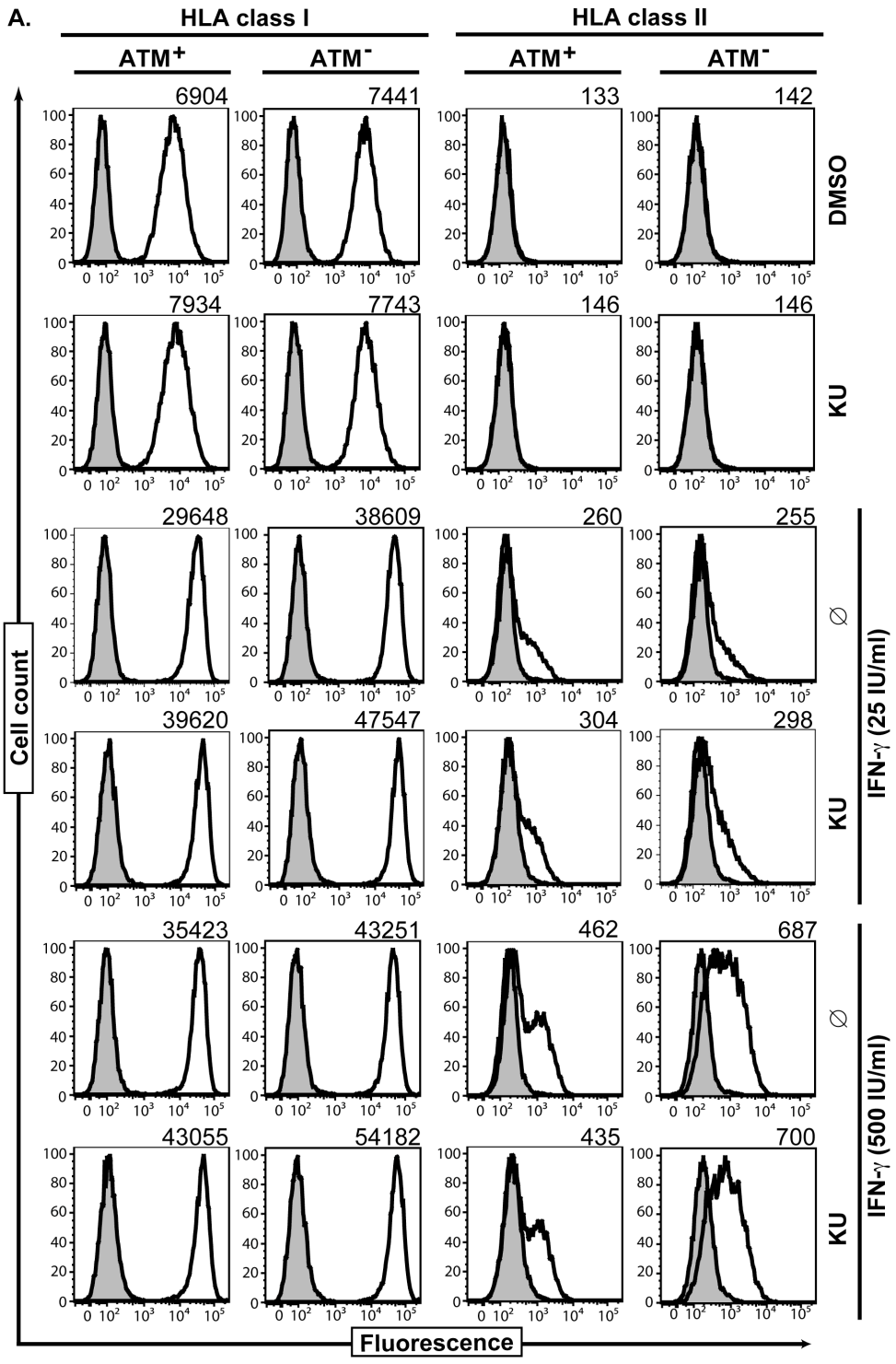


Figure 4.17. KU-55933 inhibition of ATM activity did not impair HLA Class I and II expression on ATM$^+$ and ATM$^-$ cells. (Continued on page 150).

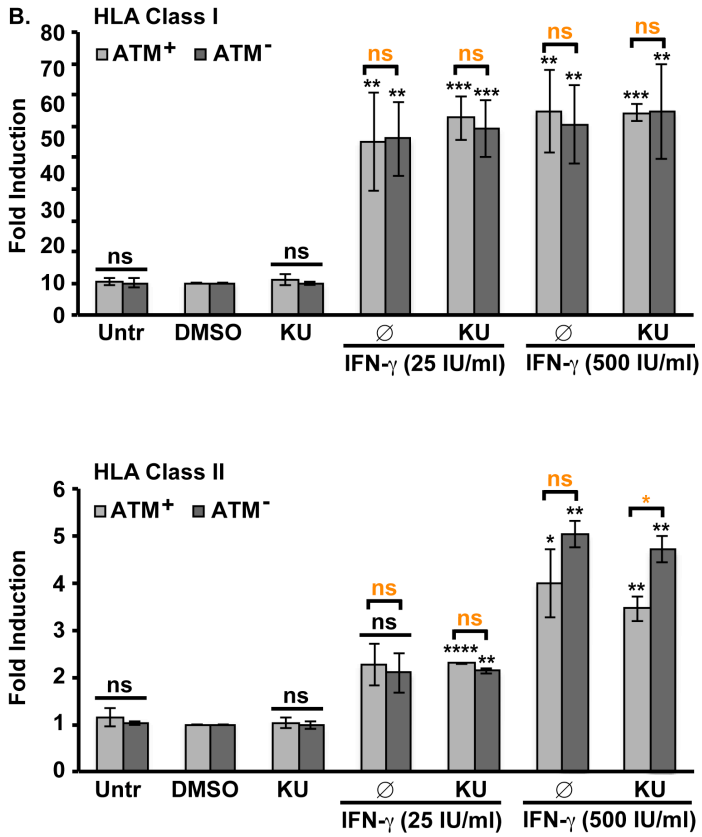


Figure 4.17. KU-55933 inhibition of ATM activity did not impair HLA Class I and II expression on ATM$^+$ and ATM$^-$ cells. ATM$^+$ and ATM$^-$ cells were pre-treated with KU-55933 for 1 hour (KU) or left untreated (∅) and then stimulated with 25 or 500 IU/mL of IFN-γ for 72 hours in the presence or absence of this inhibitor. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody (1st and 2nd vertical panels) or with a PE-conjugated anti-HLA-DRα Class II antibody (3rd and 4th vertical panels) and cell surface expression of these molecules determined by flow cytometric analysis using a FACSCanto. **A.** Results were analysed using FlowJo and are presented as the geometric means of fluorescence of stained samples (numbers on the top right hand corner of each graph). One representative experiment of three independent experiments yielding similar results is shown. The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. **B.** Each condition was normalised, dividing the value of the geometric mean of the stained sample by the value corresponding to the DMSO control. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class I (upper graph) and class II (lower graph). Values are expressed as fold change relative to DMSO control. Statistical analysis was done using Student's t-test relative to the DMSO control of each individual cell line in order to determine the profile of induction of HLA class I and II in each one of them upon IFN-γ treatment (symbols in black). This test was also used to compare between both cell lines for each time point of stimulation with this cytokine (symbols in orange) [non-significant - (ns), p>0.05, *- p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001].

4.5. Discussion

It was previously shown that ATM knockdown inhibited different aspects of IFN-γ biological activities, namely the induction of HLA Class II and the antiviral response (Watling *et al*., 2008). Nonetheless, ATM did not seem to impact on the kinetics of STAT1 activation when STAT1 tyrosine 701 phosphorylation was assessed in cells treated with IFN-γ from 15 minutes to 6 hours of stimulation (Watling *et al*., 2008). STAT1 serine 727 phosphorylation is an additional post-translational modification, which has been reported by Darnell and colleagues as necessary for STAT1 to achieve maximal transcriptional activity (Wen *et al*., 1995). However, the effect of ATM silencing on STAT1 serine 727 phosphorylation in the context of IFN-γ signalling was not addressed by Watling *et al.* (2008). Therefore, we examined the effect of ATM silencing by RNAi on IFN-γ-induced STAT1 serine 727 and tyrosine 701 phosphorylation from 5 to 60 minutes of stimulation.

The results presented here by western blot using whole cells extracts indicated that reduced levels of ATM impacted on the kinetics of IFN-γ-induced STAT1 serine 727 phosphorylation and this was visible at 10 minutes of stimulation (**Figures 4.1**). This phosphorylation was not prevented from occurring, but rather delayed, and by 30 minutes it was similar to the phosphorylation observed in untransfected cells and in cells transfected with siNS or siATR (**Fig. 4.1**). The lysis buffer used (Schindler's buffer) was stringent enough to allow a good degree of rupture of the nuclear membrane, so that the whole cells extracts represented a mixture of cytoplasmic and nuclear proteins (**Fig. 4.1**). However, for gaining a better insight to the phosphorylation pattern and distribution of STAT1 across the cytoplasmic and nuclear compartments, protein fractionation was performed and again analysed by western blot during a more extensive time course of IFN-γ treatment (**Fig. 4.2**).

The reduction/delay in STAT1 serine 727 phosphorylation caused by ATM silencing was also observed and statistically significant in the nuclear compartment at 10 and 20 minutes of IFN-γ treatment (**Fig. 4.2.A** and **B**), where this phosphorylation has been established to occur (Kovarik *et al*., 2001). The

decreased level of serine 727 phosphorylated STAT1 detected in the nucleus upon ATM silencing could be the result of DNA inaccessibility and occur as a consequence of impaired binding of tyrosine 701 phosphorylated-STAT1 to the chromatin. Indeed, it was reported that STAT1 tyrosine 701 phosphorylated proteins must be bound to DNA for serine 727 phosphorylation to occur (Sadzak *et al*., 2008). The observed reduction of STAT1 serine 727 phosphorylated in the cytoplasm could thus be a consequence of STAT1 not being able to access the DNA as readily upon siATM. This could also explain why it was subsequently observed in the cytoplasm upon shuttling of STAT1.

The reason why the impairment of STAT1 serine 727 phosphorylation in the cytoplasm was only significant after 30 minutes of stimulation with IFN-γ (**Fig. 4.2.B**), although clearly visible at 10 and 20 minutes in the western blots (**Fig.4.2.A**), could be attributed to the way quantification had to be done in this compartment. The antibody used against STAT1 serine 727 phosphorylation detected two bands. The lower molecular weight band was always present regardless of stimulation, while the upper band was only detected upon IFN-γ treatment. This was quite striking when comparing cytoplasmic versus nuclear extracts, with the lower band visible only in the cytoplasmic compartment and the IFN-γ-inducible upper band appearing upon cytokine treatment in both compartments, but always being considerably less intense than the lower band in the cytoplasm. The two bands were specific, since STAT1 silencing resulted in the disappearance of both of them (**Fig. 4.1** and **4.2**). The lower band could correspond to an additional serine residue phosphorylated in response to growth factors in the culture media, which could justify its constant presence, with or without IFN-γ stimulation. In fact, several reports have referred serine phosphorylation to occur in response to growth factors without concomitant tyrosine phosphorylation and, in addition, that phosphorylation and dephosphorylation of both tyrosine and serine could be regulated independently (Ceresa and Pessin, 1996; Chung *et al*., 1997; Gotoh *et al*., 1996; Jain *et al*., 1999; Kovarik *et al*., 2001; Ng and Cantrell, 1997; Yamashita *et al*., 1998). For the cytoplasmic extracts, both bands had to be quantified given their close proximity (**Fig.4.2.B**) and this most likely obscured the changes and differences observed on

the cytoplasm regarding STAT1 serine 727 phosphorylation induced by IFN-γ (upper band) when silencing ATM (**Fig.4.2.A**). Nonetheless, ATM regulation of STAT1 serine phosphorylation was demonstrated by western blot, using whole cell extracts and fractionation samples, and additionally by flow cytometry (**Fig. 4.3**).

A decrease in tyrosine 701 phosphorylated STAT1 was also noticeable upon ATM silencing when studying whole cell extracts, particularly at 5 minutes of IFN-γ stimulation (**Fig. 4.1**). ATM knockdown could affect STAT1 tyrosine 701 phosphorylation either in the cytoplasm, the compartment where this event takes place, and/or affect directly or indirectly the nuclear distribution of tyrosine phosphorylated STAT1. When assessing the effect of ATM silencing on STAT1 tyrosine phosphorylation using cytoplasmic and nuclear fractionation, this phosphorylation event seemed to be impaired in the nucleus at the 10 minutes time point (**Fig.4.2.A** and **4.2.C**). This reduction was reproducible and consistently observed in all three experiments and we attributed the fact that it did not stand as statistically significant (**Fig. 4.2.B**) to inter-experimental variation regarding the level of IFN-γ-induced STAT1 tyrosine 701 phosphorylation. While a decrease in STAT1 serine 727 phosphorylation occurring in the nucleus was also seen in the cytoplasm upon STAT1 shuttling, the same was not observed regarding tyrosine 701 phosphorylation. No tyrosine phosphorylation increase was detected for STAT1 in the cytoplasmic compartment, where this initial phosphorylation occurs, as it could be expected if nuclear translocation was impaired. However, even when STAT1 nuclear accumulation peaked, around 30 minutes of stimulation, as seen in time course experiments (**Fig. 4.2.A**) this fraction would only account for a quarter of the total STAT1 cellular pool as previously mentioned in (Haspel *et al*., 1996). Therefore, a small decrease in the nuclear amount of tyrosine phosphorylated STAT1, would be difficult to detect upon shuttling into the cytoplasm considering the much higher percentage of tyrosine phosphorylated STAT1 present in that compartment.

STAT1 nuclear localization when ATM was silenced was not impaired ad shown by Image J quantification of western blots. This could be due to variations between experiments or the fact that, the sensitivity of this method is not enough to

discriminate such impairment. However, if our hypothesis is right and ATM is indeed necessary to allow tyrosine phosphorylated STAT1 binding to the DNA by regulating the condensation status of chromatin, then the absence of ATM could affect both tyrosine and serine phosphorylation in different ways, as well as ultimately STAT1 accumulation in the nucleus. ATM silencing could potentially result in a delay, or impairment, of serine phosphorylation and, in addition, nuclear STAT1 tyrosine phosphorylated unbound to DNA would potentially be more exposed to the action of nuclear tyrosine phosphatases, resulting in its dephosphorylation (Meyer *et al*., 2003), which would lead to a reduction in STAT1 levels in the nucleus as a consequence of increased export to the cytoplasm. The data obtained by flow cytometry determined that the impairment observed in STAT1 tyrosine 701 phosphorylation, caused by ATM silencing, was significant (**Fig.4.3**) and the western blots suggest that it most likely occurred as a consequence of increased dephosphorylation in the nuclear compartment (**Fig.4.2**).

Interestingly, the increase in the amount of serine 727 phosphorylated STAT1 in the nucleus from 10 to 60 minutes, (**Fig. 4.2.C**) was not accompanied by a similar increase in the levels of phosphorylated STAT1 tyrosine 701 in this compartment, even though it decreased from 10 to 60 minutes time point in the cytoplasm. It is possible that only part of the pool of tyrosine phosphorylated STAT1 present in the nucleus was also phosphorylated on the serine residue at 10 minutes post IFN-γ treatment. In addition, at the 60 minutes time point, part of the initially nuclear translocated tyrosine 701 phosphorylated STAT1 molecules may have been dephosphorylated and the remaining STAT1 molecules could all be bound to DNA and phosphorylated in both residues.

The effect of siATM at very early time points after IFN-γ treatment may be enough to perturb the efficient assembly of STAT1 together with the transcription machinery on the DNA and severely impair the transcription of a specific subset of target genes. Reports seem to agree, for instance, that serine 727 transcriptional dependence differs among ISGs (Kovarik *et al*., 2001; Varinou *et al*., 2003). However, the ISGs that were found to be reliant on this post-translational modification for transcription and the level of this dependence varied between

reports. As an example, work done by Kovarik and co-workers (2001) using fibroblasts obtained from STAT1-deficient mice complemented with a wild-type or a serine 727 mutated STAT1, demonstrated only a small dependence for IRF-1 on serine 727 phosphorylation, while Varinou and colleagues (2003) found IRF-1 transcription to be profoundly reduced in STAT1 mutant mice unable to phosphorylate this residue. These disparities may be explained by differences across the mice models used and variations should be expected accordingly to the cellular background each model used. This could ultimately explain why ATM silencing exerted a sustained, prolonged and more intense reduction over IFN-induced expression of HLA Class II than on HLA class I. Indeed, while HLA class II was strongly impaired 24 and 72 hours after treatment with IFN-γ, HLA class I was only modestly impaired at 24 hours and it reached normal levels of expression by prolonging stimulation to 72 hours (**Fig.4.4** and **Fig.4.5**).

ATM was shown to be important in the regulation of IFN-γ signalling at the transcriptional level. ATM silencing did not result in a similar phenotype to STAT1 knockdown and it was crucial only for a subset of ISGs, namely CIITA, Ii and HLA-DRα (**Fig.4.7**). An interesting comparison could be made between, for instance, SOCS1, SOCS3 and CIITA transcription requirements. Expression of both these ISGs depends on the combined effort of STAT1 and IRF1, however, despite this similarity, only CIITA transcription was affected upon ATM silencing.

With regards to IRF-1 transcription, siATM had no effect after 1 hour of stimulation, but it did result in a partial reduction of IRF-1 mRNA after 6 hours of IFN-γ treatment (**Fig. 4.7.1.A**). It could be that as IRF-1 induction weakens at later time points, possibly due to up-regulation of negative regulators of the pathway, transcription occurring at the promoter of IRF-1 gene becomes more susceptible to ATM absence. This could account for the transient impairment caused by ATM silencing over HLA class I expression at cell surface after 24 hours of IFN-γ treatment (**Fig.4.4**), which then subsided to normal levels after a 72 hour period of cytokine stimulation (**Fig. 4.5**), despite considerable amounts of IRF-1 (**Fig. 4.7.1.B**). Despite the efficient reduction on STAT1 protein levels by RNAi, IRF-1 mRNA and protein could still be detected. Acknowledging that the proportion of

STAT1 that is translocated into the nucleus upon stimulation with IFN-γ is much less than the total pool of protein, the remaining amount of STAT1 upon RNAi knockdown could still be enough to guarantee partial transcription of IRF-1 mRNA in comparison with siNS control and expression of IRF-1 protein (**Fig. 4.7.1**).

Although ATM silencing was responsible for strong and sustained reduction on HLA class II expression (**Fig. 4.4** and **Fig. 4.5**), this was not completely abrogated, which could be justified by the fact that a small proportion of CIITA mRNA was still transcribed and translated into protein (**Fig. 4.7.2**), again possibly due to incomplete knockdown. Considering that HLA-DRα transcription is dependent on the previous expression of CIITA, it was impossible to determine if the impairment observed for the expression of Class II molecules was solely the result of CIITA expression being affected or if ATM regulated both CIITA and HLA-DRα expression and, therefore, this was a combined effect.

In contrast, although also not significant, there was a tendency for ATR knockdown to increase TAP1 (**Fig. 4.7.5**) as well as IRF-1 expression (**Fig. 4.7.1.A**) and this could account for the significant rise observed on HLA class I expression at cell surface ($p<0.05$) (**Fig.4.3.B**).

Expression of SOCS1 is induced by IFN-γ, as a way of down regulating and controlling in a timely manner the biological effects of this cytokine. Its expression is dependent of STAT1 activation and subsequent STAT1-induced transcription of IRF-1, which then binds to SOCS1 promoter. ATM silencing did not affect SOCS1 transcription even though it caused a slight reduction on IRF-1 mRNA and protein levels (**Fig. 4.7.1.A and 4.7.1.B**). Therefore, ATM-induced reduction of IRF-1 was not sufficiently severe to impact on SOCS1 induction, at least under these experimental conditions.

These data support our hypothesis that ATM regulation of IFN-γ responses affords a certain degree of specificity. This could be intimately associated with either the complexity of the promoter and transcription complexes assembled, or the need for additional chromatin modifications, involving for instance histone acetylation or changes in the chromatin condensation status. An accumulating body of evidence

indicates that chromatin remodelling is a necessary and central event to several biological processes that involve DNA as a template. These include transcription, replication and DNA repair. It seems likely that these pathways may use common players regarding chromatin remodelling. The yeast INO80 and SWR1 ATP-dependent chromatin remodelling complexes have been shown to be an integral component of pathways that maintain genomic integrity. These complexes and their human counterparts additionally regulate transcriptional processes (Morrison and Shen, 2009). The elimination of nucleosomal DNA supercoiling in elongating spermatids is also facilitated by transient DNA strand breaks (Leduc *et al*., 2008), which are intimately associated with ATM recruitment. Long term IFN-β treatment has also been shown to cause DSBs and ATM activation, while large-scale chromatin decondensation and the induction of chromatin loops in the HLA region on the chromosome 6 are seen after a 10 minutes treatment with IFN-γ (Christova *et al*., 2007).

These, and other examples, prompted us to propose the hypothesis that ATM plays a central role in IFN-γ responses through a mechanism that involves chromatin decondensation or other alterations to facilitate transcription. Given the fact that some transcription factors have the capacity to induce chromatin modifications, for instance STAT1 *via* recruitment of CBP/ p300, a link between STAT1 activation and consequent recruitment of ATM, a chromatin modulator, seems highly feasible. Indeed, ATM has been reported to up-regulate gene transcription upon HDAC inhibition (Jang *et al*., 2010; Lee, 2007)). ATM has also been involved in p53 transcriptional regulation in the context of DNA damage responses and shown to reduce CREB transcriptional activity through its phosphorylation (Shi *et al*., 2004).

ATM mediates several important biological functions, which have been related to its serine/threonine kinase activity. Given the fact that ATM serine 1981 phosphorylation, associated with its kinase activity, was detected as soon as 15 minutes after IFN-γ treatment (Watling *et al*., 2008), we further assessed the relevance of ATM enzymatic activity. Nonetheless, the inhibition of ATM kinase activity by the ATP-competitive inhibitor KU-55933 had no effect on any of the

analysed IFN-γ-induced responses, contrarily to what was observed when silencing ATM by RNAi. STAT1 serine 727 and tyrosine 701 phosphorylation were not decreased (**Fig. 4.9**), HLA class II expression at cell surface was not impaired (**Fig. 4.11**) and transcription of CIITA, HLA-DRα and Ii was also not reduced when ATM enzymatic activity was inhibited. It is, therefore, possible that ATM played a structural role, independent of its kinase activity, in IFN-γ responses. Divergences between inhibiting ATM kinase activity and eliminating the protein have already been encountered (Choi *et al*., 2010).

However, it has been suggested that ATM could also become catalytically active by a conformational change, possibly without involving autophosphorylation on serine 1981 (Lavin and Kozlov, 2007). It is thus possible that in this cellular context and within IFN-γ responses, a conformational change, different from that taking place during the process of DSB repair, may be unaffected by the ATP-competitive inhibitor used here. In order to firmly establish ATM kinase activity irrelevance for IFN-γ signalling, additional work would be necessary and bearing that in mind ATM wild type and kinase-dead mutants green fluorescent protein (GFP)-tagged were obtained. Unfortunately, due to time restrictions, it was not possible to transfect U2OS cells with the ATM plasmids and to assess if differences would result from the absence of kinase activity regarding HLA class II expression, which would be once again used as a read-out.

After establishing that the levels of ATM mRNA and protein between the ATM^+ and ATM^- cell lines differed (**Fig. 4.13**) STAT1 serine 727 and tyrosine 701 phosphorylation were analysed and found to be decreased in the ATM^- cell line compared to the complemented one (**Fig. 4.14** and **Fig. 4.15**). However, no significant differences were detected in terms of HLA class I and II expression (**Fig.4.16**). The shape of the population expressing HLA class II was clearly distinct between both cell lines, possibly indicating the existence of a non-homogenous level of expression of ATM on the individual complemented cells. If this was the case, which could have been assessed by flow cytometry, by detecting ATM in individual cells, an ATM-high expressing population could have been sorted and then compared with the parental cell line. As an alternative, co-

staining of ATM and HLA class II could have been used to determine the relation between the expression of these molecules and the amount of ATM. Another possibility would be that ATM presence for a correct expression of these molecules in these cells is only needed in suboptimal conditions of stimulation, or that these cell lines have adapted and possess counter-acting mechanisms perhaps involving the recruitment of other proteins to substitute for ATM. Therefore, it was decided to continue the experiments using ATM silencing by RNAi and stop using the ATM^+ and ATM^- in this study.

In summary, data presented in this chapter support a role for ATM in the regulation of IFN-γ signalling pathway. We have shown that ATM modulated STAT1 phosphorylation/activation and localisation. Expression of HLA class II, but not of HLA I, was strongly dependent on ATM. This distinctive effect was caused at the transcriptional level, with ATM regulating transcription of a specific subset of ISGs necessary for HLA class II transcription and processing or of HLA-DRα itself. ATM enzymatic activity did not seem to be involved in any of these effects, which suggested that ATM's role in the regulation of IFN-γ biological responses did not depend of its canonical kinase and may be dependent solely of its structure.

CHAPTER 5

Results

Mechanistic insights into the role of ATM in the modulation of IFN-γ signalling pathway: from transcription-induced DSBs to promoter binding and protein-protein interactions

5.1. Introduction

Having established the physiological importance of ATM in IFN-γ signalling we proceeded to explore the underlying molecular mechanism of action of ATM. We focused our attention in two important questions, namely, how is ATM recruited upon IFN-γ signalling and how does ATM affect the outcome of this cytokine's biological activity at the level of transcription. We hypothesized that ATM could act in a similar way in the regulation of IFN-γ signalling as it does in the realm of DDR, where ATM is a key player.

The DDR is a mechanism of tumour suppression that acts by preventing genomic instability (Fan *et al*., 2006). ATM is known to be a crucial mediator of responses to DSBs, becoming rapidly recruited and activated following DNA damage (Shiloh, 2006). Upon DNA DSBs formation, human ATM, a serine-threonine kinase that exists as an inactive dimer, undergoes auto-phosphorylation of serine 1981, which results in the formation of ATM enzymatically active monomers (Bakkenist and Kastan, 2003; Helt *et al*., 2005; Kastan and Lim, 2000; Rogakou *et al*., 1998). This activity was not, however, according to our data, necessary for IFN-γ signalling. Upon localization at the DSBs sites, ATM then initiates a complex cascade of phosphorylation events of downstream targets, ultimately leading to the repair of these lesions (Bakkenist and Kastan, 2003).

The MRN complex, formed by MRE11, RAD50 and NBS1, acts as an important sensor of DNA damage. This complex, which accumulates in nuclear foci upon detection of DSBs, seems to be responsible for recruiting ATM to these foci and for guaranteeing ATM's stable association with DNA ends and its activation (Dupre *et al*., 2006; Lavin and Kozlov, 2007; Srivastava *et al*., 2009; Sun *et al*., 2005). ATM is the main mediator of histone H2AX phosphorylation on serine 139 residue (referred to as γ-H2AX) on the chromatin flanking the DSB (Bakkenist and Kastan, 2003; Hakem, 2008; Helt *et al*., 2005; Kastan and Lim, 2000; Rogakou *et al*., 1998). γ-H2AX has an important role in chromatin reorganisation and remodelling by establishing a docking platform that ensures accumulation of ATM downstream effectors, such as BRCA1, 53BP1 and the MRN complex *via* the recruitment of the

scaffold protein MDC1 (Kolas *et al*., 2007; Reina-San-Martin *et al*., 2003; Sakamoto *et al*., 2007; Spycher *et al*., 2008; Stucki *et al*., 2005).

H2AX phosphorylation on serine 139 is an early event in the DSB-induced DDR. γ-H2AX accumulation on each side and in adjacent areas of the DSBs, can be visualised as discrete foci by immunofluorescence microscopy and this approach has been used to determine the occurrence of these DNA lesions (Bonner *et al*., 2008; Vasireddy *et al*., 2010). γ-H2AX foci can be generated not only by DNA damage inflicted by exogenous agents like ionizing radiation, but also as a consequence of several intrinsic metabolic cellular processes, associated with production of reactive oxygen species ROS, (Bonner *et al*., 2008). A discrete amount of γ-H2AX foci can occur, for instance, during differentiation of reproductive cells (Hamer *et al*., 2003), in the mitosis phase (McManus and Hendzel, 2005) and during meiotic recombination (Lange *et al*., 2011). Importantly, H2AX phosphorylation can also occur as a consequence of transcription-induced DSBs and activation of ATM (Sordet *et al*., 2009). To determine if IFN-γ stimulation led to an increase in the number an/or area of γ-H2AX foci, we analysed the occurrence of these foci in U2OS cells, as a commonly accepted and used sensitive marker for DSBs formation and ATM activation.

Topoisomerase II is an enzyme that introduces transient discrete DSBs in the context of several biological processes (Nitiss, 2009; Pommier *et al*., 2010). Occurrence of transcription-induced DSBs has been reported (Guirouilh-Barbat *et al*., 2008) and topoisomerase II is known to be necessary to allow decondensation of regions where coiling of DNA increases, as it happens during active transcription (Williamson and Lees-Miller, 2011). In addition, binding of topoisomerase II on gene promoters and the introduction of DSB by this enzyme are known to be involved in transcriptional initiation and regulation in response to nuclear receptors and transcription factors. A connection was established between topoisomerase II-induced DSBs, the subsequent activation of the DDR effector poly[adenosine diphosphate-ribose] polymerase-1 (PARP1), and changes on chromatin structure by histone H1-high-mobility group B (HMGB1) (Ju *et al*., 2006). PARP1 has also been implicated in NF-κB-mediated apoptosis in

response to genotoxic stress, as it is necessary to recruit ATM upon DSBs (Stilmann *et al*., 2009). In the context of DDR, ATM has been shown to increase transcription of genes as a result of histone deacetylase activity inhibition (Jang *et al*., 2010).

Several reports have linked transcription-induced DSBs, recruitment of ATM by these DNA lesions, the activation of DDR effectors by transcription and the regulation of this process by the repair machinery in chromatin remodelling. Therefore, in addition to monitoring γ-H2AX formation in response to IFN-γ, a topoisomerase inhibitor was used to determine if blocking chromatin remodelling by this enzyme, necessary to remove excessive coiling of DNA during transcription, would have a similar effect on IFN-γ responses as those seen when ATM was silenced.

It is now well established that chromatin condensation status must be regulated so that transcription and other processes that depend on DNA accessibility can occur (Downey and Durocher, 2006; Morrison and Shen, 2009). Condensed chromatin generally inhibits transcription by not allowing the binding of transcriptional regulators, as well as the process of elongation carried out by RNA polymerase II. This can be reverted by cellular molecular mechanisms capable of altering the nucleosomal structural organisation and consequently, chromatin condensation (Downey and Durocher, 2006). DNA processing and metabolism rely, therefore, on chromatin modifying factors. Interestingly, ATM role in DSBs repair has also been attributed to its ability to promote heterochromatin decondensation (Goodarzi *et al*., 2008).

It is conceivable that the capacity of ATM to temporarily perturb heterochromatin condensation status (Goodarzi *et al*., 2008), changing its topology and increasing DNA accessibility, could also be relevant and necessary for adequate transcription in response to IFN-γ. ATM may regulate chromatin accessibility upon IFN-γ stimulation, affecting STAT1 binding at the level of ISGs promoters and/or the recruitment of the transcription machinery along the ISGs. The ability of ATM to specifically alter STAT1 binding to the promoters of a subset of ISGs could explain the impairment observed in IFN-γ-induced transcription and expression of specific

ISGs caused by ATM silencing. We assessed this possibility by performing STAT1 chromatin immunoprecipitation assays at the level of the promoter region of IRF-1, TAP1, CIITA and HLA-DRα upon IFN-γ treatment and by silencing ATM with RNAi. Moreover, we assessed if ATM role in IFN-γ-mediated biological responses involved direct binding of ATM to the promoter regions of the above-mentioned ISGs.

Transcription initiation and elongation involve the recruitment and assembly of multi-protein complexes. No reports were found in the literature regarding interactions between ATM and STAT1 proteins. It is possible, however, that ATM-mediated regulation of the IFN-γ/STAT1 signalling occurs, for example, through a post-translational modification of STAT1, involving an interaction with ATM, which could alter its activity or DNA binding ability. Therefore, we performed a double immunofluorescence staining of STAT1 and ATM, and assessed if these proteins co-localised in the absence of or upon IFN-γ treatment by confocal microscopy. We additionally investigated using immunoprecipitation if ATM and STAT1 interacted and if this was inducible by IFN-γ.

5.2. Transcription-induced DSBs were not involved in IFN-γ signalling

As mentioned before, DSBs can occur to remove excessive transcription-induced coiling of DNA and these transient lesions are introduced by the activity of topoisomerase II. If DSBs formed as a consequence of IFN-γ-mediated transcription, and this was the cue for ATM recruitment, then an increase in DSBs should be expected shortly after cytokine treatment. Taking into consideration that silencing ATM by RNAi affected IFN-γ-induced phosphorylation of STAT1 after 10 to 20 minutes of stimulation, cytokine-induced DSBs could signal ATM to these damage sites quickly, thereby maintaining a relaxed conformation of the chromatin suitable for transcription. On the contrary, if γ-H2AX foci were induced by IFN-γ at later points, then this would likely be the result of cumulative coiling of DNA subjected to continuous and prolonged transcription and unlikely to link ATM to IFN-γ signalling, since we see this being affected by ATM silencing by RNAi after short periods of stimulation.

5.2.1. γ-H2AX foci, which signal the location of DSBs, were not increased in response to IFN-γ treatment

In order to determine γ-H2AX foci formation by immunofluorescence microscopy, different fixation/permeabilization options and primary antibody concentrations were tested, using cells treated with etoposide as a positive control for DSBs formation. Etoposide stabilizes the cleavage complexes formed by topoisomerase II and the DNA, which results in permanent DSBs signalled by γH2AX by preventing progression of either DNA replication forks or RNA polymerase (Pommier, 2006; Pommier, 2009).

U2OS cells were treated with IFN-γ in an extensive time course, fixed using PFA, permeabilized with triton X-100 and stained for γ-H2AX, initially using glass coverslips (**Fig. 5.1.A** and **B**). The immunofluorescence images of the unstained controls and of the controls for the secondary antibody unspecific binding (**Fig. 5.1.A**) provided information regarding the specificity and quality of the γ-H2AX staining (**Fig. 5.1.B**). γ-H2AX foci localised in the nucleus of cells, which is expected as this histone functions in the packaging and structural organisation of the DNA into nucleosomes. Foci of γH2AX phosphorylated on serine residue 139 were present in all IFN-γ-treated samples; however, γH2AX phosphorylation was equally visible in untreated cells, which could be the result of transcription and replication of these highly proliferative cells (**Fig. 5.1.B**).

Given the diversity in the number and area of γ-H2AX foci, in order to allow their counting in a statistically significant number of cells, the staining was done in clear-bottomed 96-well plates instead of glass coverslips and a screening microscope was used for image acquisition. Analysis of the images was done with Methamorph software. Data relative to the number (**Fig. 5.1.C**) and area (**Fig. 5.1.D**) of γ-H2AX foci of an average of 3150 cells *per* condition was compared to the stained untreated condition using Student's t-test.

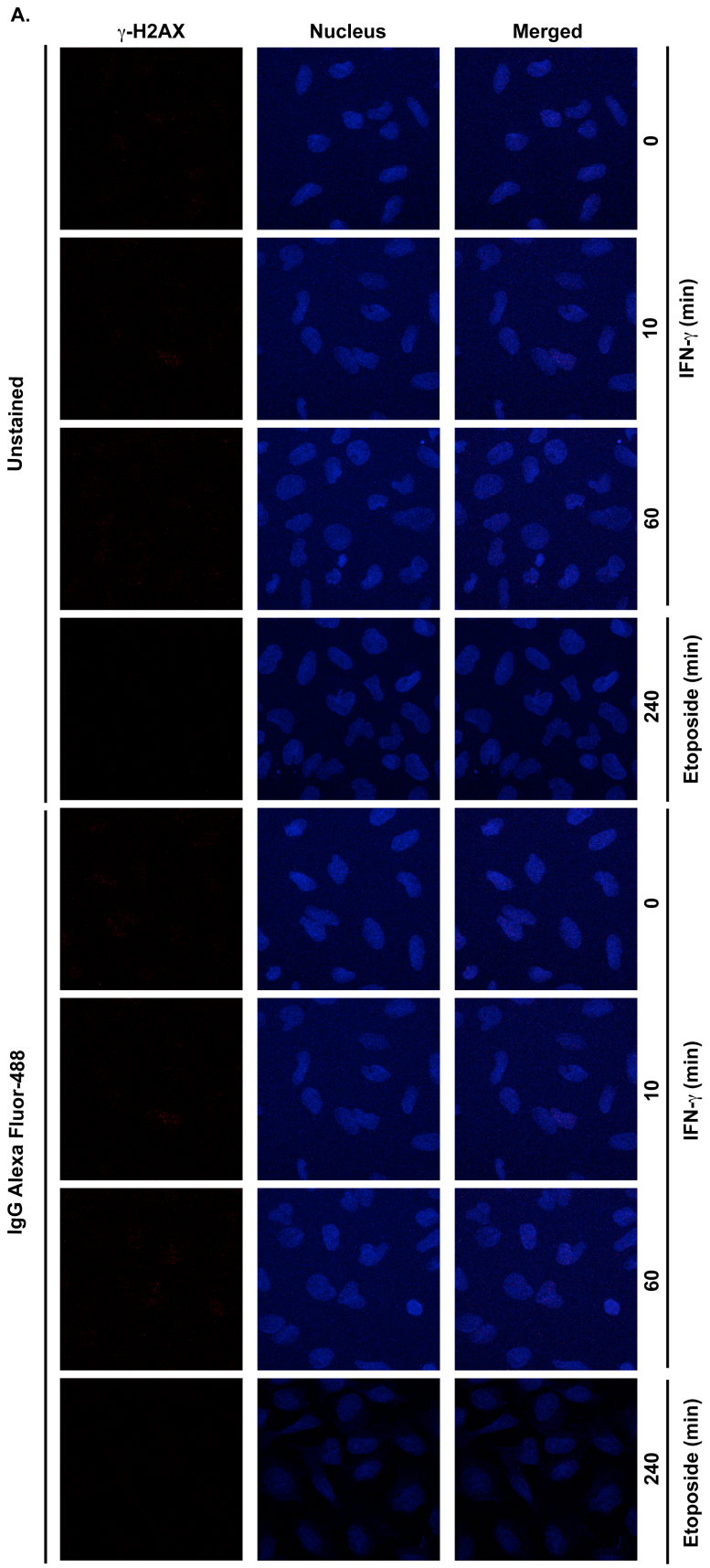


Figure 5.1. γ-H2AX foci number and area were not increased in U2OS cells as a consequence of IFN-γ treatment. (Continued on pages 167 and 168).

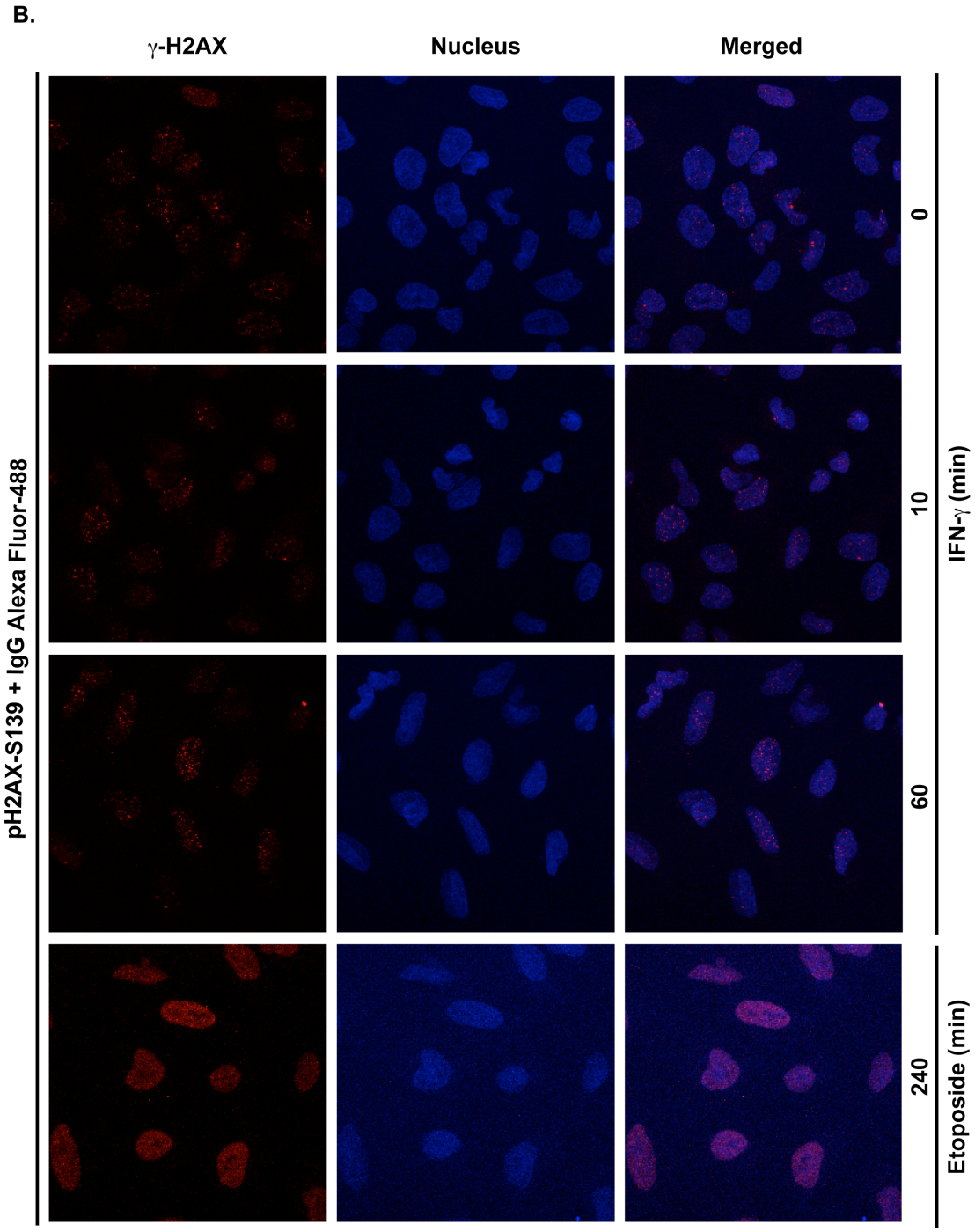


Figure 5.1. γ-H2AX foci number and area were not increased in U2OS cells as a consequence of IFN-γ treatment. (Continued on page 168).

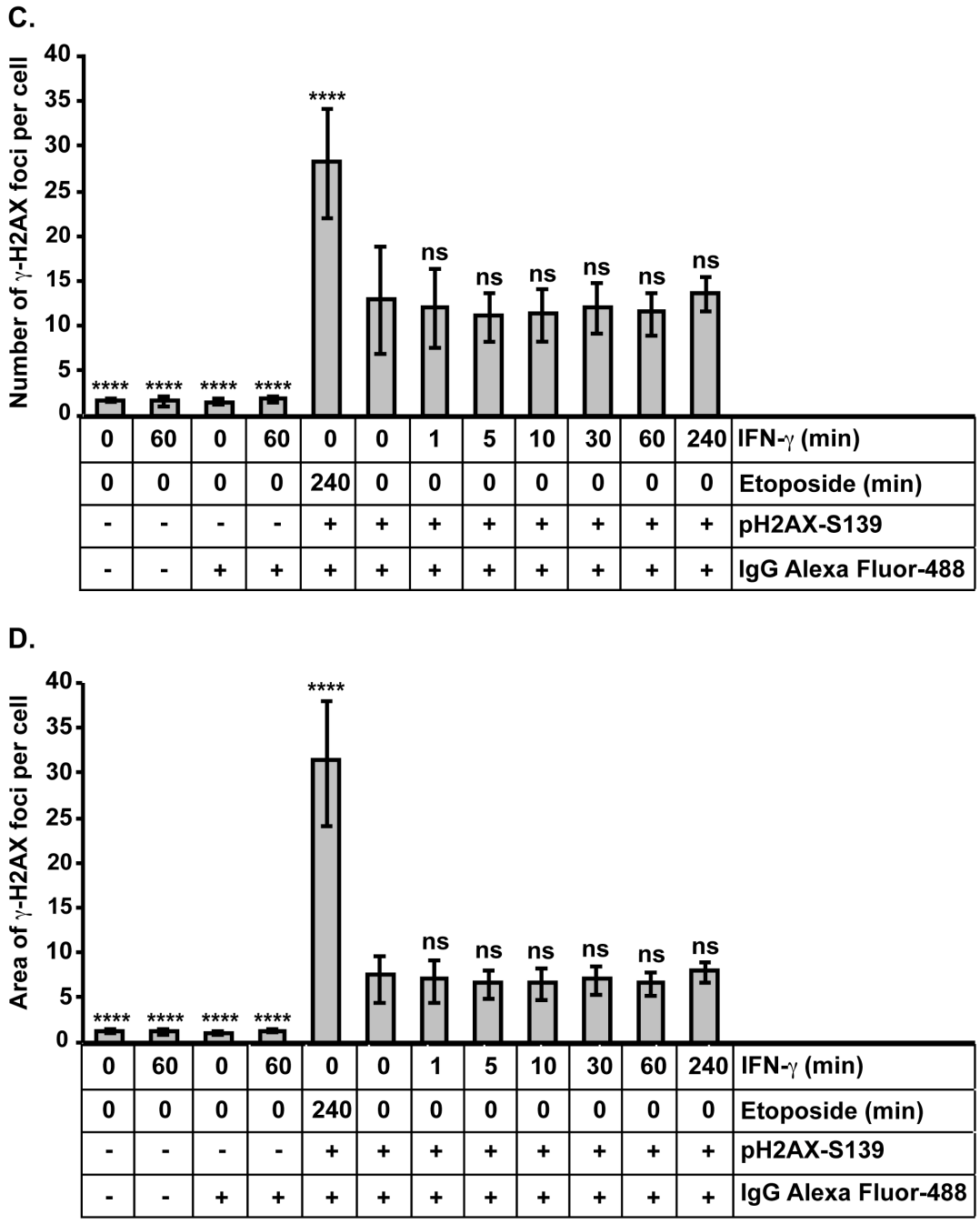


Figure 5.1. γ-H2AX foci number and area were not increased in U2OS cells as a consequence of IFN-γ treatment. A. U2OS cells were plated on a transparent surface and stimulated with IFN-γ (500 IU/ml) or with etoposide for the indicated time (minutes - min). Cells were fixed with 4% PFA and permeabilized with 0.1% of Triton-X in PBS. Staining was performed using a pH2AX-S139 uncoupled primary antibody and an anti-rabbit-IgG Alexa Fluor-488. Nuclear counterstaining was carried out with TO-PRO-3. **A** and **B.** Examples of the quality of γ-H2AX optimised staining are shown for U2OS cells plated on glass coverslips (A. unstained and secondary antibody only stained controls; B. pH2AX-S139 staining). Images were visualised under a Zeiss LSM 510 Meta Confocal microscope with a 20x/1.4 oil immersion objective lens using

Immersol 518 oil. Images composites of approximately 20 x 0.5 μm Z-stacks were obtained using Zeiss LSM 510 software. Overlapping images of pH2AX-S139 and TO-PRO-3 are shown on the 3th vertical panel. **C** and **D.** U2OS cells were plated on clear-bottom 96-well plates, stimulated and stained with pH2AX-S139 antibody and counterstained with DAPI to visualize the nuclei. Image acquisition was done using a Molecular Devices ImageXpress Micro Screening Microscope. A total of 42 fields *per* condition were acquired, each field containing an average of 75 cells, and were analysed using Methamorph software. Data are presented as average *per* cell of γ-H2AX foci number (C) and area (D). This experiment was repeated twice with similar results. Statistical analysis was performed using Student's t-test relative to the untreated stained condition [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$].

Foci of phosphorylated H2AX on serine 139 were clearly observed in both untreated and IFN-γ-treated cells. No difference regarding either the number (**Fig. 5.1.C**) or the area (**Fig. 5.1.D**) of γ-H2AX foci was noticeable upon IFN-treatment, independently of the period of stimulation. The number and area of γ-H2AX foci induced by etoposide was significantly higher in comparison with the level of foci in untreated cell ($p<0.0001$), as well as in IFN-γ-stimulated cells. The unstained and IgG-stained controls presented basal levels of auto-fluorescence, significantly inferior to the levels of γ-H2AX staining ($p<0.0001$).

5.2.2. Inhibition of chromatin remodelling and DSBs formation by topoisomerase II: a similar effect to ATM silencing on ISGs transcription?

Deciphering how ATM is recruited upon IFN-γ signalling, as an important modulator of this pathway, could facilitate understanding ATM's mechanism of action. The role of ATM as a chromatin modulator has been so far associated with this protein's function as a central coordinator for DSDs repair mechanisms. ATM recruitment to chromatin has been demonstrated to involve detection of disturbances, for instance, through occurrence of DSBs (Bakkenist and Kastan, 2003; Lavin and Kozlov, 2007). DSBs are known to occur also as a consequence of active transcription mediated by topoisomerase II action. Thus, we wondered if very early (and, presumably, low level) transcription-induced DSBs could mediate ATM recruitment and enable IFN-γ signalling. In addition to IFN-γ-induced γH2AX foci, we analysed the effect inhibiting the catalytic activity of topoisomerase II had on the levels of mRNA of several ISGs (**Fig.5.2**). In order to do so, we used a

chemical compound named merbarone, which blocks topoisomerase II enzymatic DNA cleavage and thus prevents DSBs formation (Fortune and Osheroff, 1998), contrarily to the action of etoposide, which blocks the dissociation of the cleavage complex from the DNA, leading to permanent DSBs (Kusari *et al*., 2009; Pommier, 2006). U2OS cells were pre-treated with merbarone for 1 hour, to guarantee appropriate inhibition of topoisomerase II prior to IFN-γ stimulation. ISG transcription was assessed by measuring mRNA levels using qPCR as described before. For each individual experiment mRNA induction was compared between merbarone pre-treated cells stimulated with IFN-γ and cells treated only with the cytokine, using fold induction values normalised to the untreated condition (**Fig. 5.2**).

Transcription kinetics of IRF-1, TAP1, CIITA, HLA-DRα and Ii in cells treated with IFN-γ were similar to what had been previously observed for similar time courses (**Fig. 4.12**). The use of merbarone had no significant effect on IFN-γ-driven transcription of IRF-1, which mimicked what was observed when silencing ATM (**Fig. 4.7.1**). TAP1 transcription was essentially not affected by merbarone and although a significant reduction could sometimes be seen for a particular time-point, this was not consistent across experiments and the effect was no longer obvious upon prolonged cytokine treatment (**Fig. 5.2.2**).

Surprisingly, in contrast with ATM knockdown (**Fig. 4.7.2**), inhibition of topoisomerase II catalytic activity by merbarone had no effect on CIITA mRNA levels after cytokine treatment (**Fig. 5.2.3)**. Although a consistent and statistically significant impairment could be observed for IFN-γ-induced transcription of Ii and HLA-DRα upon treatment with merbarone (**Fig. 5.2.3** and **Fig. 5.2.4**, respectively), this reduction was not as striking/ severe as when silencing ATM for the same periods of stimulation (**Fig. 4.7.3** and **Fig.4.7.4**). Indeed, ATM silencing consistently impaired IFN-γ-induced production of HLA-DRα mRNA after 6 hours of stimulation to non-significant levels (**Fig. 4.7.4**), while the fold reduction in Ii mRNA levels was also higher than with merbarone (**Fig. 4.7.3**). The inhibition of topoisomerase II catalytic activity by merbarone, preventing chromatin structure re-organisation through the introduction of transient DSBs did not, therefore, completely recapitulate the effects of ATM silencing by RNAi.

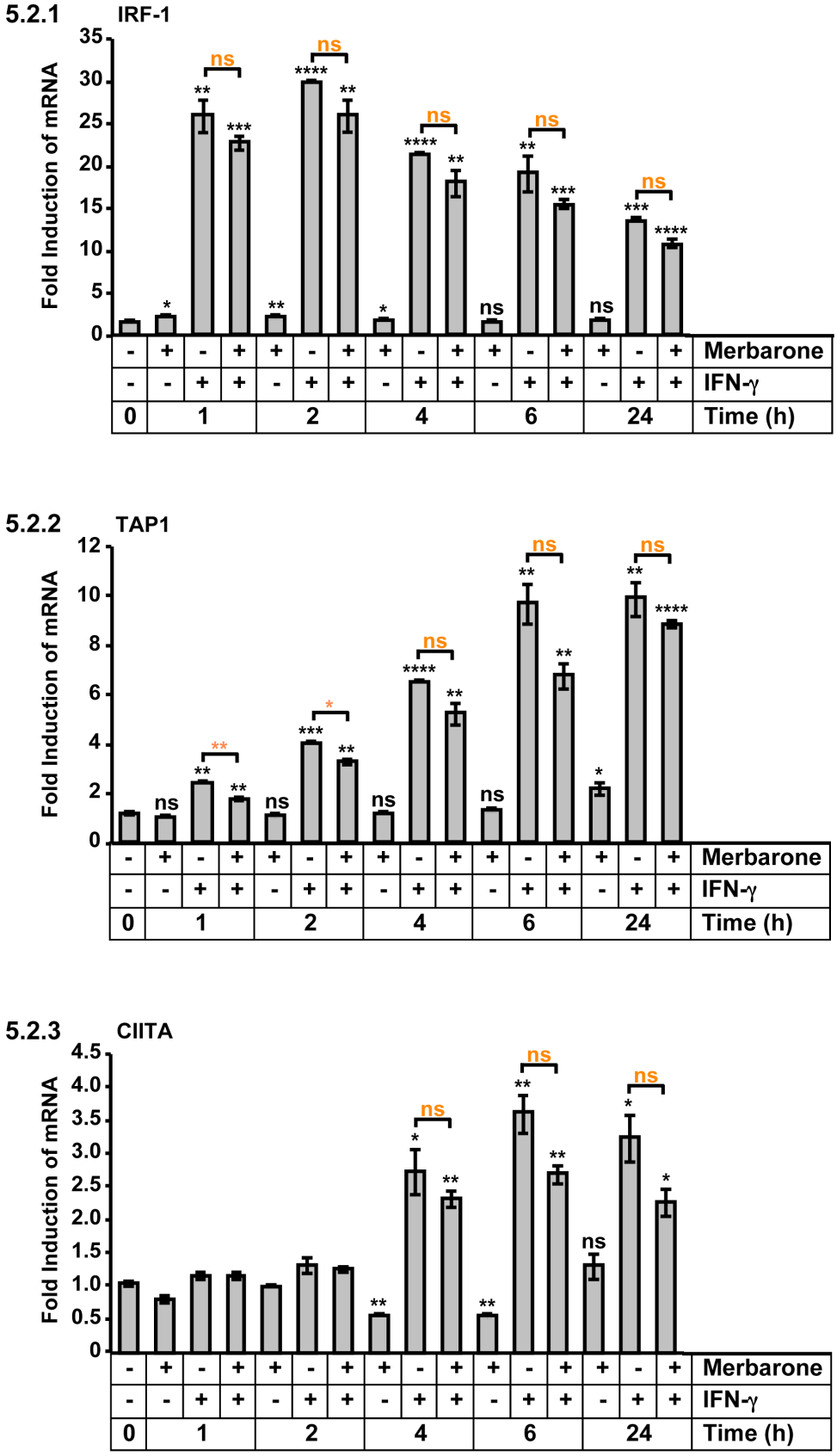


Figure 5.2. Inhibition of topoisomerase activity did not fully recapitulate the effect of silencing ATM. (Continued on page 172).

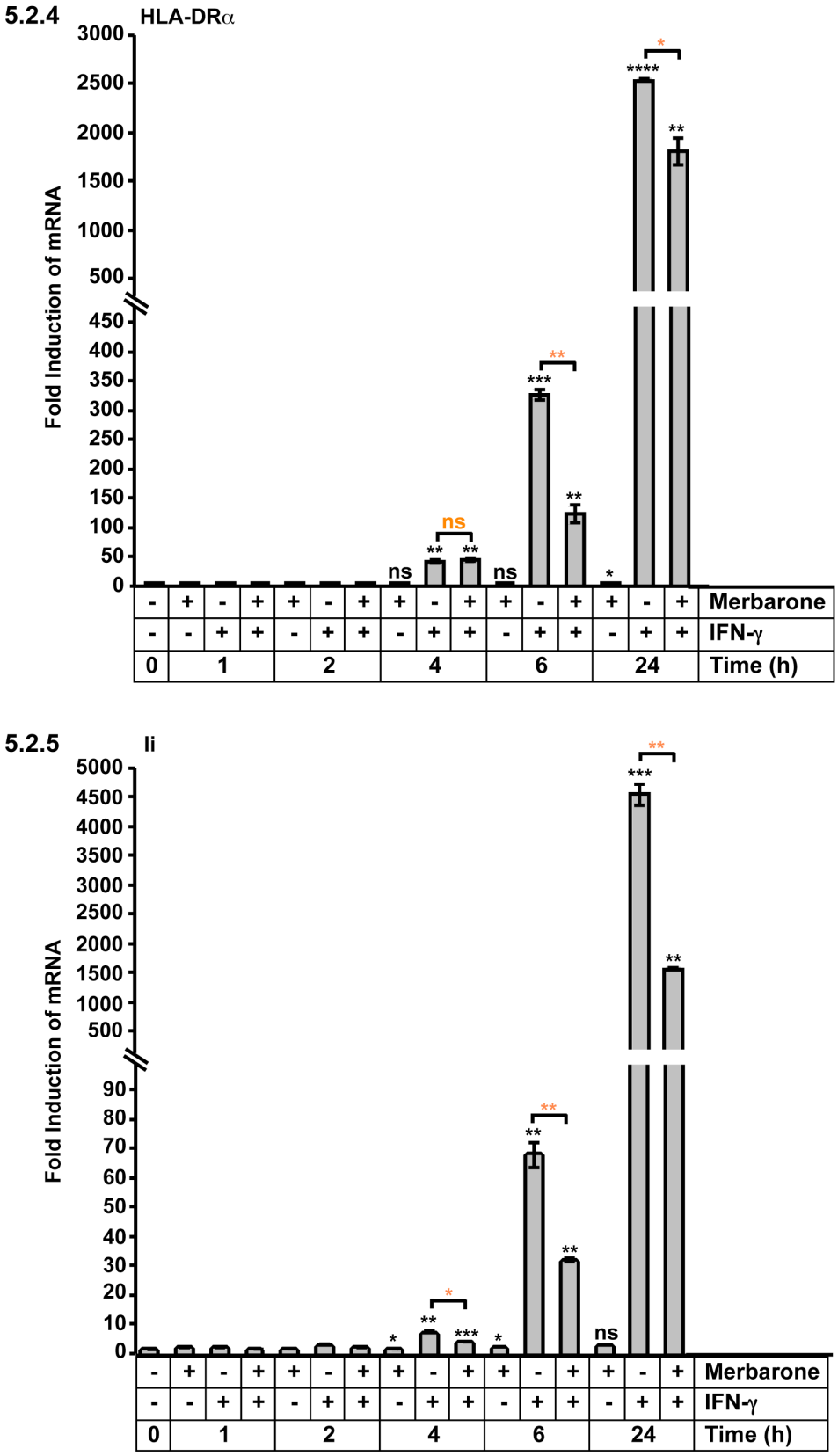


Figure 5.2. Inhibition of topoisomerase activity did not fully recapitulate the effect of silencing ATM. Merbarone did not affect IFN-γ-induced transcription of CIITA and the impairment in Ii and HLA-DRα mRNA transcripts levels was less pronounced than the one observed upon

siATM. Cells were pre-treated for 1 hour (h) with merbarone (10 μM) or were left untreated. Cells were then stimulated with IFN-γ (500 IU/ml) for the indicated periods of time (hours - h). RNA extraction and reverse transcription was performed and the obtained cDNA was amplified with primers specific to detect mRNA of known ISGs namely, IRF1, TAP1, CIITA, HLA-DRα and Ii by qPCR as described before. Each reaction was performed in triplicate. Data was analysed and first normalised to the HPRT reference gene. Fold induction values were then obtained upon normalisation, by dividing the values of the treated by the untreated conditions (0 h) for each individual experiment. Mean ± SD values were obtained for at least two independent experiments done in triplicates and yielding identical results. Statistical analysis was done using Student's t test relative to the untreated control (black symbols). This test was also used to compare between cells treated only with IFN-γ or cells treated with merbarone prior to stimulation with IFN-γ for each time point where significant mRNA expression was detected (orange symbols) [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$].

5.3. ATM regulation of IFN-γ-induced binding of STAT1 to ISGs promoters

ChIP is a widely used and useful technique to study *in vivo* interactions between proteins and DNA. In order to assess if ATM was involved in regulating IFN-γ-induced binding of STAT1 to the promoters of different ISGs, we immunoprecipitated the chromatin corresponding to selected ISGs using a STAT1 antibody. Concomitantly, we also used RNAi to modulate the levels of ATM.

5.3.1. Optimisation of STAT1 chromatin immunoprecipitation parameters and ISG enrichment detection

Optimisation of the crucial steps of ChIP procedure, namely fixation or protein-DNA crosslinking, DNA shearing into small fragments, immunoprecipitation and analysis by PCR of the protein-bound DNA that is immunoprecipitated, is required as it is dependent, for instance, on the cell line used.

Crosslinking by formaldehyde allows quick fixation of the chromatin structure and associated proteins so that the interaction can be preserved during the procedure (Das *et al*., 2004; Solomon *et al*., 1988; Solomon and Varshavsky, 1985). Fixation was done using the time of exposure to and concentration of formaldehyde as indicated in the protocol provided by Professor Pavel Kovarik. These parameters

were also in accordance with the conditions used for U2OS cells in the literature consulted [e.g. (Hancock *et al*., 2010)].

Appropriate shearing of the chromatin is one of the crucial steps in a ChIP protocol (Bellard *et al*., 1989; Dingwall *et al*., 1981; Reneker and Brotherton, 1991). DNA must be sheared into fragments that range from 200 to 1000 base pairs and this was achieved by sonication. Different periods of sonication and sample volume were tested when implementing the method in our laboratory, to guarantee that a suitable size of DNA fragments would be obtained with the available sonicator, which was different from the sonicator used in the protocol provided by Professor Pavel Kovarik. The length of sheared DNA in these samples was compared with a control sample, which was obtained when performing ChIP in Professor Pavel Kovarik's laboratory. Upon sonication, samples were decrosslinked, DNA was isolated and the efficiency of sonication determined by agarose gel electrophoresis (**Fig. 5.3.A**). A period of 25 minutes of sonication using a volume of 0.5 ml of chromatin was determined to give the most suitable DNA shearing, and was, therefore, used in all ChIP experiments performed.

PCR primers were obtained to determine the enrichment of a set of ISGs to which STAT1 is known to bind upon IFN-γ treatment in order to assess the efficiency of STAT1 ChIP. These ISGs included IRF-1, GBP-1 and CIITA pIV. A temperature gradient was performed to determine the ideal temperature of amplification of each ISG primer set using RT-PCR (**Fig. 5.3.B**). A temperature of amplification between 59-62 ˚C was considered adequate for assessing enrichment of STAT1 binding to the promoters of the analysed ISGs upon cytokine treatment, since it resulted in maximal PCR amplification.

For the immunoprecipitation of STAT1, two commercial antibodies raised against this protein were tested and titrated using chromatin samples obtained from U2OS cells treated with IFN-γ for 30 minutes (**Fig. 5.3.C**). This time point has been frequently used to detect binding of IFN-γ-activated STAT1 to the IRF-1 promoter (Buro *et al*., 2010; Sadzak *et al*., 2008; Snyder *et al*., 2005).

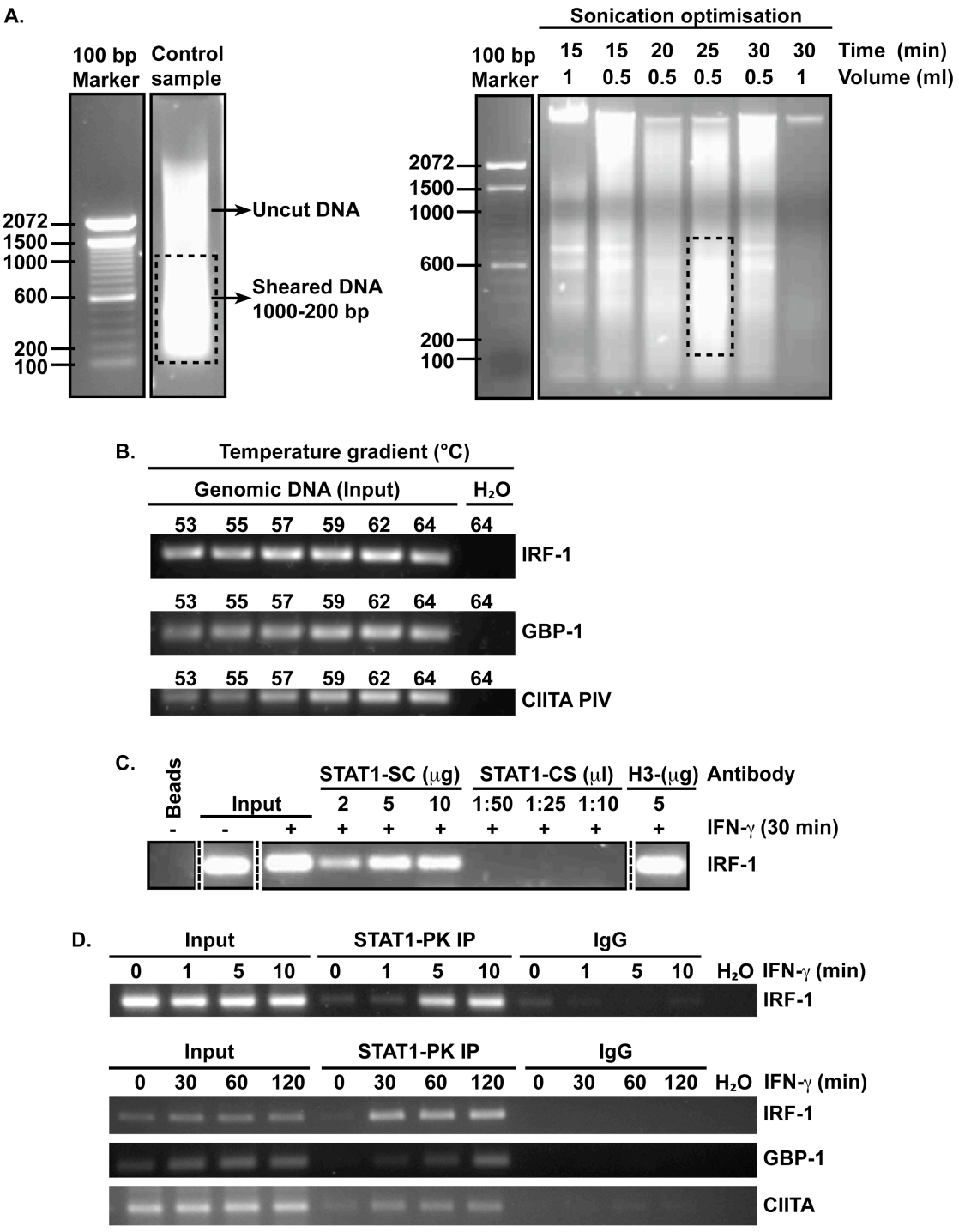


Figure 5.3. Optimisation of the STAT1 ChIP protocol using RT-PCR. A. The sonication step was optimised on U2OS cells regarding the sonicator and solutions used, by testing the indicated volumes of cell extracts (ml) and different times of sonication. Sheared DNA was purified and run in a 2% agarose gel to determine the size of the fragments. **B.** RT-PCR amplification temperatures were optimised for primers amplifying promoter regions of the ISGs IRF-1, GBP-1 and CIITA pIV. **C.** U2OS cells were stimulated with IFN-γ for 30 minutes, cell lysates were sheared by sonication and different amounts of two commercially available antibodies for STAT1 were tested for ChIP

(SC - Santa Cruz; CS - Cell signalling). An antibody against histone 3 (from Upstate technology) was used as a positive control for the efficiency of the ChIP protocol. STAT1 binding to IRF-1 promoter was detected by running the amplified RT-PCR sequences on a 2% agarose gel and was used to determine the usefulness of the antibodies for this assay and what would be the saturating concentration. **D.** A ChIP assay was done using the optimised conditions for sonication of unstimulated and stimulated U2OS cells with IFN-γ for the indicated time points (minutes - min), described in the literature as suitable to detect STAT1 binding to the promoters of IRF-1, GBP-1 and CIITA pIV. Immunoprecipitation was done using the STAT1 antibody (STAT1-PK) kindly provided by Professor Pavel Kovarik and the amplified RT-PCR products were run in a 2% agarose gel, confirming that the experimental conditions were suitable and the ChIP assay worked.

Efficiency of STAT1 immunoprecipitation was determined by assessing the enrichment in IRF-1 recovered DNA when compared to the condition where no antibody was used but only beads were added, by running the amplified RT-PCR sequences on an agarose gel. Histone 3 immunoprecipitation was additionally used as a positive control for the ChIP procedure, as this protein is permanently associated with DNA. The STAT1 antibody obtained from Cell Signalling, although specified as suitable for ChIP, did not efficiently immunoprecipitate this protein in contrast to the antibody purchased from Santa Cruz, which could be used at 5 μg *per* chromatin sample, as it was deemed to be a saturating concentration.

Additionally, a non-commercial antibody was also used for the STAT1 ChIP assays, at the amount recommended by Professor Pavel Kovarik, who kindly provided us this antibody (**Fig. 5.3.D**). ChIP assays were done using the optimised conditions for sonication of U2OS cells and using this antibody (STAT1-PK). STAT1 binding to the promoters of IRF-1, GBP-1 and CIITA pIV was determined in IFN-γ-stimulated samples for several time points, such as 30, 60 and 120 minutes, which are commonly used in the literature for these ISGs. In addition, shorter IFN-γ stimulation periods were also used to detect enrichment upon STAT1 ChIP of the early-transcribed ISG IRF-1, in order to determine if this could be detected earlier than frequently seen in the literature. IFN-γ-inducible enrichment of all these ISGs was indeed observed in the STAT1 immunoprecipitated samples upon 30, 60 and 120 minutes and when controlling for the initial amount of chromatin used in each condition (inputs) and for the background noise assessed using IgG controls. IRF-1 enrichment in the DNA pulled-down with a STAT1 antibody was detected as

early as 5 minutes after cytokine treatment, indicating that indeed recruitment of STAT1 at least for this ISG occurs very quickly. These preliminary assays confirmed that the experimental conditions were suitable for STAT1 ChIP to be performed.

When initially silencing ATM by RNAi in U2OS cells and assessing how this would impact on STAT1 binding to IRF-1, GBP-1 or CIITA pIV, we found it difficult to do so by RT-PCR, even though enrichment could easily be detected upon IFN-γ treatment (**data not shown**). Although RT-PCR is still currently used to determine association of proteins and DNA, this detection technique can be rather limiting when determining differences between conditions, since the intensity of the bands observed in the agarose gels correspond to the endpoint of the reactions of amplification. In order to be able to compare differences in the binding to specific DNA sequences by STAT1 in RNAi experiments, we decided that ISGs enrichment would have to be detected by qPCR, thus allowing us to assess the initial existent amount of a specific DNA sequence in immunoprecipitated samples.

Consequently, a number of published primer sets recognising sequences within the promoters of several ISGs, namely, IRF-1, TAP-1, GBP-1, CIITA pIV, HLA-DRα and Ii were selected and tested for SYBR Green-based qPCR detection, as previously done with the primers for the mRNA detection. Different combinations of forward and reverse primers concentrations were tested and the combination yielding the highest level of detection with less variation among triplicates was chosen. Additionally, a melting curve analysis was performed for all primer sets, to guarantee that only one fragment was being amplified. A non-template control was also included to monitor for contaminations and primer dimer formation. Suitable primers for IRF-1, TAP1, CIITA and HLA-DRα were selected and used in the following experiments (primers sequences and concentrations are summarized in Materials and Methods Section).

5.3.2. Analysis of the kinetics of IFN-γ-inducible STAT1 binding to selected ISGs promoters

Having established the STAT1 ChIP protocol in the laboratory and optimised the detection by qPCR of IRF-1, TAP1, CIITA pIV and HLA-DRα promoter sequences enrichment, STAT1 binding kinetics to these promoters was then studied. This was important to enable selection of the appropriate time points of IFN-γ stimulation at which to determine the effect of ATM silencing on STAT1 recruitment to specific ISGs. U2OS cells were initially stimulated with IFN-γ for 2.5, 5, 10 and 30 minutes. Additionally, an IFN-γ time course was repeated twice including stimulation periods of 10, 30 and 60 minutes. Cytokine stimulation was followed by crosslinking with formaldehyde, DNA shearing by sonication and immunoprecipitation of STAT1-bound DNA sequences. After decrosslinking and DNA purification, enrichment for the selected ISGs was analysed using SYBR Green-based qPCR (**Fig. 5.4**). A standard calibration curve was included for every ISG primer set to allow quantification of the immunoprecipitated amount of DNA present in STAT1 immunoprecipitated samples, as well as in the input and IgG control samples. The input samples served the purpose of controlling for the amount of chromatin used in each immunoprecipitation and allowed the normalisation of the data. Samples where a non-specific IgG was added instead of a specific antibody were used to detect the background noise associated with the ChIP protocol, which varied for different primers.

IFN-γ-induced phosphorylation and activation of STAT1 is a fast event, which is reflected by the fact that this transcription factor also quickly translocates into the nucleus (**Fig. 4.2**). Considering that STAT1 serine 727 phosphorylation was detected as early as 5 to 10 minutes after stimulation (**Fig. 4.1.** and **Fig.4.2**) and that this event can only occur after tyrosine 701-phosphorylated STAT1 is bound to the DNA, we hypothesised that STAT1 recruitment to the promoters of at least the early-transcribed genes, such as IRF-1, would occur shortly after treatment. STAT1 binding to ISGs was, therefore, initially assessed during a time course of IFN-γ treatment that included time points as short as 2.5 minutes (**Fig.5.4**).

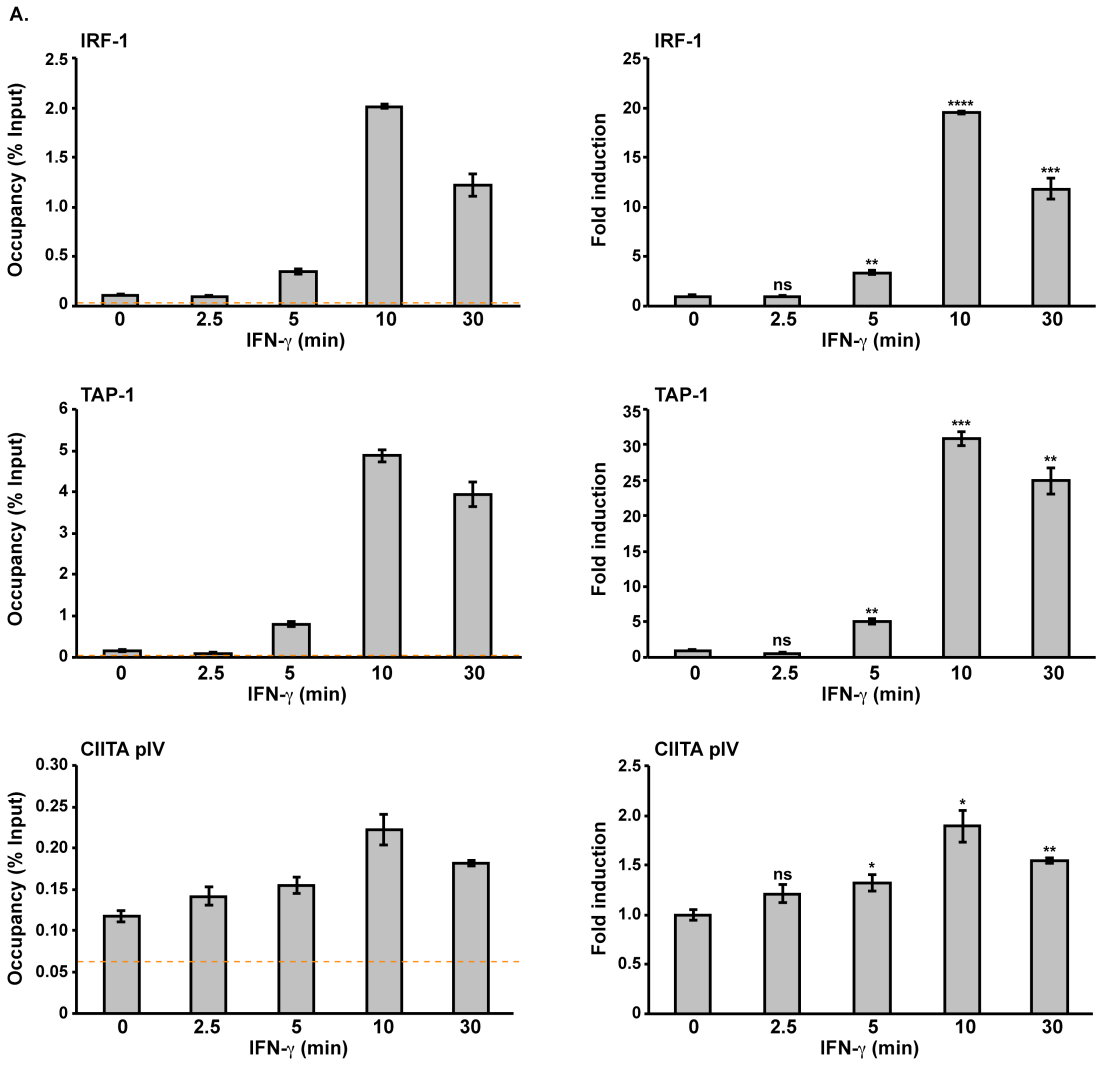


Figure 5.4. Kinetics of IFN-γ-inducible binding of STAT1 to the promoters of IRF-1, TAP-1 and CIITA pIV. STAT1 ChIP assay was performed using unstimulated and stimulated U2OS cells with IFN-γ for the indicated time points (minutes - min). Immunoprecipitation was done using the STAT1 antibody provided by Professor Pavel Kovarik. STAT1 binding to the promoter region of the ISGs IRF-1, TAP1, CIITA pIV was analysed using qPCR. Data is presented as occupancy of the promoter, upon normalisation to the input material (left graphs) and as fold change after normalising the % of input of each sample to the basal untreated level (right graphs). The dashed line corresponds to the average value of occupancy of the IgG controls. Each PCR condition was run in triplicates. Statistical significance was calculated using Student's t-test relative to the untreated condition [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. **A.** IFN-γ-inducible binding of STAT1 to the analysed ISGs promoters is sown for periods of 2.5, 5, 10 and 30 minutes of stimulation. This was done only once. **B.** STAT1 binding to IRF-1, TAP-1 and CIITA pIV was assessed after IFN-γ stimulation for periods of 10, 30 and 60 minutes. This time course was performed twice.

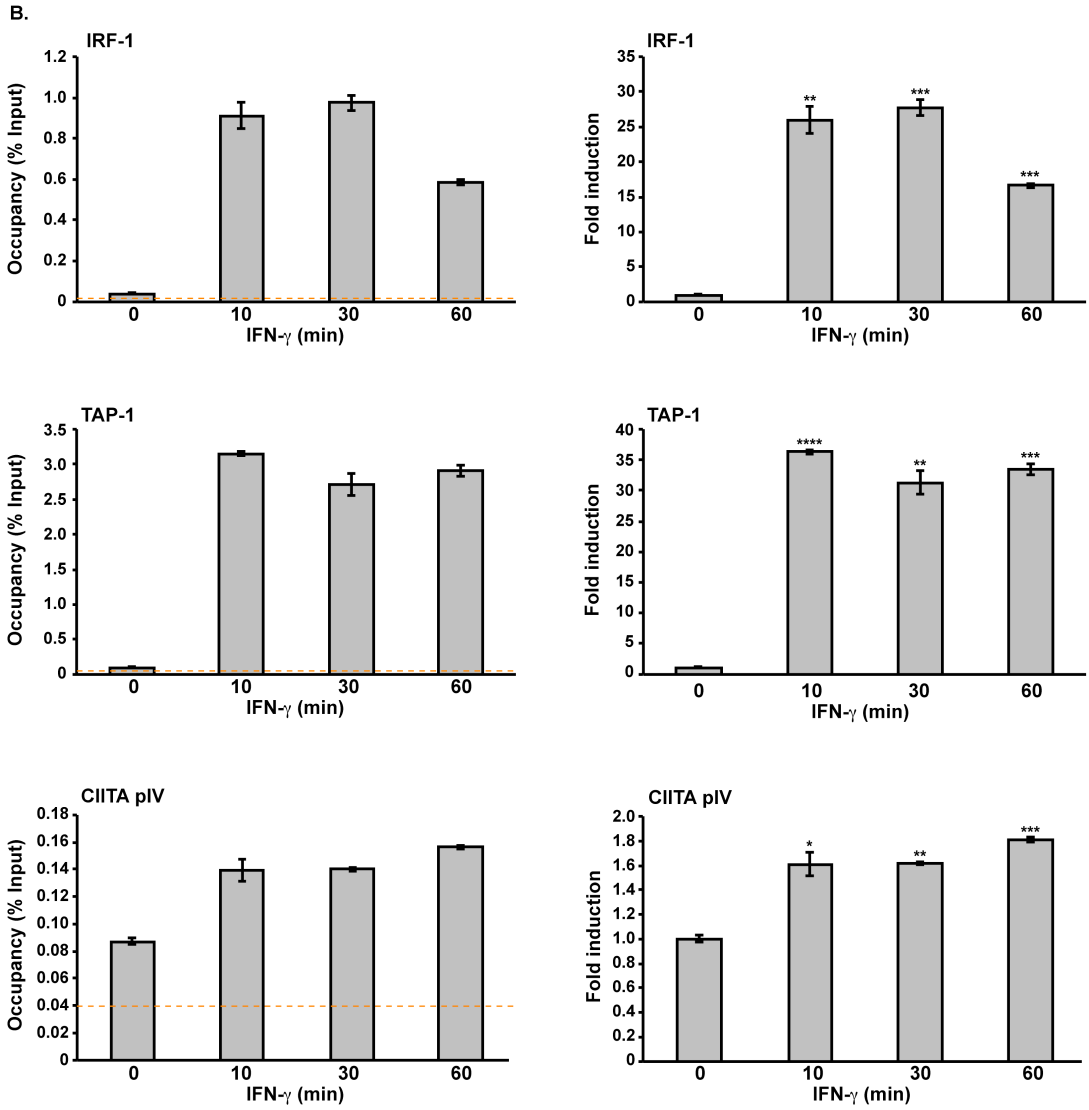


Figure 5.4. Kinetics of IFN-γ-inducible binding of STAT1 to the promoters of IRF-1, TAP-1 and CIITA pIV. (Continued from page 179).

Significant binding of STAT1 to IRF-1, TAP1 and CIITA pIV promoters was clearly detected as soon as after 5 minutes of stimulation with IFN-γ (**Fig. 5.4**). IFN-γ-induced recruitment of STAT1 to these ISGs promoters seemed to occur during the 5 to 10 minutes period of stimulation. Although significant, STAT1 occupancy (percentage of input) of these ISGs promoters after 5 minutes of stimulation was quite low when compared to the maximum value of binding upon 10 minutes of cytokine treatment. This was particularly true for CIITA pIV, where STAT1 occupancy after 5 minutes of IFN-γ stimulation was statistically significant, but it was very close to the basal level of IgG noise and was quite small even at its maximal recruitment after 10 minutes of cytokine treatment.

For the purpose of assessing if ATM silencing would selectively decrease STAT1 binding to ISGs promoters we needed to ensure that STAT1 binding at the selected time of IFN-stimulation would be consistently significant above basal noise levels and to an extent that a reduction would be visible. We thus assessed STAT1 recruitment to IRF-1, TAP-1 and CIITA pIV promoters twice more in a time course extended from 10 to 60 minutes (**Fig. 5.4.B**) and observed consistently significant levels of promoter occupancy at 10 minutes by STAT1, even for CIITA pIV. STAT1 binding upon ATM silencing was, therefore, analysed after a period of IFN-γ treatment of 10 minutes. This time point was selected as we reasoned that it was still early enough for any difference in promoter occupancy to be detected, since STAT1 serine 727 phosphorylation in the nucleus was shown to be impaired up to 20 minutes after cytokine treatment in the absence of ATM (**Fig.4.2**).

5.3.3. STAT1 binding to specific ISGs promoters was affected by ATM silencing

U2OS cells were transfected with a non-targeting siRNA sequence (siNS) and with siRNA targeting ATM. Forty-eight hours after transfection cells were treated for 10 minutes with IFN-γ or left untreated. STAT1 ChIP was performed as previously described and enrichment for IRF-1, TAP1 and CIITA pIV was determined by qPCR. In addition, STAT1 binding to HLA-DRα at this time point of cytokine treatment was also assessed. Results are presented both as occupancy of the promoters by STAT1 (% of input) and as fold induction of STAT1 binding upon normalisation by the untreated value of the respective transfection condition (**Fig. 5.5**).

STAT1 binding to the promoters of IRF-1 ($p<0.001$), TAP-1 ($p<0.001$), CIITA pIV ($p<0.01$) and HLA-DRα ($p<0.001$) was statistically significant at 10 minutes after stimulation with IFN-γ in siNS-transfected cells in the representative experiment shown (**Fig. 5.5.A**). IFN-γ-induced recruitment of STAT1 to IRF-1 and TAP1 promoters was always considerably above IgG background noise levels, which translated into fold inductions of 7 to 12 and 20 to 35, respectively, across several experiments in the siNS control. The level of STAT1 occupancy was much smaller at the CIITA pIV and HLA-DRα promoters in siNS-transfected cells, as previously

observed, although always statistically significant and this translated into fold inductions ranging from 1.6 to 6 and 1.6 to 8.5, respectively, across several experiments performed.

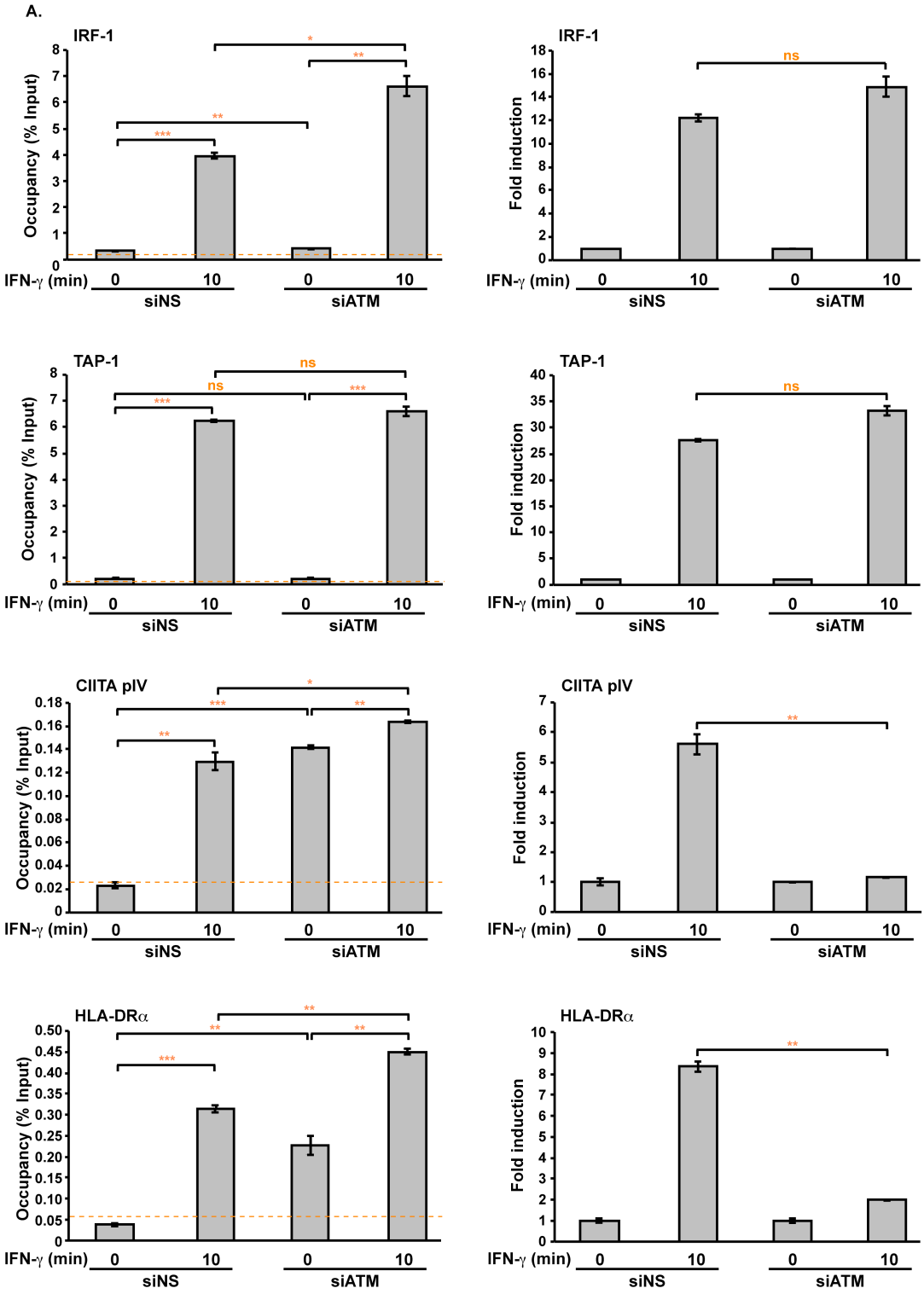


Figure 5.5. ATM silencing affected differentially STAT1 binding to the promoters of distinct genes. (Continued on page 183).

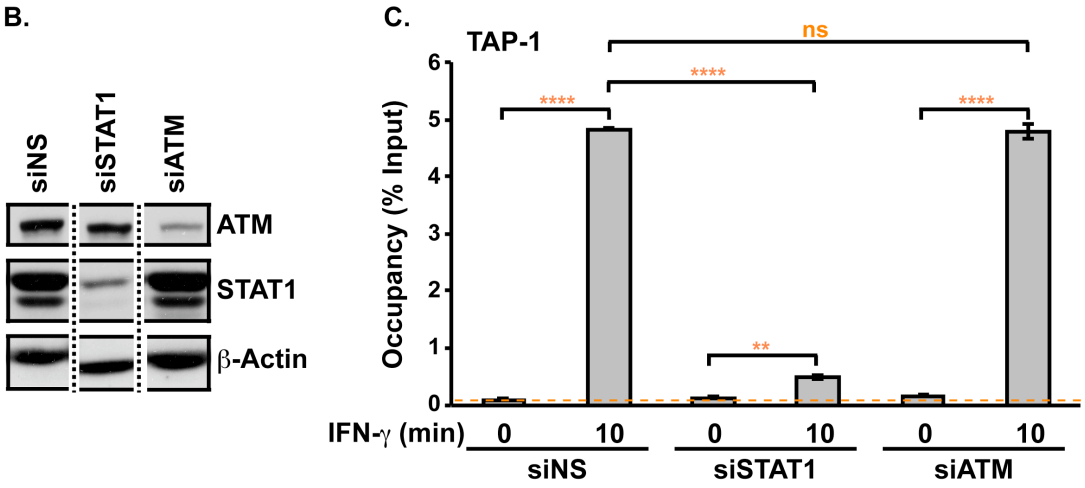


Figure 5.5. ATM silencing affected differentially STAT1 binding to the promoters of distinct genes. A. U2OS cells were transfected with 20 nM of a non-targeting sequence (siNS) or siATM. Forty-eight hours after transfection cells were treated for 10 minutes (min) with IFN-γ or left untreated. CHIP was performed using an anti-STAT1 antibody (PK or SC). Enrichment of STAT1 binding to the promoter region of IRF-1, TAP1, CIITA pIV and HLA-DRα was detected using qPCR. Data is presented as STAT1 occupancy of the promoter, upon normalisation to the input material (left) and as fold change after normalising to the basal untreated level of each transfection condition (right). The dashed line corresponds to the average value of occupancy of the IgG controls. Each PCR condition was run in triplicates. One representative experiment of three independent experiments yielding similar results is shown. Statistical significance was calculated using Student's t test [non-significant (ns) - p>0.05, * - p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001]. **B.** and **C.** U2OS cells were transfected as before. **B.** Efficient protein silencing by RNAi was verified forty-eight hours after transfection, concomitantly with ChIP assays. Total cell lysates were prepared with Schindler's buffer and proteins were separated in a 6% SDS-PAGE gel. Western blotting analysis was performed using antibodies against ATM and STAT1. β-actin was used as a loading control. **C.** STAT1 ChIP was performed in cells transfected siNS, siATM or siSTAT1. IFN-γ induced STAT1 binding to TAP-1 promoter after 10 minutes treatment was detected by qPCR.

ATM silencing increased the basal level of STAT1 occupancy at IRF-1 ($p<0.01$), CIITA pIV ($p<0.001$) and HLA-DRα ($p<0.001$) promoters as shown when compared to the basal level of the siNS condition (**Fig. 5.5.A**). Upon normalisation to the respective transfection control, a similar fold induction of IFN-γ-stimulated STAT1 binding was observed for IRF-1 and TAP1 promoters, between siNS and siATM conditions. Reduction of ATM protein levels did not affect the level of STAT1 occupancy of TAP-1 promoter in the absence of cytokine stimulation (compared to siNS, $p>0.05$). When silencing ATM the IFN-γ-driven STAT1

recruitment for CIITA pIV ($p<0.05$) and HLA-DRα ($p<0.01$) promoters was almost abrogated, in comparison to the siNS control possibly as a consequence of the increased STAT1 occupancy in the absence of stimulation (**Fig. 5.5**, graphs on the left). This effect could be responsible for the differences observed at the transcriptional and translational levels when comparing IRF-1 and TAP1 with CIITA and HLA-DRα (**Fig. 4.5, Fig. 4.7.1**, **Fig. 4.7.2**, **Fig. 4.7.7**).

Besides ATM, STAT1 was also silenced by RNAi, as a positive control for the technique and for the results obtained above. Following 48 hours of transfection, ATM and STAT1 protein expression was reduced, as determined by western blotting analysis (**Fig. 5.5.B**). As an example, STAT1 ChIP resulted in an enrichment of TAP-1 promoter sequence in the recovered STAT1-bound DNA in the siNS-transfected cells compared to untreated cells ($p<0.0001$). This enrichment was almost abrogated by reduction of STAT1 protein levels (compared to induction in siNS, $p<0.0001$), but not significantly affected by ATM silencing by RNAi (compared to untreated siATM, $p<0.0001$; compared to induction in siNS, $p>0.05$) (**Fig. 5.5.C**).

5.4. Binding of ATM to ISGs promoters was detected upon IFN-γ stimulation

ATM silencing specifically affected the transcription of CIITA, Ii and HLA-DRα ISGs. We observed that this reduction in ATM protein levels increased STAT1 binding to CIITA and HLA-DRα promoters in the absence of stimulation, resulting in a lower IFN-γ-induced recruitment of STAT1 to these promoters. Given the apparent involvement of ATM in maximal STAT1 recruitment to specific ISGs, we wondered if this meant that ATM also physically interacted with these ISGs promoters. We used a commercial ATM antibody know to be suitable for ATM ChIP (Berkovich *et al*., 2007; Golding *et al*., 2004) and assessed if ATM bound to ISGs promoters upon IFN-γ treatment in U2OS cells (**Fig. 5.6**).

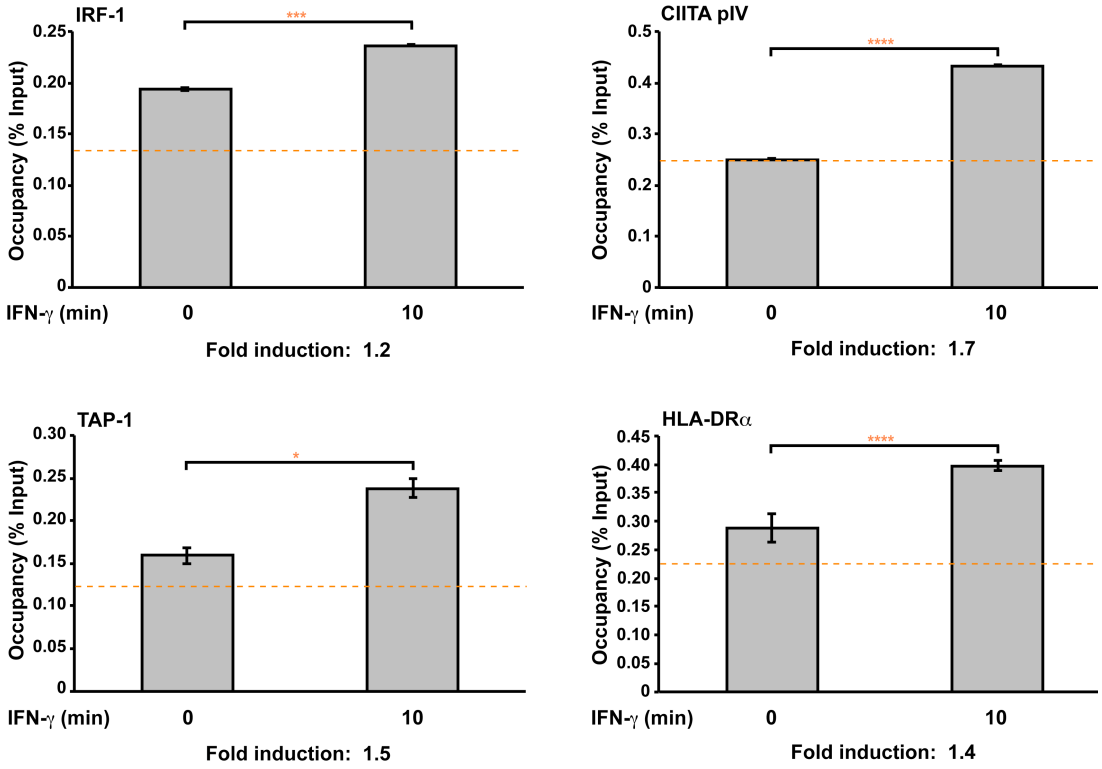


Figure 5.6. A modest binding of ATM was detected in all ISGs promoters analysed. U2OS cells were transfected using RNAiMAx reagent and 20 nM of siATM or a siNS. Forty-eight hours after transfection, cells were treated for 10 minutes (min) with IFN-γ or left untreated. CHIP was performed using an ATM antibody (Calbiochem). ATM binding to the promoter region of IRF-1, TAP-1, CIITA pIV and HLA-DRα was detected using qPCR. Data is presented as ATM occupancy of the promoter, upon normalisation to the input material (left) and as fold change, after normalising to the basal untreated level of each transfection condition (right). Each PCR condition was run in triplicates. One representative experiment of two (CIITA) or three (IRF-1, TAP-1, HLA-DRα) independent experiments yielding similar results is shown. Statistical significance was calculated using Student's t-test [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$].

ATM ChIP was done as previously described for STAT1 and we analysed enrichment at the IRF-1, TAP-1, CIITA pIV and HLA-DRα promoter DNA sequences (**Fig.5.6**). The results showed an increase in ATM occupancy of IRF-1 ($p<0.001$), TAP-1 ($p<0.05$), CIITA pIV ($p<0.001$) and HLA-DRα ($p<0.001$) induced by IFN-γ. Although ATM binding upon IFN-γ treatment to all these ISGs was statistically significant, it was consistently low and very close to the IgG background noise. Fold inductions ranged from 1.2 to 1.9 for IRF-1, 1.5 to 2.0 for TAP-1, 1.2 to 1.7 for CIITA and 1.2 to 1.5 for HLA-DRα.

5.5. ATM and STAT1 co-localise

In order to assess ATM and STAT1 subcellular localization by immunofluorescence microscopy, different conditions were tested extensively regarding fixation and permeabilisation of the cells optimal antibody concentration (**data not shown**). In brief, after stimulation with IFN-γ for short periods of time (2.5, 5, 10 and 30 minutes), U2OS cells were fixed with 4% PFA, permeabilised with 100% ice-cold methanol and stained with anti-ATM and anti-STAT1 antibodies (1:50 dilution). Representative images of ATM and STAT1 co-staining obtained for untreated and IFN-γ-treated cells for the 2.5 and 10 minutes time points are shown (**Fig.5.7**).

STAT1 is described in the literature as being predominantly cytoplasmic in the absence of stimulation but it has been shown to constantly shuttle between the cytoplasm and the nucleus. STAT1 increases rapidly, but transiently, its localization in the nucleus upon treatment with IFN-γ (Lillemeier *et al*., 2001; McBride *et al*., 2002). In agreement with these previous reports, STAT1 (green, 1st vertical panel) was detected in a more dispersed and diffused pattern in both cellular compartments before stimulation with the cytokine. ATM (red, 2nd vertical panel) is also known to be both cytoplasmic and nuclear and it was indeed observed in both locations in the absence of stimulation. Upon stimulation, both STAT1 and ATM presented a more compacted localization in the nuclear area and in the surroundings of this compartment (**Fig.5.7.A**). When images were merged, although very discrete, ATM and STAT1 co-localised under all conditions (4th vertical panel, white arrows). Co-detection of these proteins seemed to occur preferentially at the periphery of the nuclear compartment. Although this observation cannot be used to conclude that there was a physical interaction between ATM and STAT1, their proximal localisation suggested that this was a possibility (**Fig.5.7.A**).

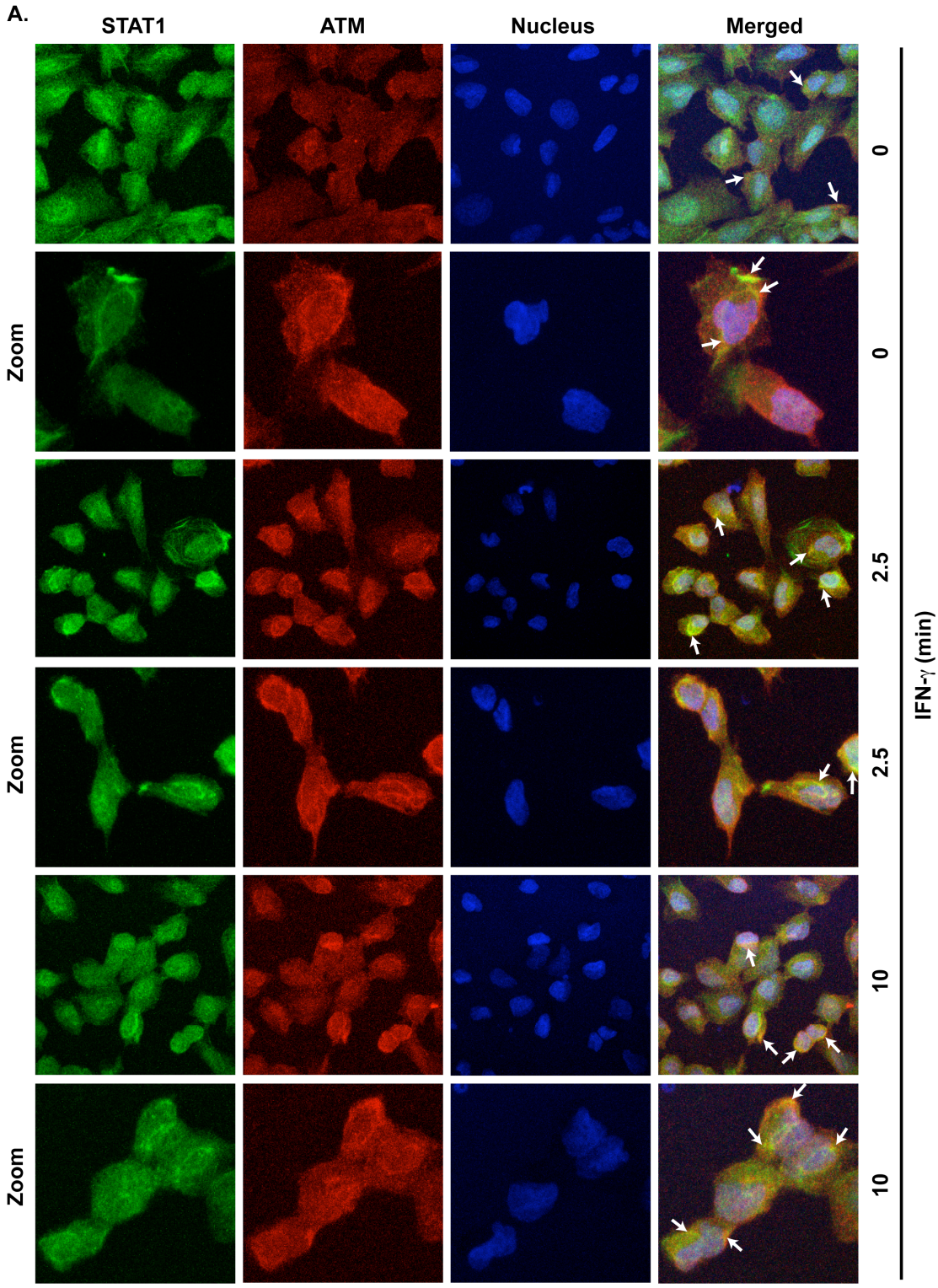


Figure 5.7. STAT1 and ATM appear to co-localise. (Continued on page 188).

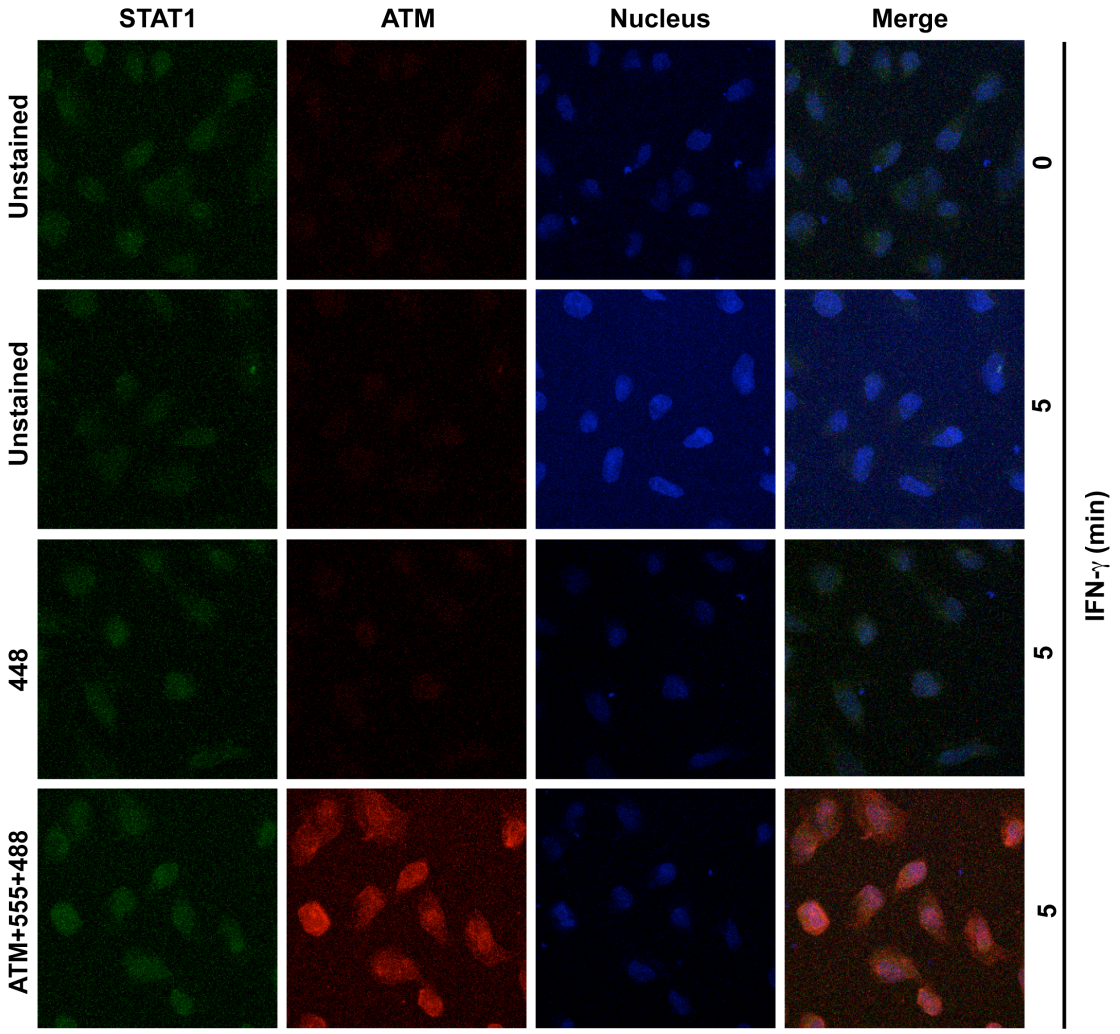


Figure 5.7. STAT1 and ATM appear to co-localise. U2OS cells were plated on glass coverslips and stimulated with IFN-γ (500 IU/ml) for the indicated time points (minutes - min). Cells were fixed with 4% PFA and then permeabilized with 100% ice-cold methanol. **A.** ATM and STAT1 subcellular localisation was visualised by immunofluorescence co-staining using specific anti-ATM and anti-STAT1 (STAT1α/β N-terminus) antibodies. Images were visualised under a Zeiss LSM 510 Meta Confocal microscope with a 20x/1.4 oil immersion objective lens using Immersol 518 oil. Immunoreactivity against STAT1 primary antibody was detected with an anti-rabbit- IgG Alexa Fluor-488 (green) and ATM detection was performed using an anti-mouse-Alexa Fluor-555 labelled IgG. Nuclei were counterstained with TO-PRO-3. Images composites of approximately 20 x 0.5 μm Z-stacks were obtained using Zeiss LSM 510 software. Overlapping images of ATM, STAT1 and TO-PRO-3 are shown on the 4th vertical panel. The white arrows signal some of the overlapping areas (yellow) for ATM (red) and STAT1 (green) staining. **B.** Examples of the controls prepared for assessing auto-fluorescence, non-specific staining of antibodies and bleed-through across channels. Untreated or IFN-γ treated cells were used to determine the levels of auto-fluorescence in unstained cells (1st and 2nd horizontal panel), unspecific binding (3rd horizontal panel) and bleed-through (4th horizontal panel) using the same settings as in **A**.

Auto-fluorescence of unstained cells, either untreated or treated with the cytokine, was determined as a control. Cells stained solely with IgG Alexa Fluor-488 secondary antibody (used to detect STAT1 and shown in green) were analysed. This allowed us to control for unspecific binding and fluorescence emission in the channel used for STAT1 detection. Single STAT1 staining was performed to determine bleeding through effects into the channel used for ATM detection. As an additional control cells were first stained with the primary ATM antibody, then this antibody was detected by conjugation with the IgG Alexa Fluor-555 (shown in red) and finally cells were incubated with the IgG Alexa Fluor-488 (used to detect STAT1 and shown in green) in the absence of STAT1 primary antibody. This was done to control for bleeding through the red into the green channel as well as for possible reactivity of secondary antibodies. These controls were done to guarantee that when staining samples simultaneously for ATM and STAT1, an overlap of the fluorescence of both antibodies would be real and not a consequence of unspecific fluorescence bleaching, as this could be misinterpreted as a proximal localization of these proteins (**Fig.5.7.B**).

5.6. ATM protein interactions mediated by IFN-γ

5.6.1. ATM physically interacted with non-phosphorylated STAT1 and with tyrosine 701-phosphorylated STAT1 upon IFN-γ stimulation

To assess the existence of an interaction between ATM and STAT1 co-immunoprecipitation of these proteins was performed. U2OS cells were treated with IFN-γ for short periods of time (2.5, 5 and 10 minutes) or left untreated and co-immunoprecipitation of ATM and STAT1 was performed using whole cell extracts. The immunoprecipitated samples were separated in 6% SDS-PAGE gels and western blot analysis performed (**Fig. 5.8**).

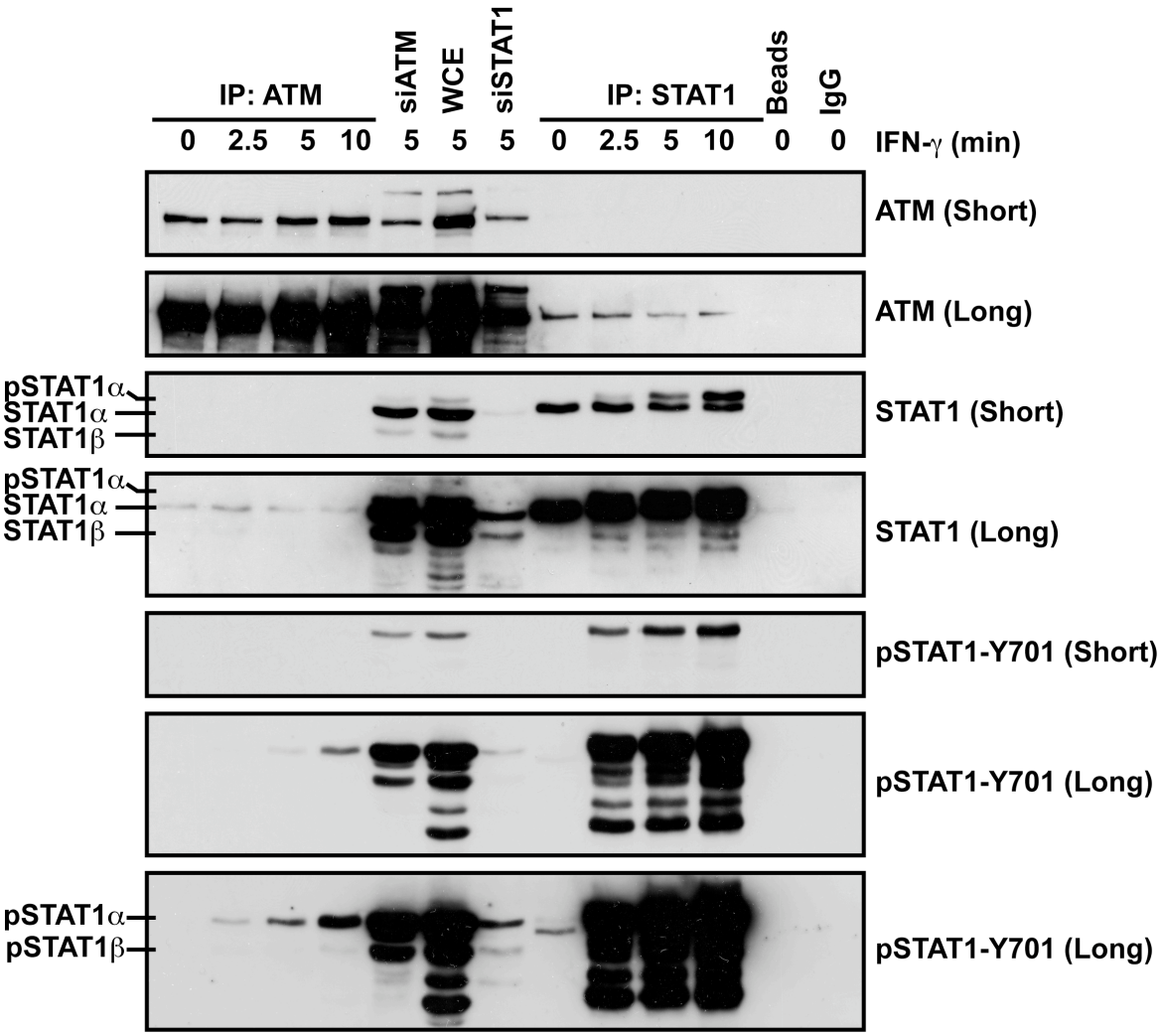


Figure 5.8. ATM interacted with non-phosphorylated STAT1 proteins in the absence of IFN-γ stimulation and with tyrosine phosphorylated STAT1 upon cytokine treatment. U2OS cells were stimulated with IFN-γ (500 IU/ml) for the indicated time points (minutes - min), after which cell lysates were prepared using a co-IP lysis buffer. Immunoprecipitation was carried out using 1 mg of protein and antibodies against ATM or STAT1α [STAT1α (NCL-STAT1A) and STAT1α p91 (C-1110)]. An IgG and a beads only control were always included together with a whole cell extract sample. Additionally, controls with silenced ATM and STAT1 were also included. Immunoprecipitates were separated in a 6% SDS-PAGE gel and, after wet transfer, ATM, pSTAT1-Y701 and STAT1 (STAT1α/β N-terminus) proteins were detected by western blotting (long - long exposure, short - short exposure). These results are representative of three independent experiments yielding identical results.

Immunoprecipitation of STAT1 was done using antibodies that targeted only the alpha isoform of the protein, which is considered to be the one responsible for IFN-γ-induced transcription. Western blot analysis was performed using a STAT1 antibody that detected the N-terminal region of the protein and, therefore, detects

both alpha and beta isoforms. Three bands were visualised for STAT1 in IFN-γ-treated whole cell extracts, namely, STAT1β (smaller molecular weight), STAT1α (immediately above) and finally a phosphorylated STAT1α band. In the STAT1α immunoprecipitated conditions (**Fig. 5.8,** right panel), these three higher molecular bands were detected in the samples treated with IFN-γ, with the top phosphorylated band (STAT1α) not being present in the unstimulated condition. With increased time of stimulation a shift was observed between the amounts of unphosphorylated and phosphorylated STAT1α protein that was pulled down, with the amount of phosphorylated STAT1α clearly augmenting (upper band *versus* second band of STAT1α).

Whole cell extracts of ATM and STAT1 RNAi silenced samples were used, together with an untransfected whole cell extract, all stimulated for 5 minutes with IFN-γ, to confirm the identity of ATM and STAT1 bands. Immunoprecipitations of a control IgG and beads were also included and no ATM or STAT1 could be detected in those samples.

The data thus suggests an interaction between ATM and unphosphorylated STAT1 in the absence of IFN-γ stimulation and, upon cytokine treatment, between activated STAT1 and ATM. This protein-protein interaction to the best of our knowledge has never been reported in the context of cytokine signalling. Immunoprecipitation of ATM pulled down STAT1 throughout all time points. By using an antibody that targeted STAT1 phosphorylated on tyrosine 701 we observed that ATM interacted with both unphosphorylated and phosphorylated forms of STAT1α (**Fig. 5.8,** left panel).

5.6.2. RNA polymerase II was recruited upon IFN-γ stimulation and associated with ATM and STAT1

STAT1-induced activation by IFN-γ determines its nuclear translocation and DNA binding to the promoters of ISGs, located at a distance from the RNA polymerase II transcription initiation site. Transcription of these ISGs involves additional co-activators, such as CBP and/or p300, which are necessary for RNA polymerase II

association to be achieved. These co-activators guarantee the efficient recruitment of the transcription machinery by spanning the distance between RNA polymerase II and the transcription activator, ensuring that transcription is initiated at a specific location (Darnell, 1997; Hiroi and Ohmori, 2003). Consequently, RNA polymerase II has been found together with STAT1 at the promoter region of ISGs (Hiroi and Ohmori, 2003; Snyder *et al*., 2005). We, therefore, investigated whether a multi-protein complex involving ATM, STAT1 and RNA polymerase II formed upon IFN-γ treatment. This possibility was assessed by reciprocal co-immunoprecipitation using whole cell extracts of U2OS cells upon stimulation with IFN-γ (**Fig. 5.9**).

STAT1 was pulled down when RNA polymerase II was immunoprecipitated as a consequence of IFN-γ stimulation **(Fig. 5.9.A**). This interaction was not detected in unstimulated cells but was visible as soon as after 2.5 minutes of treatment with the cytokine. The amount of STAT1 associated with RNA polymerase II increased with time of stimulation. STAT1α seemed to be the isoform interacting with RNA polymerase II inducible by IFN-γ and was found phosphorylated on tyrosine 701. ATM could also be seen in RNA polymerase II immunoprecipitated samples. It is hard to say whether this association was inducible as the amount of ATM pulled down was very small. In addition, the untreated sample seems underloaded. RNA polymerase II could also be detected in the reverse co-immunoprecipitations for ATM (**Fig. 5.9.B**) and STAT1 (**Fig. 5.9.C**).

Overall, the data suggests that ATM and STAT1 associated even before cytokine stimulation. Treatment with IFN-γ leads to increasing STAT1 phosphorylation and ATM may preferentially bind to tyrosine-phosphorylated STAT1 (**Fig. 5.8**). In addition, IFN-γ treatment led to an increasing association of STAT1 with RNA polymerase II (**Fig. 5.9.C**), but was without effect on the association between ATM and RNA polymerase II (**Fig. 5.9.B**), if one takes into consideration the protein loading in this particular experiment.

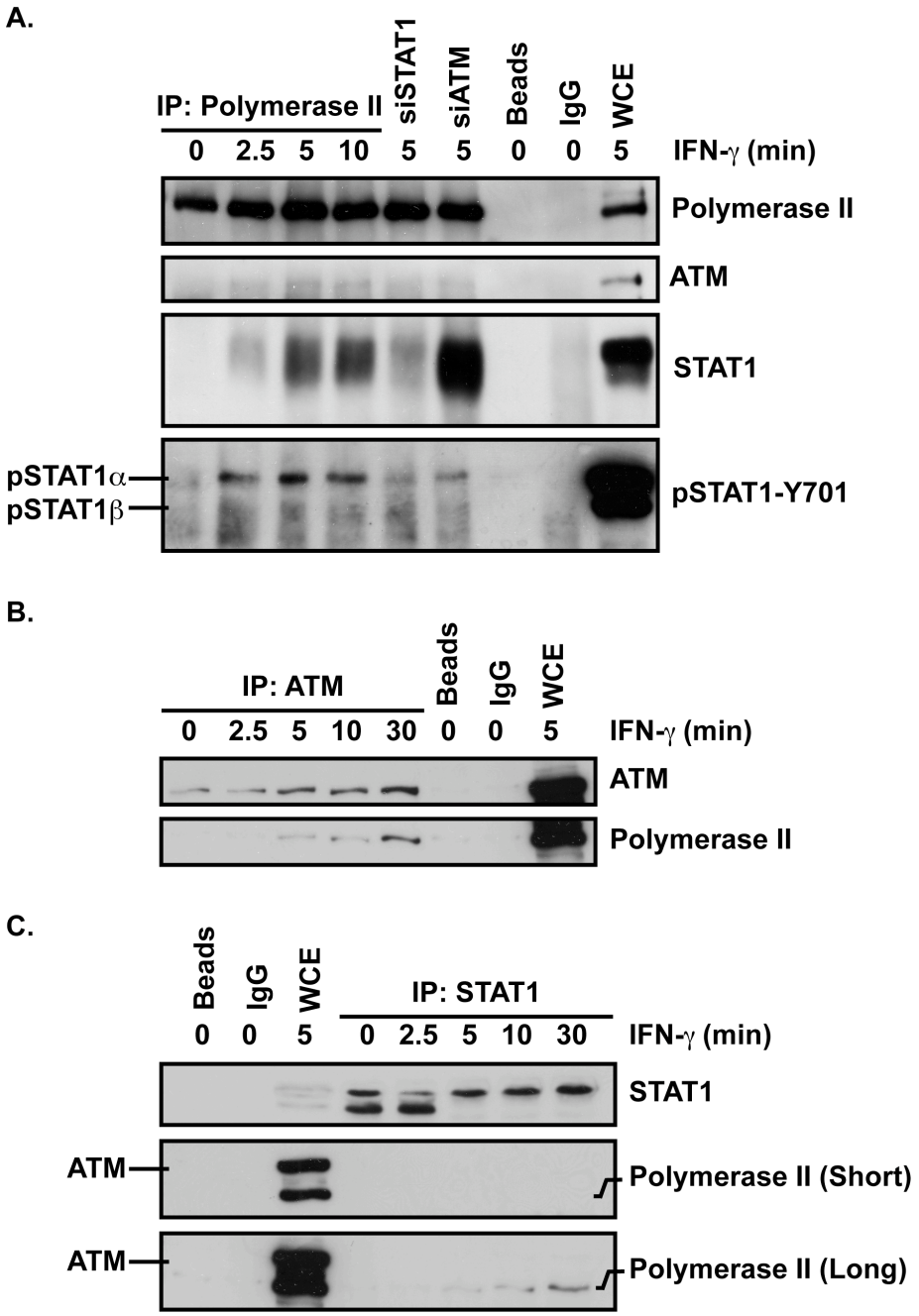


Figure 5.9. RNA polymerase II interacted with both STAT1 and ATM upon IFN-γ treatment. U2OS cells were stimulated with IFN-γ (500 IU/ml) for the indicated time points (minutes - min) and cell lysates were prepared using a co-IP lysis buffer. Immunoprecipitation of RNA polymerase II (**A.**), ATM (**B.**) and STAT1α [STAT1α (NCL-STAT1A), STAT1α p91 (C111)] (**C.**) proteins was performed using 1 mg of protein. An IgG and a beads only control were always included together with a whole cell extract sample, as were samples with silenced ATM and STAT1. Additionally, controls with silenced ATM and STAT1. Immunoprecipitates were separated in a 6% SDS-PAGE gel and, RNA polymerase II, ATM and STAT1 (STAT1α/β N-terminus) detected by western blotting (Long - long exposure, Short - short exposure). These results are representative of two independent experiments.

5.7. Discussion

The canonical IFN-γ pathway is well studied and described. This cytokine biological activities are mediated by the JAK/STAT signalling pathway. After being phosphorylated by JAK1 and JAK2, STAT1 is responsible for transducing the signal from the membrane to the nucleus, where it binds the promoters of ISGs and activates their transcription in a timely tuned sequential process, depending on the need for additional newly synthesized transcription factors and co-activators. IFN-γ signalling is, however, more complex than envisaged, since recent studies support that many additional signals are required in order to obtain the full range of responses to this cytokine (Briscoe *et al*., 1996; Costa-Pereira *et al*., 2005; Ramana *et al*., 2002).

This is particularly emphasised regarding STAT1 serine 727 phosphorylation, which is necessary for obtaining maximal transcriptional activity for a specific subset of genes (Kovarik *et al*., 2001; Varinou *et al*., 2003; Wen *et al*., 1995). This post-translational modification has been shown to be regulated by several different kinases in different experimental and cellular contexts, (Nguyen *et al*., 2001; Sadzak *et al*., 2008) (Goh *et al*., 1999); (Nair *et al*., 2002); (Deb *et al*., 2003). Another study suggests, for example, that the AKT pathway activation by IFNs acts together with and independently of the JAK/STAT pathway to induce the biological effects of these cytokines by promoting mRNA translation of ISGs (Kaur *et al*., 2008).

Evidence also indicates that other additional unidentified pathways exist and are important for the modulation of IFN-γ-induced biological responses (Briscoe *et al*., 1996; Watling *et al*., 2008) and ATM is one such example. Investigating how these STAT1-dependent or -independent pathways are involved in IFN-γ signalling could help future development of new therapeutic approaches involving the use of this cytokine in cancer treatment.

After establishing the relevance of ATM as a modulator of IFN-γ-induced signalling it was important to understand how it operated. Taking into consideration that ATM

acted as a positive modulator of STAT1 serine 727 phosphorylation, it is reasonable to assume that ATM most likely regulated STAT1-dependent IFN-γ-induced signalling and that it did that in the nuclear compartment of the cells, possibly through association with DNA.

ATM is recruited to the DNA and activated upon detection of DSBs (Shiloh, 2006), a DNA lesion that can also occur as a consequence of active transcription. We, therefore, assessed if DSBs formed after IFN-γ treatment, as this could be a common link or DNA cue for the recruitment of ATM in different biological contexts. We determined DSBs formation upon IFN-γ treatment both directly by detecting γ-H2AX foci, known to surround both ends of these lesions, and indirectly by preventing transcription-induced DSBs by inhibiting topoisomerase II, the enzyme responsible for such activity. Since H2AX phosphorylation on serine 139 is a target of ATM in DSBs context, detection of γ-H2AX foci formed upon IFN-γ treatment could additionally indicate the involvement of ATM in the repair of IFN-γ-induced DSBs (Bakkenist and Kastan, 2003; Hakem, 2008; Helt *et al*., 2005; Kastan and Lim, 2000; Rogakou *et al*., 1998).

No increase on the number and area of γ-H2AX foci was observed by immunofluorescence (**Fig. 5.1**). Preventing transcription-induced DSBs by topoisomerase II (**Fig. 5.2**) did not impair ISGs transcription in response to IFN-γ in a comparable way to ATM silencing (**Fig. 4.7**). The impairment observed in the transcription of Ii and HLA-DRα, but not consistently in CIITA expression, caused by merbarone, almost certainly resulted from the accumulation of excessive coiling in these highly transcribed genes and not from the absence of ATM recruitment, which was expected to occur quicker than transcription-induced DNA coiling.

These data indicate that the factor determining ATM recruitment to IFN-γ pathway was not the occurrence of DSBs. However, these may not be the only DNA alterations capable of recruiting ATM activity. Indeed, ATM activation has been shown to occur not only in response to DSBs, but also in cells subjected to hypotonic stress (Earnshaw and Laemmli, 1983; Jeppesen *et al*., 1992) or treated with drugs that can alter chromatin structure without introducing DSBs, namely

chloroquine (Jensen *et al*., 1994; Krajewski, 1995; Snyder, 2000) and histone deacetylase inhibitors (Krajewski, 1999; Kuo and Allis, 1998). HDACs could be involved in ATM recruitment by IFN-γ signalling, given their important role in regulation of transcription. In addition, HDAC inhibition was shown to up-regulate ATM-induced transcription in the context of DDR (Jang *et al*., 2010). Nonetheless, at this point how ATM becomes involved as a modulator of IFN-γ activities remains elusive.

STAT1 binding to the promoter region of ISGs precedes and determines the recruitment of RNA polymerase II and the assembly of a multi-protein transcription complex. In order for transcription to occur, chromatin condensation status must be regulated and a relaxed conformation adopted. ATM has been shown to promote chromatin decondensation and to be a transcriptional regulator in the context of DSBs repair. Our data shows that it is also a modulator of IFN-γ signalling at the level of transcription. Therefore we hypothesised that ATM could be necessary to promote appropriate relaxation of the chromatin to allow IFN-γ-induced STAT1 binding to the promoters of ISGs and progression of the transcription machinery along the genes. If this was the case, reduction of ATM protein level should impair differentially STAT1 binding to the promoters of certain ISGs, which we assessed by performing STAT1 ChIP.

We initially optimized the STAT1 ChIP protocol by using IFN-γ-stimulation periods of 30 minutes to look at IRF-1 enrichment. In the literature, binding of STAT1 to ISGs like IRF-1 is frequently assessed after 30 minutes or more of treatment with IFN-γ, at which a high degree of occupancy of the promoter by STAT1 can be detected. Binding of STAT1 to CIITA and HLA-DRα is frequently analysed at even later time points, such as 1 to 4 hours. This makes sense as it may result from the fact that initiation of transcription for CIITA is dependent not only of STAT1 binding but also association of newly synthesised IRF-1, while for HLA-DRα it involves additionally the binding of CIITA to its promoter. Therefore, this order of events has led to STAT1 recruitment to the promoters of these genes to be commonly analysed much later after the initial event of STAT1 translocation into the nucleus and DNA binding. In fact STAT1 could be found associated with the promoters of

all the ISGs analysed as early as after 5 minutes of cytokine treatment and increasing up to 10 minutes (**Fig. 5.4.A**). This indicates that STAT1 binding occurs quickly after IFN-γ stimulation through all ISGs, independently of transcription being promptly initiated, such as for IRF-1, or delayed by the need of newly synthesized proteins as it happens for CIITA. We determined if ATM silencing affected STAT1 recruitment to ISGs promoters upon 10 minutes of stimulation, to ensure a consistent detection of STAT1 at an early time where any disturbances caused by ATM absence should not be overcome by prolonged cytokine treatment.

STAT1 occupancy of IRF-1 and TAP-1 promoters was considerably higher than at CIITA pIV promoter. This could reflect the existence of stronger recognition sequences in the first two ISGs, while weak STAT1 recognition sites in CIITA pIV and HLA-DRα promoters lead to a small and more difficult to detect recruitment of STAT1, although this will probably be sustained for a period of hours. Curiously, STAT1 level of occupancy of CIITA pIV and HLA-DRα promoters detected upon cytokine treatment was not decreased *per se*, but a consistent and significantly higher level of unphosphorylated STAT1 was observed without stimulation. This effect could be responsible for the differences observed at the transcriptional and translational levels between molecules like IRF-1 and CIITA (**Fig. 4.7.1** and **Fig. 4.7.2**). In other words, in the absence of stimulation, upon ATM silencing, an accumulation of unphosphorylated STAT1 at the level of CIITA and HLA-DRα promoters would prevent recruitment of tyrosine 701 phosphorylated/active STAT1. These data also indicated that the effect observed in HLA-DRα expression could be more than a consequence of reduced CIITA mRNA and protein levels, since STAT1 promoter recruitment was affected for both ISGs.

In the attempt to test the hypothesis that ATM silencing increased unphosphorylated STAT1 mobilization, thereby preventing phosphorylated STAT1 recruitment to CIITA pIV and HLA-DRα promoters, we used a pSTAT1-Y701 antibody to immunoprecipitate STAT1. Only two experiments were performed and although we detected enrichment of IRF-1 and TAP1 sequences recovered upon IFN-γ treatment, for both siNS and siATM conditions, the same did not always

happen for CIITA pIV and HLA-DRα. These variations prevent taking any conclusions about the possible differential recruitment of unphosphorylated/phosphorylated STAT1 to different ISGs promoters.

ATM is known to indirectly associate to DNA, *via* association with MRN complex proteins. We tested the possibility of ATM binding to ISGs promoters in response to IFN-γ. We observed a rather low occupancy level of ATM at the promoters of IRF-1, TAP-1, CIITA pIV and HLA-DRα, but consistent and significant (**Fig. 5.6**). However, we were unable to assess how physiologically relevant this binding is, especially considering that CIITA and HLA-DRα transcription is strongly dependent on ATM, but not IRF-1 and TAP-1 transcription and we could not detect a preference for ATM binding to CIITA pIV and HLA-DRα promoters after IFN-γ treatment. Therefore, in order to determine if ATM is recruited to ISGs, further evidences are needed. Performing an ATM ChIP using transfected cells with either a siATM or with a non-specific siRNA could clarify if indeed this binding is real. Nonetheless, we may infer that the low level of binding detected could reflect a very transient event, in which a small amount of ATM binding to DNA could be needed only shortly and locally at the ISGs to induced adequate chromatin remodelling. ATM could promote suitable chromatin opening for tyrosine 701-phosphorylated STAT1 binding, being quickly released after that and bound and release again sequentially. It could also be that it is instead the structural complexity of the genes that will determine the importance of ATM regulation of transcription and not a preferential binding of ATM.

A reciprocal interaction between ATM and STAT1 was confirmed by co-immunoprecipitation (**Fig. 5.7**). This interaction was not IFN-γ-inducible, in the sense that ATM was found associated to unphosphorylated STAT1 in the absence of stimulation. Upon IFN-γ treatment, STAT1 bound to ATM was found to be phosphorylated on tyrosine 701. While the level of total STAT1 protein associated with ATM was quite similar across different periods of IFN-γ stimulation, the amount of tyrosine 701 phosphorylated STAT1 increased with time of cytokine treatment. A way of interpreting these results is to consider that ATM and STAT1 form a stable complex, independent of stimulation and where the ratio between the

two proteins is maintained. Upon IFN-γ treatment, the phosphorylation status of ATM-bound STAT1 is changed. One possibility is that the ATM-STAT1 protein complex gets recruited to the IFN-γ-receptor complex and becomes phosphorylated by the JAKs, translocating then into the nucleus. The other plausible explanation would be that as ATM unbound STAT1 dimers become tyrosine 701 phosphorylated in response to IFN-γ and the concentration of the post-translational-modified STAT1 increases, the unphosphorylated STAT1 initially bound to ATM is exchanged by activated tyrosine 701 phosphorylated STAT1, while the proportion of ATM and STAT1 molecules is maintained during the process.

Regarding the co-immunoprecipitation results we suggest that using a STAT1α antibody to immunoprecipitate STAT1 and the use of an antibody that recognises both isoforms of the protein for western blot analysis, allowed us to not only to determine that ATM interacted with active STAT1α even prior to treatment with IFN-γ, but also that STAT1β was bound to STAT1α, particularly upon stimulation with cytokine, and, therefore, was pulled down together with STAT1α. When immunoprecipitating ATM, STAT1 was reciprocally found in the immunoprecipitated samples, through out all conditions. By using an antibody that targeted STAT1 701 residue tyrosine phosphorylation, we further confirmed that indeed ATM interacted with both unphosphorylated and phosphorylated forms of STAT1α (**Fig. 5.7,** left panel).

Co-immunoprecipitation data also indicated that RNA polymerase II was bound to ATM and to STAT1 (**Fig. 5.9**), which probably means that perhaps an interaction between the three proteins can occur in the nucleus. Other proteins, such as CBP and/or p300 co-activators, which are know to be necessary for RNA Polymerase II association with STAT1 and to be necessary to guarantee that transcription is initiated at an appropriated location, could additionally be involved (Darnell, 1997) (Hiroi and Ohmori, 2003; Snyder *et al*., 2005).

This interaction between ATM and RNA polymerase II sheds some light over the mechanism of action of ATM in regulating IFN-γ responses. It is possible that the

defect observed in CIITA, Ii and HLA-DRα at the transcriptional level, when silencing ATM, results not from a STAT1 binding impairment to these ISGs promoters, but from an inadequate recruitment of RNA polymerase II. The reason why this should only impact on a certain subset of genes remains unclear. It is possible though that the distance needed to be bridged between STAT1 GAS binding site and the polymerase binding site by additional molecules, in order for transcription to take place, is too long in the absence of ATM for genes like CIITA. It is also plausible that transcription of these ATM-dependent genes and RNA polymerase II is actively repressed in resting cells, contrarily, for instance, to what has been described to IRF-1 gene (Wormald *et al*., 2006).

CHAPTER 6

Results

Identification of potential additional DNA repair molecules involved in the regulation of IFN-γ responses

6.1. Introduction

An accumulating body of evidence indicates that chromatin remodelling is a necessary and central event to several biological processes that involve DNA as a template. These include transcription, replication and DNA repair. It thus seems likely that these pathways may use common players for remodelling of chromatin. Yeast INO80 and SWR1 ATP-dependent chromatin remodelling complexes have been shown to be integral components of pathways that maintain genomic integrity. These complexes and their human counterparts can also regulate transcriptional processes (Morrison and Shen, 2009). The elimination of nucleosomal DNA supercoiling in elongating spermatids (Leduc *et al*., 2008) is also facilitated by transient DNA strand breaks. Interestingly, long term IFN-β treatment has also been shown to cause DSBs and ATM activation (Molseeva *et al.*, 2006). In addition, large-scale chromatin decondensation and the induction of chromatin loops in the HLA region on the chromosome 6 have been observed after a 10 minutes treatment with IFN-γ (Christova *et al*., 2007) and our results have shown that ATM silencing impaired HLA-DRα expression at the transcriptional level.

It is conceivable that the capacity of ATM to temporarily perturb heterochromatin condensation status in the context of DBSs repair (Goodarzi *et al*., 2008), changing its topology and increasing DNA accessibility, may be also relevant and necessary for adequate gene transcription in euchromatin regions. The transcriptional silencing complex formed by the heterochromatin-building factors KRAB domain-associated protein 1 (KAP1), heterochromatin protein (HP1) and/or histone deacetylase (HDAC) 1/2 was also shown to be disturbed by ATM signalling and phosphorylation of KAP1. ATM is thought to be responsible for transiently weakening the interactions between KAP1 and heterochromatin to permit DSBs within compacted chromatin regions to be repaired (Goodarzi *et al*., 2008). It is also assumed that, as consequence of this post-translation modification, KAP1 interactions with HDACs and methyltransferases are changed, leading to relaxation of chromatin (Li *et al*., 2007; Li *et al*., 2010; Zeng *et al*., 2008). The concept that KAP1 acts as a repressor of transcription, by inducing histone

modification through association with HDACs and methyltransferases, is supported by several reports (Sun *et al*., 2009) (Chodaparambil *et al*., 2007; Ferreira *et al*., 2007). KAP1 ability to modulate HDACs activity and influence transcription through modifications of the acetylation pattern of histones has been reported at the promoter level. Nonetheless, KAP1 as also been reported as a co-activator of transcription [reviewed in (Xu and Price, 2011)].

In addition to KAP1 regulating transcription *via* chromatin remodelling, this protein has been shown to physically interact with members of the STAT family, such as STAT3 and STAT1 (Kamitani *et al*., 2008; Kamitani *et al*., 2011; Tsuruma *et al*., 2008). It has also been reported that KAP1 acts as a repressor of transcription induced by TNF-α by inhibiting NF-κB/p65 acetylation by a mechanism that involves disruption of the interactions between this protein and STAT1/p300 (Kamitani *et al*., 2011). This same manuscript also reported STAT3 serine 727 to be negatively regulated by KAP1. Moreover, it appears that STAT1-induced expression of IRF-1, in response to type I IFNs is also regulated by KAP1 (Kamitani *et al*., 2008). Considering the evidence that KAP1 can interact with several STAT family members and the prominent role of this protein as a regulator of transcription, we next analysed KAP1 involvement in IFN-γ-mediated responses. Involvement of KAP1 in IFN-γ signalling was analysed by studying IFN-γ-induced expression of HLA class I and II by flow cytometry upon KAP1 silencing by RNAi. We also sought to determine if KAP1 was a negative regulator of STAT1 phosphorylation. To do so, we analysed STAT1 serine 727 and tyrosine 701 phosphorylation by western blotting upon silencing of KAP1. This was done upon KAP1 silencing, either alone or in combination with ATM knockdown by RNAi, which could provide an indication regarding the nature of the impairment caused by the absence of ATM and if it reverted the phenotype seen with siATM.

Besides HDAC1/2 involvement in ATM-mediated regulation of heterochromatin condensation status in DSBs (Goodarzi *et al*., 2008), HDACs have also been implicated in ATM-regulated transcription in the realm of DDR. In fact, the use of HDAC inhibitors was responsible for up-regulating ATM-mediated transcription of certain genes such as MCL1, growth arrest and DNA damage (Gadd45) and p21

(Ju and Muller, 2003; Jang *et al*., 2010). Moreover, STAT1 acetylation was shown to promote de-phosphorylation of this transcription factor by promoting TCP45 phosphatase binding to STAT1. STAT1 acetylation was responsible for its impaired transcriptional activity in response to IFN-α. HDAC3 was shown to be relevant for the regulation of STAT1 acetylation (Kramer *et al*., 2009), but, in fact, STAT1 has been shown to interact both with HDAC1 and HDAC3 (Kramer *et al*., 2006).

One report by Klampfer and colleagues (2004) showed HDAC1, HDAC2 and HDAC3 positively regulated IFN-γ-induced transcription by STAT1. As such, several connections can be established involving STAT1 transcriptional activity, the activity of HDACs and ATM. Therefore, we analysed the impact of silencing different combinations of HDAC1, HDAC2 and HDAC3 alone or with ATM by RNAi, using HLA class I and II expression. Our aim was to understand if HDAC activity was important for IFN-γ-induced responses, if any of the HDACs tested would have a predominant role and if silencing these HDACs would recapitulate the effects of ATM knockdown.

The ATM substrate BRCA1 was also shown to possess large-scale chromatin unfolding activity (Ye *et al*., 2001), while the MRN complex is known to be involved in the recruitment and association of ATM with DNA extremities in DSBs (Lavin and Kozlov, 2007). We therefore, investigated several proteins involved with ATM in the repair response to DSBs, namely, the members of the MRN complex, p53 and BRCA1. The relevance of all these proteins was also analysed by screening the IFN-γ-induced expression of HLA class I and II by flow cytometry upon silencing by RNAi.

6.2 A role for KAP1 in ATM-mediated regulation of IFN-γ biological responses

6.2.1. KAP1 knockdown counteracted ATM silencing in IFN-γ responses: a novel negative regulator of STAT1 phosphorylation?

As mentioned already, KAP1 has been known to interact with STAT1 and STAT3, and to act as a negative regulator of STAT3 serine 727 phosphorylation. In addition, a functional link has been established between ATM and KAP1, with ATM inhibiting KAP1 repressor activity over the chromatin structure in order to allow relaxation to occur. This relaxation is an essential requisite for transcription, including IFN-γ-induced transcription of ISGs mediated by STAT1. We also know that STAT1 serine 727 phosphorylation occurs after STAT1 binding to DNA (Sadzak *et al*., 2008). Therefore, we modulated KAP1 protein levels using RNAi and assessed if this impacted on IFN-γ-induced serine 727 phosphorylation of STAT1. KAP1 knockdown was also done concomitantly with ATM silencing, with the purpose of establishing if the absence of KAP1, which in another context is negatively regulated by ATM, alleviated the impairment imposed on STAT1 serine 727 phosphorylation by ATM silencing.

U2OS cells were transfected with siRNA oligonucleotide pools against ATM and KAP1, individually or in combination. A non-targeting sequence was also transfected with two different concentrations of siRNA, to control for single and double transfection effects. Forty-eight hours after transfection, cells were stimulated for short periods of time with IFN-γ and STAT1 tyrosine 701 and serine 727 phosphorylation levels analysed by western blotting (**Fig. 6.1**).

IFN-γ-induced phosphorylation of STAT1 serine 727 residue was observed after 10 minutes of treatment (orange arrow) (**Fig. 6.1**). The level of STAT1 serine 727 phosphorylation increased with time of stimulation, being higher after 30 minutes of treatment in untransfected and both siNS conditions. ATM and KAP1 protein levels were appropriately reduced upon knockdown by RNAi. When ATM was silenced, phosphorylation of STAT1 on serine 727 was diminished/delayed

comparatively to the one seen in untransfected and siNS samples after 30 minutes of IFN-γ treatment. We have consistently observed that the impairment in STAT1 serine 727 phosphorylation in siATM-transfected cells is only seen at early time points after IFN-γ treatment (*i.e.* up to 30 minutes) and it then catches up to reach normal induction levels as detected in untransfected or siNS-transfected cells. Contrarily to the effect caused by siATM, knockdown of KAP1 increased the level of STAT1 serine phosphorylation upon IFN-γ treatment when compared to the untransfected and siNS conditions. KAP1 thus acted as a negative regulator of STAT1 serine 727 phosphorylation. When ATM and KAP1 were silenced simultaneously, the impairment on STAT1 serine 727 phosphorylation caused by siATM was abrogated by KAP1 knockdown .

These data suggested that not only was KAP1 a negative regulator of STAT1 serine phosphorylation, as it has been described for STAT3, but that it may also work in a concerted way with ATM. KAP1 phosphorylation at serine residue 824, which had been shown to be regulated by ATM kinase activity was also assessed in response to IFN-γ treatment. KAP1 was phosphorylated at serine 824 residue in the absence of stimulation. Curiously, this phosphorylation was reduced upon IFN-γ treatment in untransfected and siNS-transfected conditions. In addition, no such phosphorylation was observed when ATM was silenced. In fact, in siATM-transfected cells, KAP1 serine 824 phosphorylation was similar to that encountered in stimulated untransfected cells, or siNS-transfected cells treated with IFN-γ (**Fig. 6.1**).

Phosphorylation of STAT1 tyrosine 701 residue was clearly visible after 5 minutes of stimulation in untransfected and siNS-tranfected samples, as expected. STAT1 tyrosine 701 phosphorylation increased after longer periods of stimulation and was maximal at 10 minutes after stimulation. As previously, ATM silencing affected this post-translational modification and STAT1 tyrosine 701 was impaired, particularly at the 5 minutes time point, but reached normal levels with increasing time of stimulation. By reducing the levels of KAP1 only a slight, but consistent, increase of STAT1 tyrosine 701 phosphorylation could be seen. Such an increase may have been responsible for reverting the impairment caused by siATM on STAT1

tyrosine 701 phosphorylation, as noticed when both proteins were targeted simultaneously (**Fig. 6.1**).

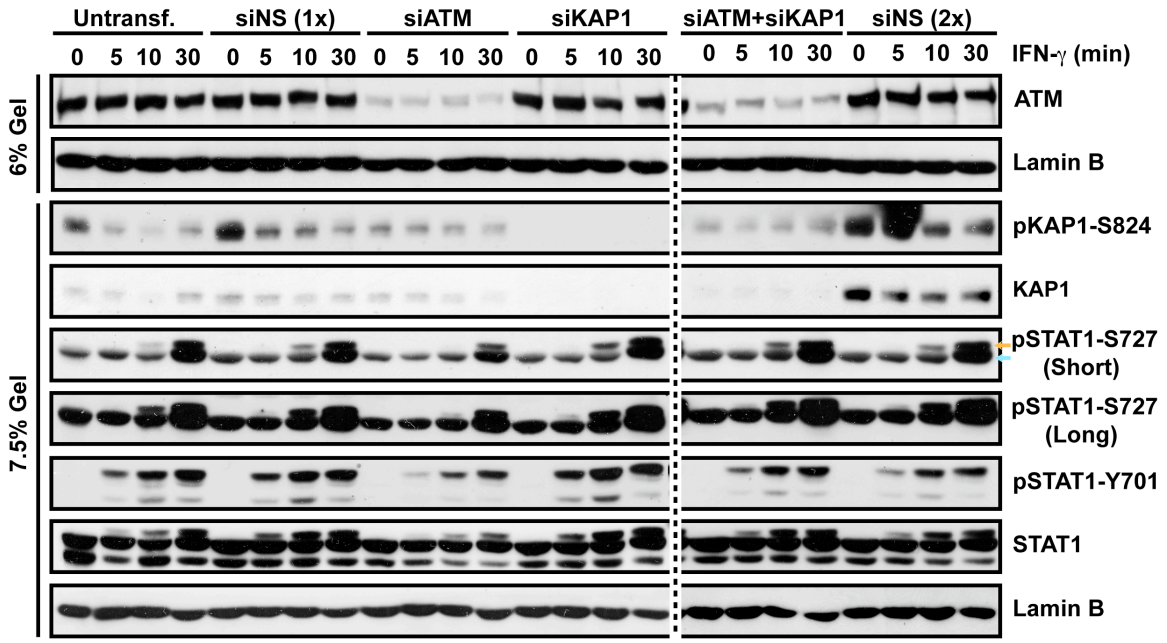


Figure 6.1. KAP1 negatively regulated IFN-γ-induced STAT1 serine 727 and tyrosine 701 phosphorylation. U2OS cells were transfected with 20 nM of KAP1 and ATM siRNAs, either individually or combined, using RNAiMAX, or were left untreated. In the case of the non-specific (NS) siRNA, cells were transfected with 20 nM [siNS (1x)] or 40 nM [siNS (2x)]. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for the time points shown in the figure (min - minutes). Total cell lysates were prepared using Schindler's buffer. Proteins were resolved using 6% and 7.5% SDS-PAGE gels and western blotting analysis was performed using antibodies against pKAP1-S824, pSTAT1-S727 and pSTAT1-Y701 to assess phosphorylation levels in response to IFN-γ (Long - long exposure, Short - short exposure). Antibodies against total ATM, STAT1 and KAP1 were used to determine the total amount of these proteins and the efficiency of ATM and KAP1 knockdown by RNAi. Lamin was used as a loading control. The orange arrow indicates IFN-γ-induced STAT1 serine 727 phosphorylation, while the blue arrow points to basal phosphorylation level detected with the same antibody. These results are representative of three independent experiments. Untransf. - untransfected, NS - non-specific.

6.2.2. KAP1 knockdown reverted the impairment caused by ATM silencing in IFN-γ-induced expression of HLA class I, but only partially reverted the effect on HLA class II expression

Our results suggest that KAP1 was a negative regulator of STAT1 phosphorylation. We, therefore, next analysed if silencing this protein also impacted on IFN-γ-induced HLA class I and II expression. U2OS cells were transfected with siRNA against ATM and KAP1, individually or in combination or left untransfected, as described before. Cells were stimulated with IFN-γ 48 hours after transfection for 24 hours and the induction of HLA class I and II was assessed by flow cytometry (**Fig. 6.2**). Results were analysed using FlowJo. In brief, IFN-γ-inducible effect on HLA class I and II expression was calculated by subtracting the corresponding basal (untreated) geometric mean value from the geometric mean of each treated condition. These values were then normalised, dividing by the value obtained for the siNS non-targeting controlling for the adequate amount of siRNA and statistical significance of the fold change relative to corresponding non-specific control was determined using Student's t-test and at least two independent experiments after normalisation (**Fig. 6.2.B**).

IFN-γ treatment induced both HLA class I and II expression as reflected by the increase in the geometric mean values depicted on the top right hand corner of each graph (**Fig. 6.2.A**). No significant differences were noticed between the siNS controls and the untransfected conditions, regarding the induction of both HLA classes, suggesting that transfection *per se* was without effect. ATM silencing significantly impaired, as previously observed for a period of 24 hours, the IFN-γ-inducible expression of HLA class I ($p<0.05$) and of HLA class II ($p<0.001$). HLA class II expression was once again shown to be more dependent on ATM, with silencing of this protein causing a higher reduction of these molecules on cell surface than what was seen for HLA class I molecules (**Fig. 6.2.A** and **Fig. 6.2.B**).

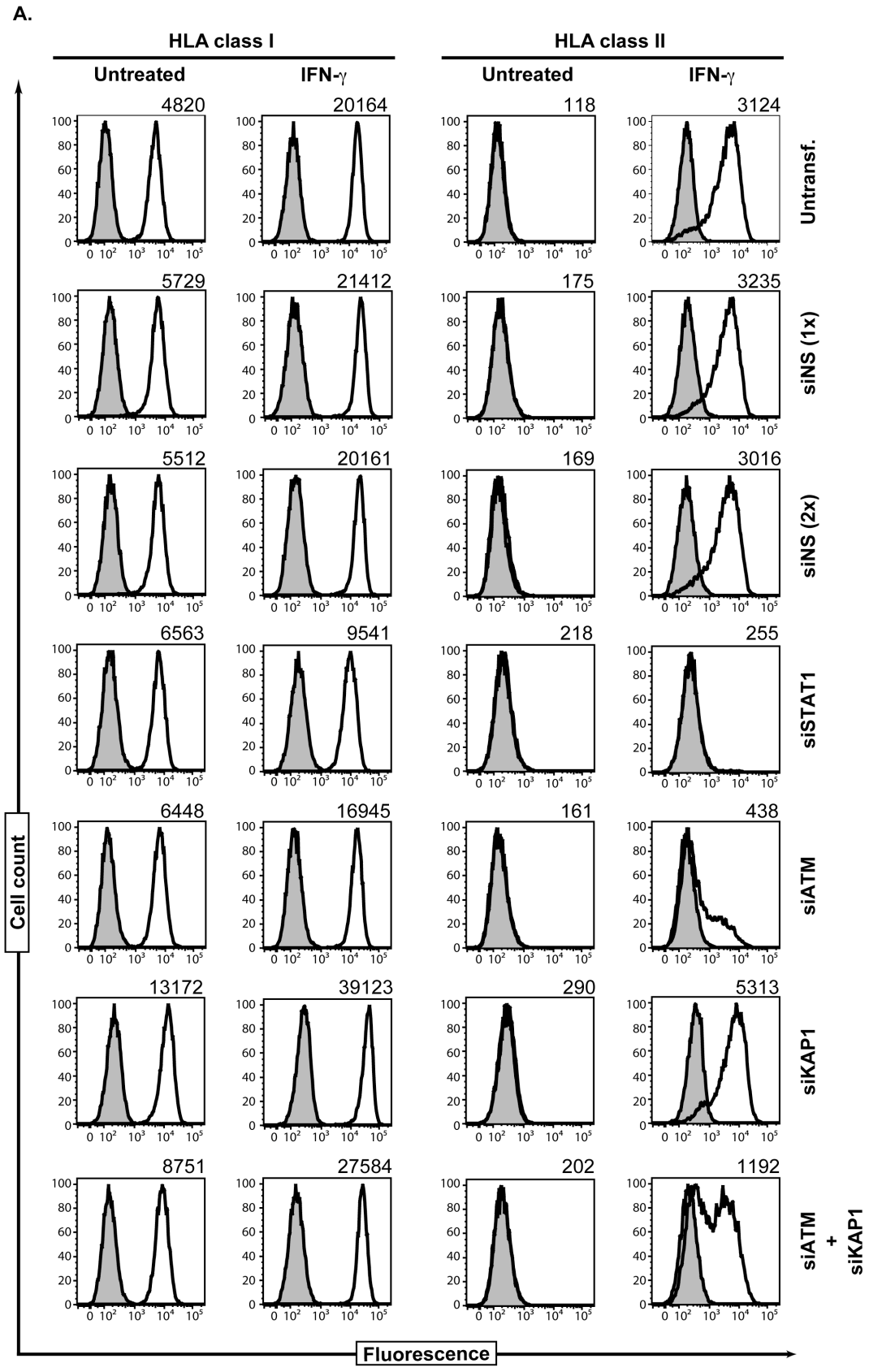


Figure 6.2. KAP1 knockdown partially rescued the impairment caused by siATM in IFN-γ-induced expression of HLA class II. (Continued on page 210).

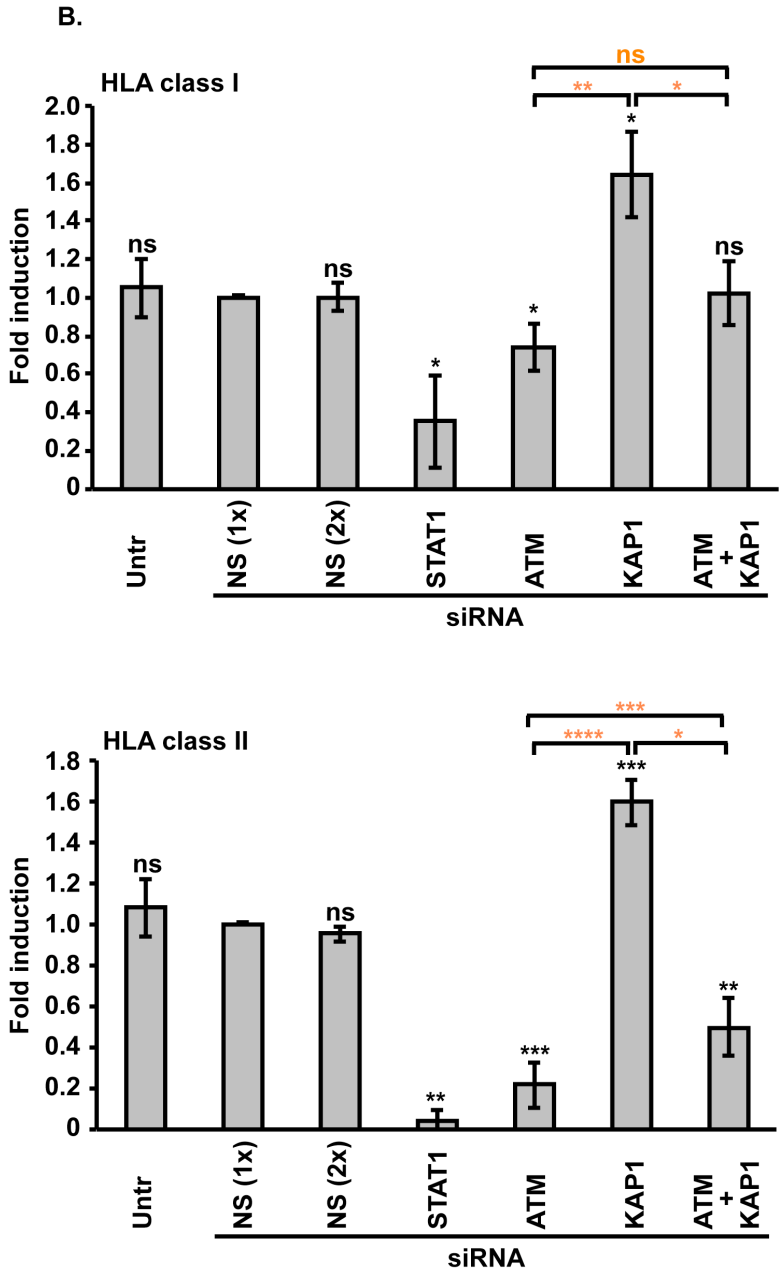


Figure 6.2. KAP1 knockdown partially rescued the impairment caused by siATM in IFN-γ-induced expression of HLA class II. U2OS cells were transfected with 20 nM of siRNAs against ATM, STAT1 or KAP1 or a non-specific sequence, using RNAiMAX. Cells were double transfected with 20 nM of both KAP1 and ATM siRNAs. In the case of the non-specific (NS) siRNA, cells were transfected with 20 nM [siNS (1x)] or 40 nM [siNS (2x)]. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for 24 hours. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody or with a PE-conjugated anti-HLA-DRα Class II antibody and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-Class I (1st and 2nd vertical panels) and HLA-DRα Class II (3rd and 4th vertical panels) expression are presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The

matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **B.** For each condition the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample. Normalisation was done dividing these values by value of the adequate siNS non-targeting control, with single transfected siRNA conditions normalised to the 20 nM siNS control, while KAP1 and ATM double transfection condition was normalised to the 40 nM siNS control. Mean ± SD obtained using three independent experiments after normalisation are shown for HLA class I (upper graph) and class II (lower graph). Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. Untr - untransfected, NS - non-specific.

In contrast to the effects caused by ATM silencing, KAP1 knockdown augmented expression of both HLA class I and II molecules after IFN-γ treatment (**Fig. 6.2.A**). siKAP1-dependent up-regulation was statistically significant for both HLA class I ($p<0.05$) and II ($p<0.001$) for 24 hours of stimulation with a saturating concentration of IFN-γ (500 IU/ml) (**Fig. 6.2.B**).

In addition, siKAP1 was able to counteract, at least partially, the siATM phenotype with regards to HLA-class II expression ($p<0.001$, in comparison to the siATM-transfected condition) when both molecules were simultaneously silenced. KAP1 silencing also counteracted the impairment caused by ATM knockdown in IFN-γ-induced HLA class I expression. Although due to inter-experimental variations this recovery does not come across as statistically significant, it was consistently observed in each experiment, and is clear when looking at the geometric mean values of siKAP1 and siATM samples versus the double-knockdown sample (**Fig.6.2.A**). Moreover, KAP1 silencing was enough to increase the basal level of HLA class I expression, independent of IFN-γ signalling, though as expected it was unable to promote HLA class II expression in resting cells.

6.2.3. KAP1 constitutively interacted with STAT1 and this interaction was disrupted upon IFN-γ stimulation

KAP1 and STAT1 association in response to type I IFNs has already been

reported. Co-immunoprecipitations of KAP1 and STAT1 were used to establish the existence of such an interaction in U2OS cells and to determine if this interaction was regulated by IFN-γ. U2OS cells were treated with IFN-γ for short periods of time (2.5, 5 and 10 minutes) or left untreated as previously described. Immunoprecipitations of KAP1 and STAT1 were carried out using whole cell extracts, followed by protein separation in 6% SDS-PAGE gels and western blotting analysis. Controls included a whole cell extract stimulated for 5 minutes with IFN-γ, IgG and beads-only samples (**Fig. 6.3**).

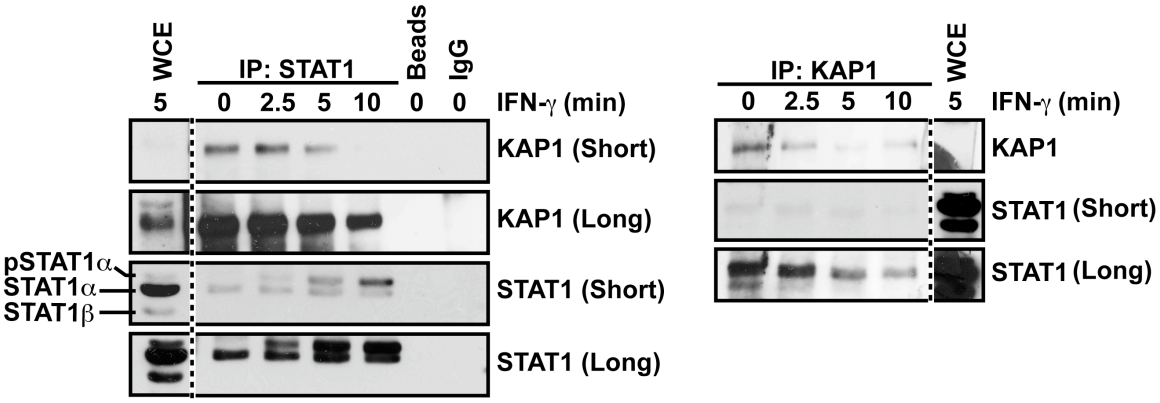


Figure 6.3. STAT1 and KAP1 interacted in the absence of IFN-γ. U2OS cells were stimulated with IFN-γ (500 IU/ml) for the indicated time points (minutes - min), after which cell lysates were prepared using a co-IP lysis buffer. Immunoprecipitation was carried out using 1 mg of protein and antibodies against STAT1α [STAT1α (NCL-STAT1A), STAT1α p91 (C111)], or KAP1 (Long - long exposure, Short - short exposure). IgG and beads-only controls were included together with a whole cell extract sample. Immunoprecipitates were separated in a 6% SDS-PAGE gel and STAT1 (STAT1α/β N-terminus) and KAP1 detected by western blotting. These results are representative of at least two independent experiments yielding identical results.

STAT1 immunoprecipitation was carried out using an antibody raised specifically against the α isoform of the protein, whilst western blotting analysis was performed using an antibody that detected both α and β isoforms of STAT1 (**Fig. 6.3**, left panel). In whole cell extracts, which had been treated for 5 minutes with IFN-γ, the total STAT1 antibody detected both STAT1β (smaller molecular weight) and STAT1α (immediately above) isoforms. A third band was detected by the total STAT1 antibody, which corresponded to phosphorylated STAT1α (higher molecular weight) (**Fig. 6.3**, left and right panel). When STAT1 was

immunoprecipitated using the STAT1α antibody, bands corresponding to unphosphorylated and phosphorylated STAT1α were observed in the samples treated with IFN-γ, whereas the top phosphorylated band was not present in the unstimulated condition. The ratio between phosphorylated and unphosphorylated STAT1α that was being immunoprecipitated increased with time of stimulation, which reflected a general increase in phosphorylated STAT1. When we previously immunoprecipitated STAT1α (**Fig. 5.8**), we also detected a small amount of STAT1β being pulled down together with the α isoform. However, because STAT1β was found associated to STAT1α in a much lower stoichiometry ratio, it was sometimes difficult to detect the truncated isoform, as it was the case for the western blots show here (**Fig. 6.3**).

KAP1 co-immunoprecipitated with STAT1 in the absence and in the presence of IFN-γ stimulation. Interestingly, the amount of KAP1 bound to STAT1α decreased with increasing phosphorylation of STAT1 (**Fig. 6.3,** left panel). This decrease in KAP1 was evident at 10 minutes after cytokine treatment when the amount of phosphorylated STAT1α being pulled-down was considerably higher than the amount of unphosphorylated STAT1. The level of KAP1 detected in whole cell extracts was much lower than in the immunoprecipitated samples and it was sometimes difficult to detect. When KAP1 was immunoprecipitated, STAT1 was reciprocally pulled-down. Despite the variation in the total amount of immunoprecipitated KAP1, after normalisation to total KAP1, the quantity of STAT1 that interacted with KAP1 decreased after 10 minutes of stimulation with IFN-γ. This is the time point at which STAT1 serine 727 phosphorylation becomes clearly noticeable. STAT1α was the predominant STAT isoform being pulled down with KAP1. However, the KAP1 immunoprecipitation did not seem to be particularly efficient and it may be that STAT1β interacting with KAP1 was below the limit of detection. In addition, it is still not clear how much KAP1 mRNA and protein are truly expressed in U2OS cells. These data confirmed that KAP1 and STAT1 interact with each other in resting cells. Importantly, and in contrast to Kamitani *et al*. study we have observed this interaction between endogenous proteins and not in an over-expressing system. In accordance with a repressor role, this association is disrupted upon tyrosine phosphorylation of STAT1.

6.3. Inhibition of HDACs impacted differentially on IFN-γ responses

It has been shown that KAP1 activity is modulated by ATM to de-repress chromatin condensation prior to the repair of DSBs (Goodarzi *et al*., 2008; Ziv *et al*., 2006). This process involves a transcriptional repressor complex where KAP1 and HDAC1/2 participate. Furthermore, the ability of KAP1 to regulate transcription at gene promoter level has been intimately associated with histone acetylation changes (Xu and Price, 2011). In addition, KAP1, HDAC1 and HDAC3 are known to interact with STAT1 (Kamitani *et al*., 2008; Kamitani *et al*., 2011; Klampfer *et al*., 2004; Kramer *et al*., 2006; Kramer *et al*., 2009; Tsuruma *et al*., 2008). These studies led us to hypothesise the existence of a transcriptional regulator complex involving STAT1, ATM, KAP1 and members of the HDAC family.

Thus, using U2OS cells we determined the relevance of HDAC1, HDAC2 and HDAC3 for the expression of HLA class I and II in response to IFN-γ. Individual silencing of HDAC1, HDAC2 and HDAC3 was done by RNAi. In addition, double knockdowns using all possible combinations between siRNA oligonucleotides pools targeting individually each one of these HDACs (HDAC1 and HDAC2, HDAC1 and HDAC3, and HDAC2 and HDAC3) were also performed. These analyses sought to identify if these HDACs had non-redundant or redundant roles in IFN-γ responses. Cells were also transfected with different HDACs siRNA combinations together with ATM siRNA in order to determine if these combinations could counteract or recapitulated the siATM phenotype and if any additive or synergistic effects could be observed. A non-targeting siRNA was transfected using three different concentrations to account for transfection effects of single, double and triple knockdowns. In addition, siSTAT1 was used as a positive control for IFN-γ biological responses. IFN-γ-induced expression of HLA class I and II was calculated and normalised as before. Student's t-test was used to determine statistical significance of the fold change relative to the corresponding non-specific control, based on two (HLA class II) or three (HLA class I) independent experiments (**Fig. 6.4**).

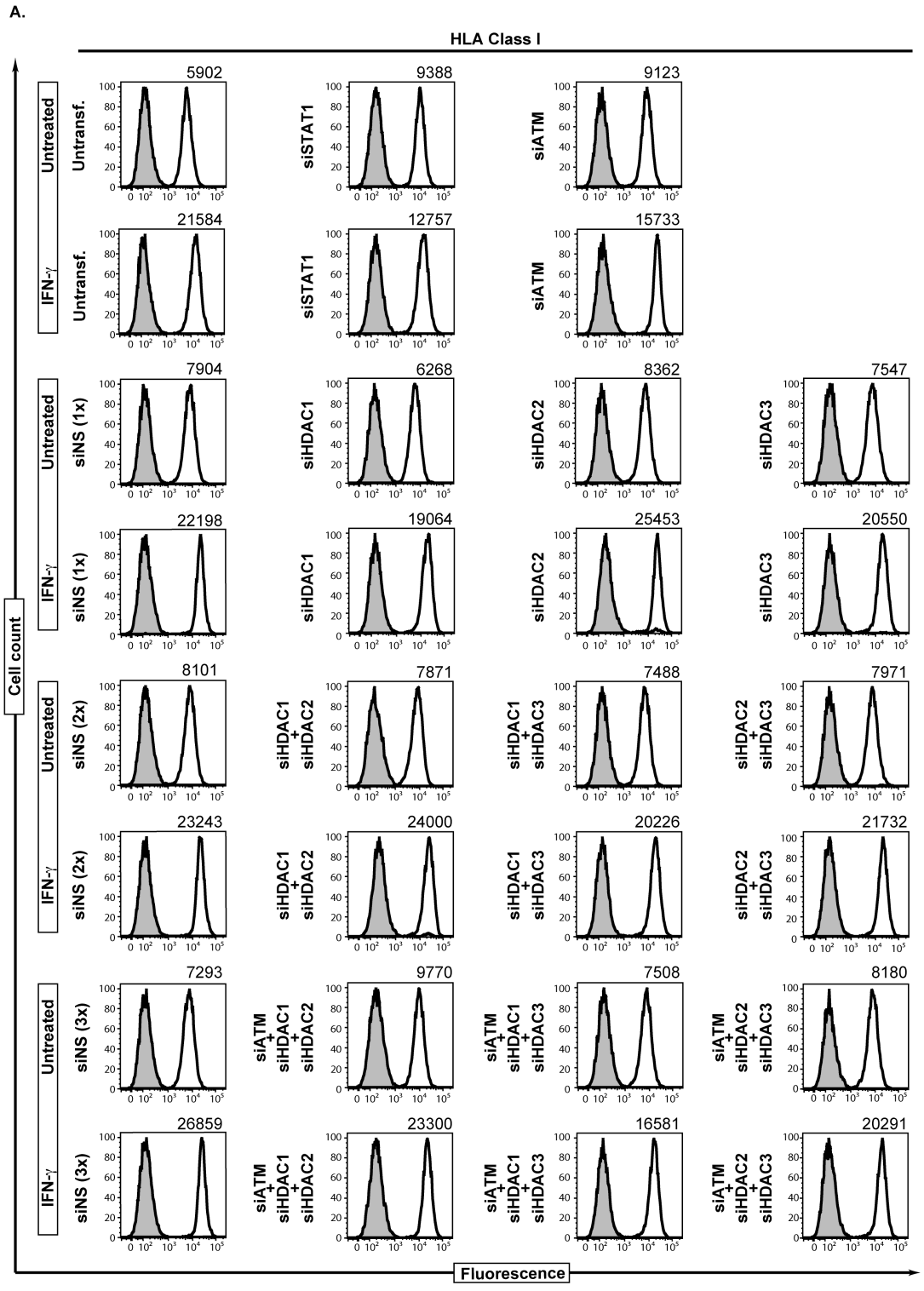


Figure 6.4. HDAC1 and HDAC3, but not HDAC2, silencing by RNAi significantly impaired expression of HLA II molecules after 24 hours of IFN-γ treatment. (Continued on pages 216 and 217).

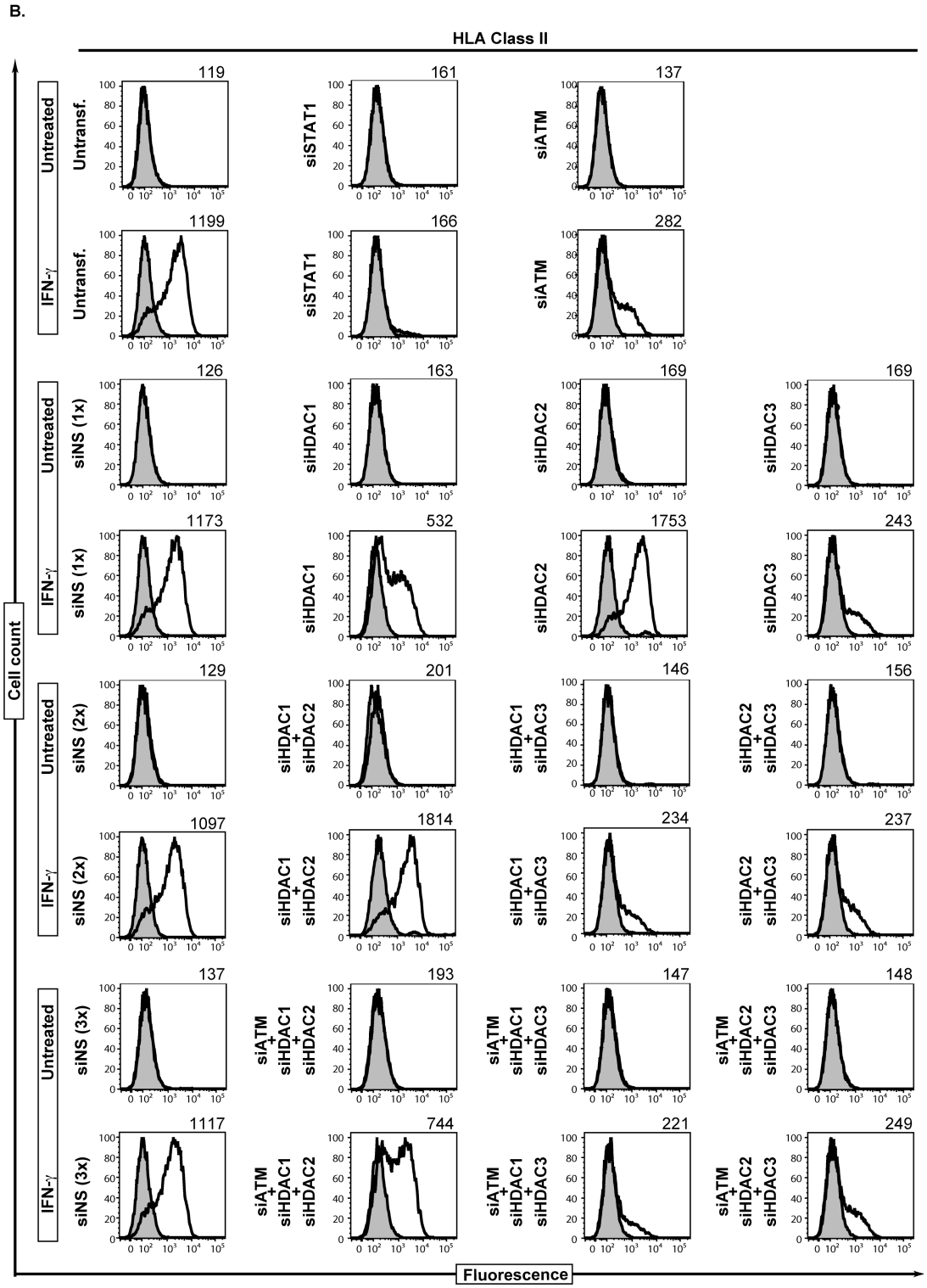


Figure 6.4. HDAC1 and HDAC3, but not HDAC2, silencing by RNAi significantly impaired expression of HLA II molecules after 24 hours of IFN-γ treatment. (Continued on page 217).

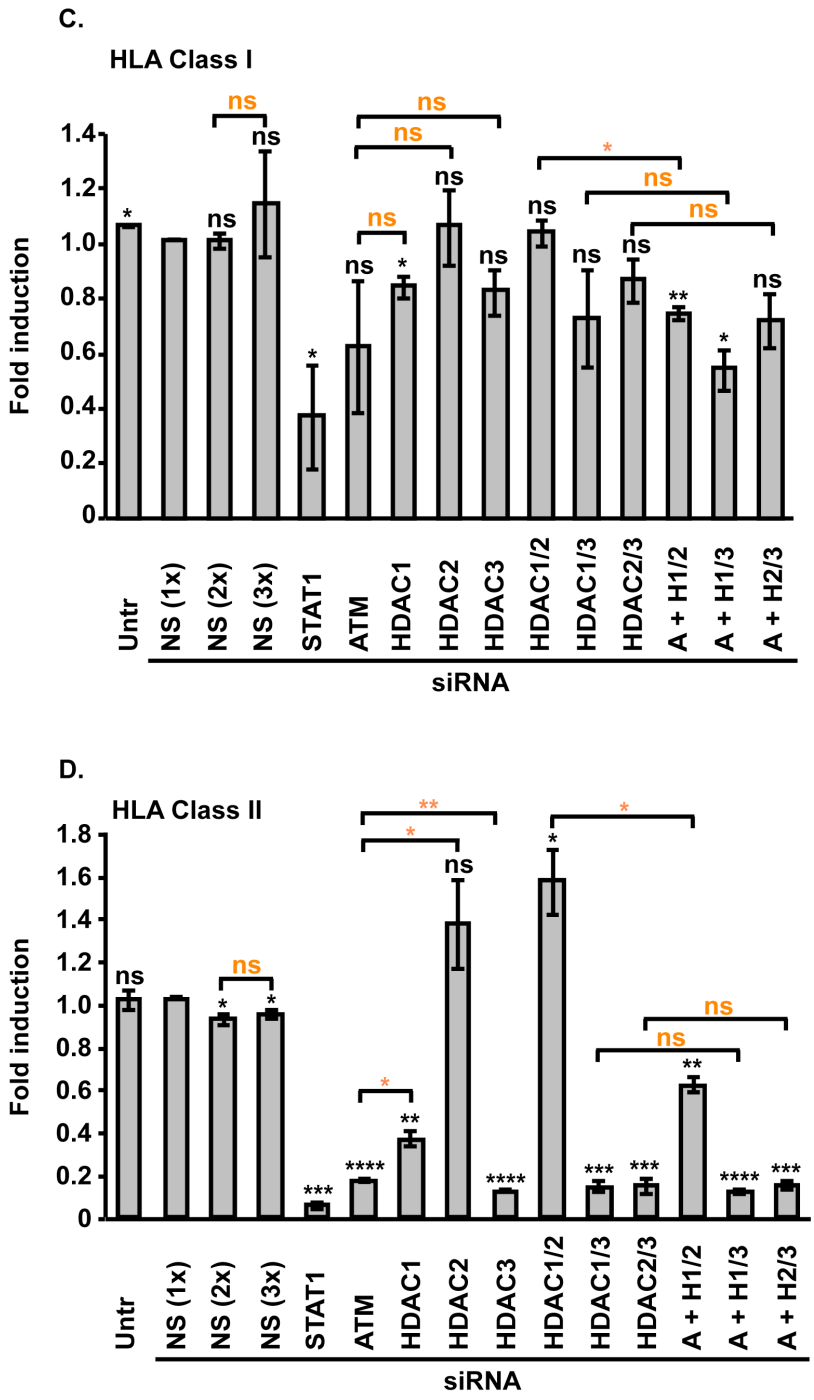


Figure 6.4. HDAC1 and HDAC3, but not HDAC2, silencing by RNAi significantly impaired expression of HLA II molecules after 24 hours of IFN-γ treatment. U2OS cells were subjected to single, double and triple transfections using 20 nM of each individual targeting siRNAs against ATM, STAT1, HDAC1, HDAC2 and HDAC3 or a non-specific sequence, using RNAiMAX, or were left untransfected. In the case of the non-specific (NS) siRNA, cells were transfected with 20, 40 or 60 nM, to control for the effects of transfection of one targeting siRNA or for the combined transfection of two or three different siRNAs. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for 24 hours. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody or with a PE-conjugated anti-HLA-DRα Class II antibody and cell

surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** and **B.** HLA-Class I (**A.**) and HLA-DRα (**B.**) expression are presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **C.** and **D.** For each condition the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample. Normalisation was done dividing these values by the value of the adequate siNS non-targeting control, with single transfected siRNA conditions normalised to the 20 nM siNS control, double transfected conditions normalised to the 40 nM siNS control and the triple transfections normalised to the 60 nM siNS control. Mean ± SD values obtained using three or two independent experiments after normalisation are shown for HLA class I (**C**) and class II (**D**), respectively. Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control with adequate amount of siRNA [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. Untr. - untransfected, NS - non-specific, A - ATM, H - HDAC.

HLA class I was induced by IFN-γ after 24 hours of treatment (**Fig. 6.4.A**) in untransfected and siNS-transfected conditions. No significant difference was observed between the controls transfected with different concentrations of the non-specific, non-targeting, siRNA. Variations in HLA class I expression in these controls in comparison to untransfected cells was too small to be responsible for the impairment observed in response to targeting specific molecules, and also because all conditions were normalised to the respective siNS control. Silencing of STAT1 was used as a control as before. In all three experiments performed, silencing of ATM caused a small reduction in HLA class I, as previously seen. In this case, however, inter-experimental variation made it impossible for this reduction to be statistically significant.

Out of the three HDACs, only HDAC1 affected IFN-γ-induced HLA class I expression, ($p<0.05$) (**Fig. 6.4.C**). No impairment resulted from simultaneously inhibiting two different HDAC proteins, even in combinations where HDAC1 was included. This could be due to inter-experiments variations or more likely because the effect with siHDAC1 was very small. No effects were seen when any two HDACs were silenced. Silencing ATM with HDAC1 and HDAC2 or HDAC3, however, led to statistically significant reductions in HLA class I expression. It thus

appears that HDACs do not play a major role in the regulation of HLA class I expression.

HLA class II expression was detected at the surface of cells stimulated with IFN-γ for 24 hours, either when cells were left untransfected or transfected with three different concentrations of the non-specific siRNA to account for the variable amount of siRNA used in single, double and triple transfections (**Fig. 6.4.B**). siSTAT1, used as positive control for the effects of IFN-γ, abrogated almost completely the expression of HLA class II (**Fig. 6.4.B** and **Fig. 6.4.D**) ($p<0.001$). This was also true for ATM silencing ($p<0.0001$) (**Fig. 6.4.B** and **Fig. 6.4.D**).

In contrast to what was observed with regards to HLA class I expression, HLA class II inducibility was reduced upon RNAi against HDAC1 and HDAC3 (**Fig.6.4.B**). While the reduction caused by HDAC1 silencing was significant ($p<0.01$, compared to siNS control), it was not as striking as the seen after silencing ATM ($p<0.05$, when compared to siATM condition) (**Fig. 6.4.D**). HDAC3 silencing, however, caused a significant and more pronounced impairment of IFN-γ-induced HLA class II ($p<0.0001$), similar in magnitude to that obtained upon transfecting siATM ($p>0.05$, compared to siATM condition) (**Fig. 6.4.D**). In all of the three experiments performed, HDAC2 knockdown led to a small increase in HLA class II expression, which given some inter-experiments variation, was not statistically significant from the siNS-transfected condition ($p>0.05$) (**Fig. 6.4.B** and **Fig. 6.4.D**). However, when HDAC1 and HDAC2 were silenced, the reduction of HLA class II expression caused by knocking down HDAC1 on its own was abrogated and the cell surface expression was similar to the one seen upon transfection with siHDAC2, when compared to the siNS control ($p>0.05$) (**Fig. 6.4.B** and **Fig.6.4.D**). When HDAC3 was silenced together with either HDAC1 (HDAC1+HDAC3) or HDAC2 (HDAC2+HDAC3) ($p<0.001$, comparatively to siNS condition), the phenotype obtained was similar to silencing HDAC3 alone. On one hand, the impairment on HLA class II induction caused by siHDAC3 was not aggravated by simultaneously silencing HDAC1; on the other hand, it counteracted and overcame siHDAC2 effect (**Fig. 6.4.B** and **Fig. 6.4.D**).

Curiously, when ATM was silenced simultaneously with siHDAC1 and siHDAC2 (siATM+siHDAC1+siHDAC2), HLA class II expression was still reduced ($p<0.01$, regarding siNS control), but not to the same extent as when ATM was silenced on its own (**Fig. 6.4.B** and **Fig. 6.4.D**). This result provides further support to the idea that HDAC2 activity differed from HDAC1 and HDAC3 in the context of IFN-γ signalling, that it had an opposing role to that of ATM. When ATM was silenced together with HDAC3 and either HDAC1 ($p<0.0001$, siATM+siHDAC1+siHDAC3) or HDAC2 ($p<0.001$, siATM+siHDAC2+siHDAC3), HLA class II expression was abrogated to a similar level to the one achieved when silencing ATM or HDAC3 on their own, or when silencing HDAC3 together with HDAC1 or HDAC2 (**Fig. 6.4.B** and **Fig. 6.4.D**). Silencing HDAC1 exerted a similar, though less pronounced effect than siATM, whilst HDAC3 silencing completely recapitulated the phenotype of siATM.

6.4. Are additional DNA damage response molecules involved in IFN-γ signalling?

An increasing number of data support the notion that the same proteins or protein complexes that are necessary for remodelling the chromatin structure in DNA repair mechanisms can also exert similar roles in replication or transcription. Proteins often recognised as being a key element in one biological process, may be essential effectors in another, distinct, process. Such is the case of ATM, which is recognised as a central coordinator of the DDR after the occurrence of DSBs and is also a modulator of the IFN-γ pathway as shown by previous work done in our laboratory. We, therefore, decided to screen other molecules of the DDR, by silencing these proteins with RNAi and using IFN-γ-induced expression of HLA class I and II as a read-out. We focussed our attention on members of the MRN complex (MRE11, NBS1 and RAD50), BRCA1 and p53.

U2OS cells were transfected using siRNA targeting individually each one of the molecules mentioned above, as well as siRNA targeting ATM or a non-targeting sequence. Forty-eight hours after transfection cells were treated for 24 hours with a non-saturating (25 IU/ml) or with a saturating concentration of IFN-γ (500 IU/ml)

(**Fig. 6.5**), as well as for 72 hours just with the saturating concentration of the cytokine, after which HLA class I and II expression was assessed as previously (**Fig. 6.6**).

HLA class I was expressed above basal levels upon treatment with IFN-γ in untransfected and siNS-transfected cells, with a higher induction being achieved at the saturating concentration of the cytokine, as it can be seen from the increase in geometric mean values after treating cells with a concentration of 500 IU/ml *versus* 25 IU/ml (**Fig. 6.5.A**).

HLA class I inducible expression was impaired by ATM silencing comparatively to the siNS-transfected condition after 24 hours of treatment with IFN-γ, as can be observed by a decrease in the geometric mean value (**Fig. 6.5.A**). The HLA class I expression was modestly, but significantly impaired in siATM-transfected samples, either in the presence of a non-saturating ($p<0.001$) (**Fig. 6.5.B**) or saturating concentrations of IFN-γ ($p<0.01$) (**Fig. 6.5.C**), and the level of impairment in terms of fold induction was similar. Of all the additional molecules tested, only RAD50 knockdown significantly reduced HLA class I expression after 24 hours of IFN-γ treatment (**Fig. 6.5.A**). This reduction was seen when using the non-saturating concentration of IFN-γ ($p<0.05$) (**Fig. 6.5.B**) and was not abrogated by increasing the amount of IFN-γ (**Fig. 6.5.C**). Silencing of p53 led to a significant up-regulation of HLA class I ($p<0.05$) upon treatment with saturating (**Fig. 6.5.C**), but not with non-saturating concentrations of IFN-γ (**Fig. 6.5.B**) for a period of treatment of 24 hours.

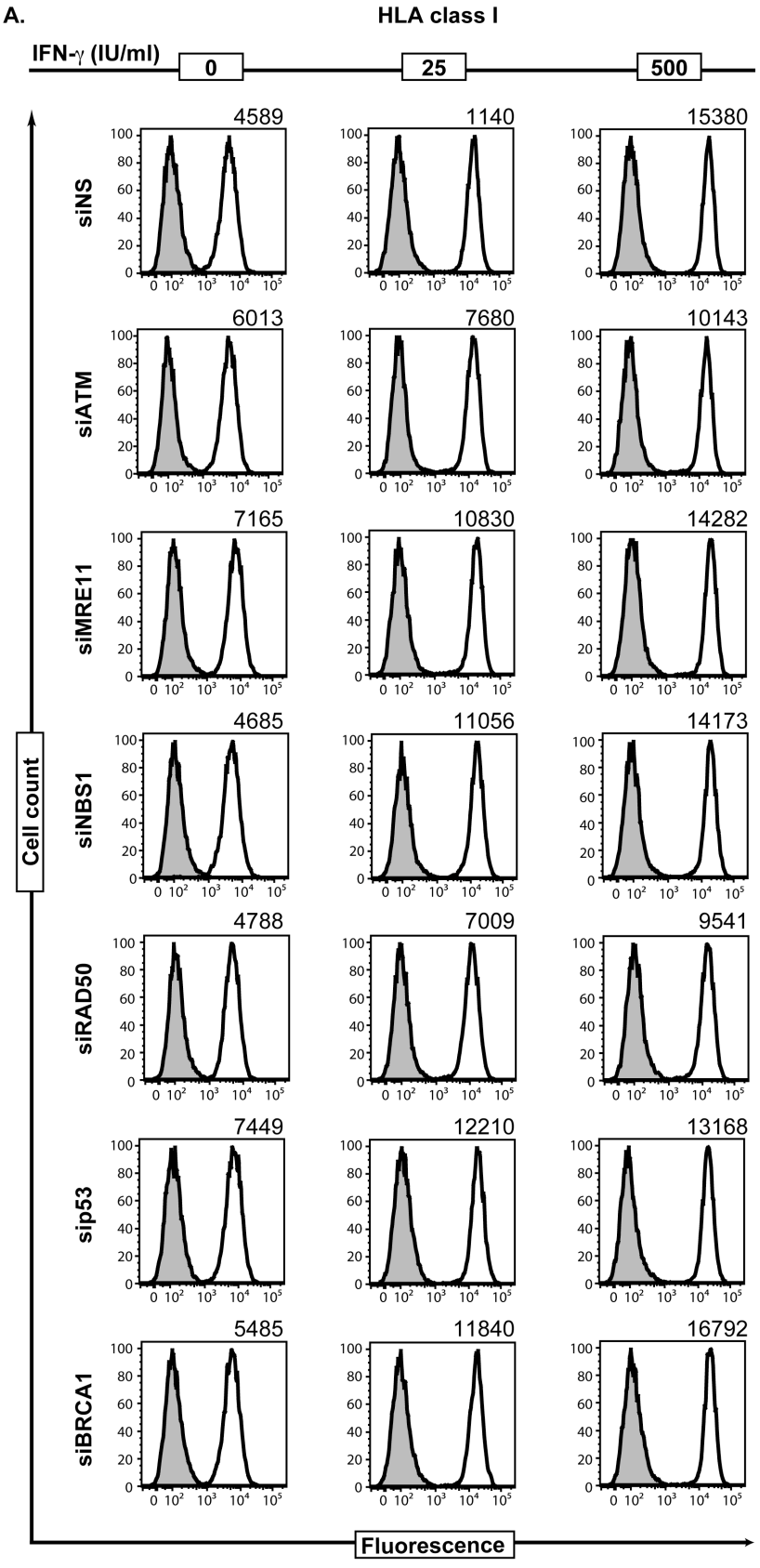


Figure 6.5. Expression of HLA Class I was reduced by ATM and RAD50 knockdown after 24 hours treatment with IFN-γ. (Continued on page 223).

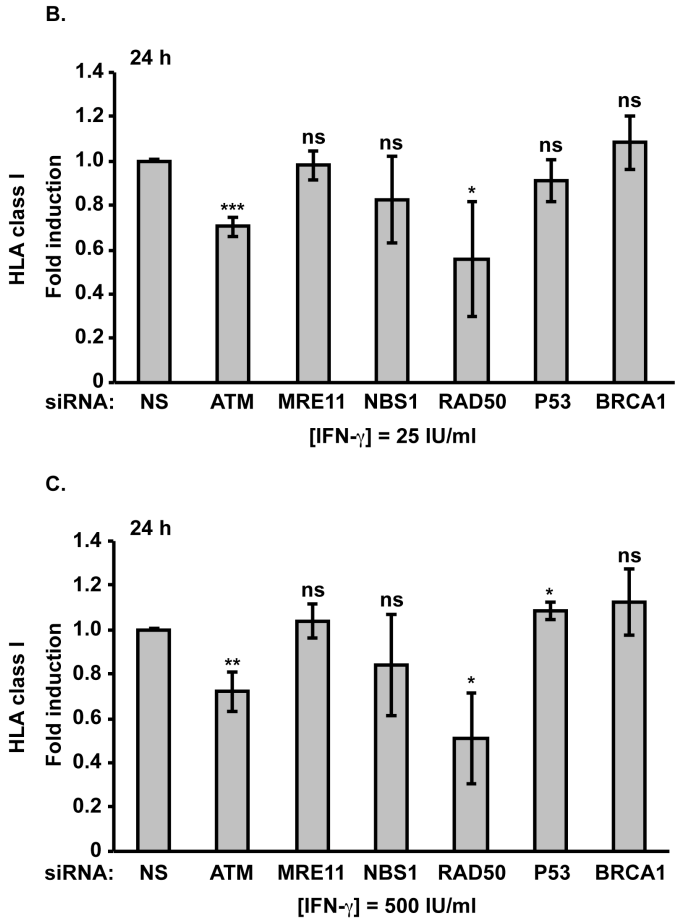


Figure 6.5. Expression of HLA Class I was reduced by ATM and RAD50 knockdown after 24 hours treatment with IFN-γ. U2OS cells were transfected with 20 nM of siRNAs against ATM, MRE11, NBS1, RAD50, p53 or BRCA1, or with a non-targeting sequence using RNAiMAX. Forty-eight hours after transfection cells were stimulated with IFN-γ (25 or 500 IU/ml) for 24 hours. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-Class I expression is presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **B** and **C.** For each condition the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample and then normalised by dividing the obtained value by the siNS non-targeting control. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class I (**B** - 25 IU/mL and **C** - 500 IU/ml of IFN-γ). Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. NS - non-specific.

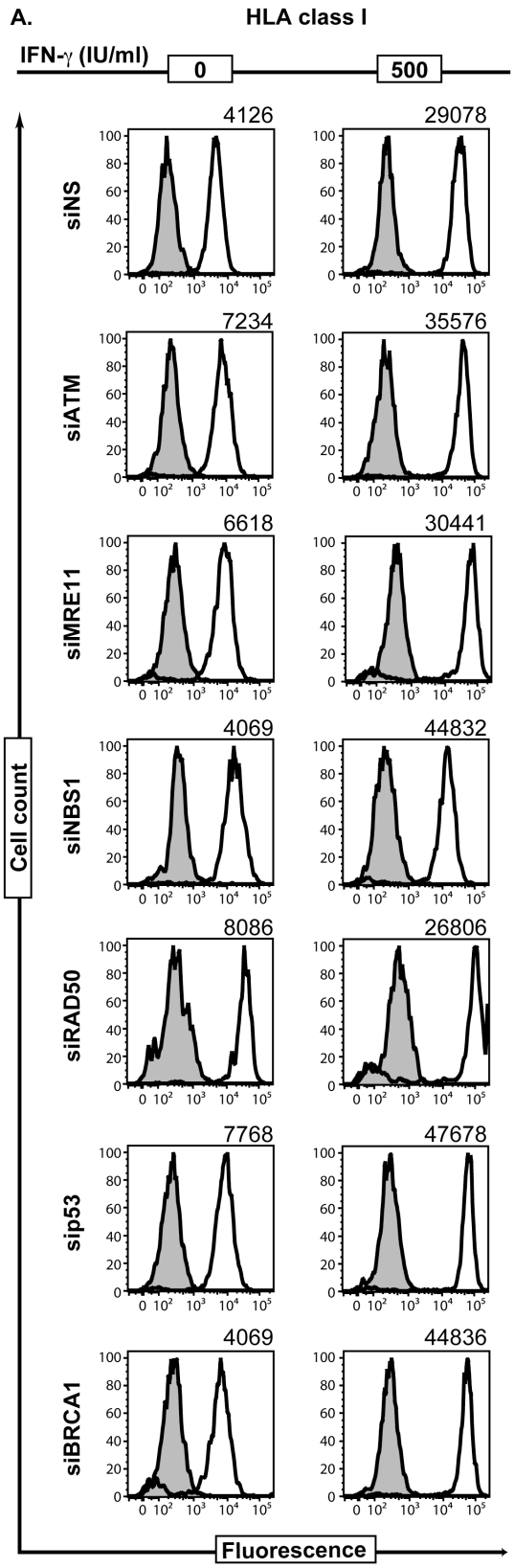


Figure 6.6. After prolonged exposure to IFN-γ for 72 hours, expression of HLA Class I was significantly reduced by RAD50 knockdown, while it was increased by BRCA1 silencing. (Continued on page 225).

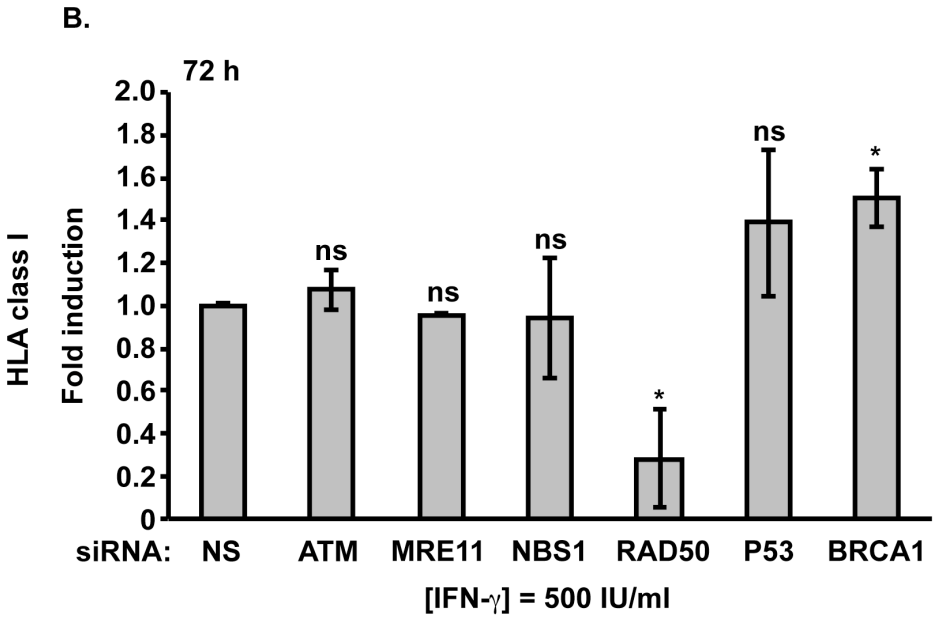


Figure 6.6. After prolonged exposure to IFN-γ for 72 hours, expression of HLA Class I was significantly reduced by RAD50 knockdown, while it was increased by BRCA1 silencing. U2OS cells were transfected with 20 nM of siRNAs against ATM, MRE11, NBS1, RAD50, p53 and BRCA1 or with a non-targeting sequence using RNAiMAX. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for 72 hours. Cells were then stained with a FITC-conjugated anti-HLA-Class I-A, -B, -C antibody and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-Class I expression is presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of two independent experiments yielding similar results is shown. **B.** For each condition the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample and then normalised by dividing the obtained value by the siNS non-targeting control. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class I. Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. NS - non-specific.

We then tested if the effect caused by silencing either RAD50 or p53 could be alleviated by increasing the time of stimulation up to 72 hours and for that purpose only the saturating concentration was used (**Fig. 6.6**). While RAD50 knockdown was still found to significantly impair IFN-γ-induced expression of HLA class I ($p<0.05$) after prolonged treatment with a saturating concentration of the cytokine, p53 silencing had no particular effect ($p>0.05$) (**Fig. 6.6.A** and **Fig. 6.6.B**). BRCA1

was seen to significantly increase these molecules at cell surface, as indicated by the increased geometric mean value upon continued stimulation for three days ($p<0.01$) (**Fig. 6.6.A** and **Fig. 6.6.B**). ATM silencing effect over IFN-γ-induced expression of HLA class I was overcome by prolonged treatment with a saturating concentration of this cytokine ($p>0.05$) (**Fig. 6.6.A** and **Fig. 6.6.B**) and, therefore, the siATM phenotype differed from both siRAD50 and siBRCA1.

With regards to HLA class II expression, treatment with IFN-γ for 24 hours, either at non-saturating or saturating concentrations, was affected by individual knockdown of two members of the MRN complex, namely MRE11 and NBS1, but not by RAD50 (**Fig. 6.7.A**). The impairment caused by MRE11 knockdown was statistically significant for both concentrations of IFN-γ ($p<0.001$) and the same was true for NBS1 silencing ($p<0.001$, for 25 IU/ml of IFN-γ; $p<0.0001$, for 500 IU/ml of IFN-γ) (**Fig. 6.7.B**). In addition, reduced levels of p53 protein also led to a small, although statistically significant, decrease of IFN-γ-induced expression of HLA class II, regardless of the amount of cytokine used ($p<0.001$) (**Fig.6.7.A** and **Fig. 6.7.B**). Interestingly, downregulation of ATM had a more pronounced effect on HLA class II expression than downregulation of any of these molecules (**Fig.6.7.A** and **Fig. 6.7.B**).

However, upon prolonged treatment for 72 hours with a saturating concentration of IFN-γ, only the silencing of NBS1 still caused a reduction on HLA class II expression ($p<0.05$), as did silencing of ATM ($p<0.01$) (**Fig. 6.8.A** and **Fig. 6.8.B**). Curiously, siBRCA1 appeared to significantly increase IFN-γ-induced HLA class II expression ($p<0.01$) (**Fig. 6.8.A** and **Fig. 6.8.B**), as it did for HLA class I (**Fig. 6.6.A** and **Fig. 6.6.B**).

According to these results, RAD50 may have a role on IFN-γ-induced expression of HLA class I and this is unlikely to be related to ATM, since silencing of this additional DDR effector did not impair on HLA class II. MRE11 and NBS1 appear to be involved in HLA class II regulation, but not with HLA class I expression. BRCA1 was the only molecule that consistently affected both HLA class I and II by increasing their expression and thus appearing to have an opposing role to the one carried out by ATM.

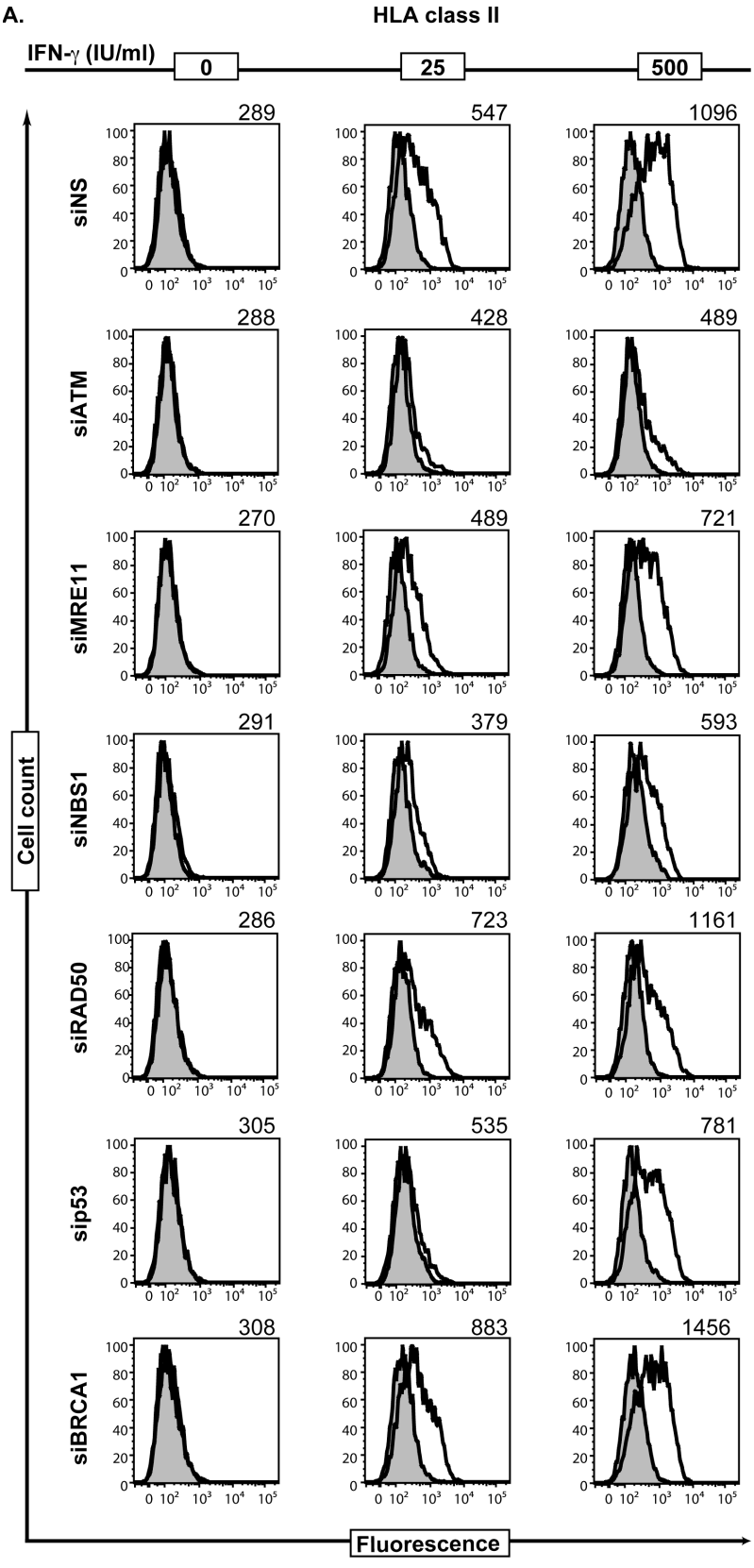


Figure 6.7. Expression of HLA class II after 24 hours treatment with IFN-γ was significantly reduced upon silencing of ATM, MRE11, NBS1 and p53 by RNAi. (Continued on page 228).

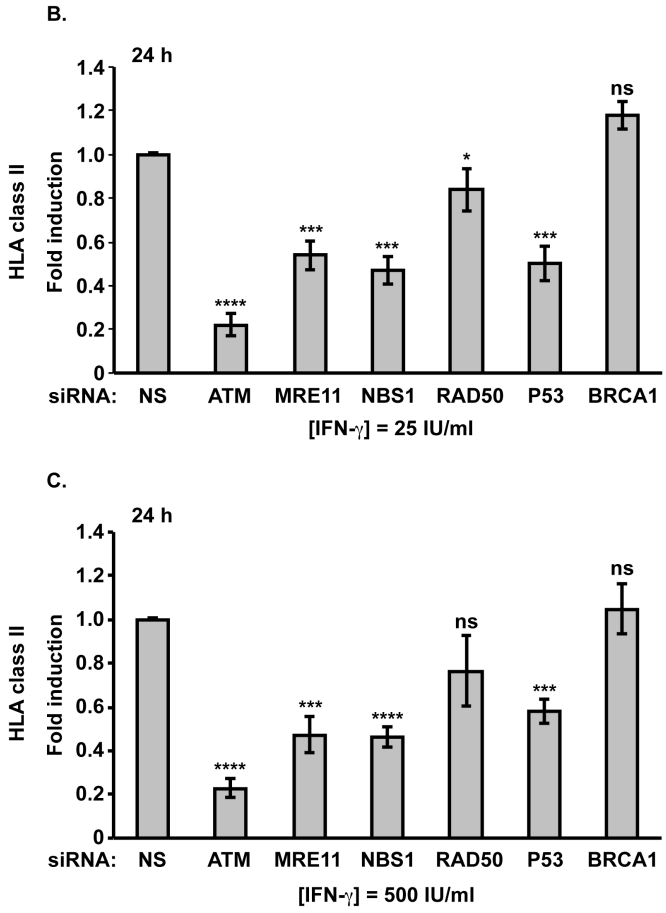


Figure 6.7. Expression of HLA class II after 24 hours treatment with IFN-γ was significantly reduced upon silencing of ATM, MRE11, NBS1 and p53 by RNAi. U2OS cells were transfected with 20 nM of siRNAs against ATM, MRE11, NBS1, RAD50, p53 and BRCA1 or with a non-targeting sequence using RNAiMAX. Forty-eight hours after transfection cells were stimulated with IFN-γ (25 or 500 IU/ml) for 24 hours. Cells were then stained with a PE-conjugated anti-HLA-DRα class II antibody and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-class II expression is presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of three independent experiments yielding similar results is shown. **B** and **C.** For each condition the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample and then normalised by dividing the obtained value by the siNS non-targeting control. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class I (**B** - 25 IU/mL and **C** - 500 IU/ml of IFN-γ). Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. NS - non-specific.

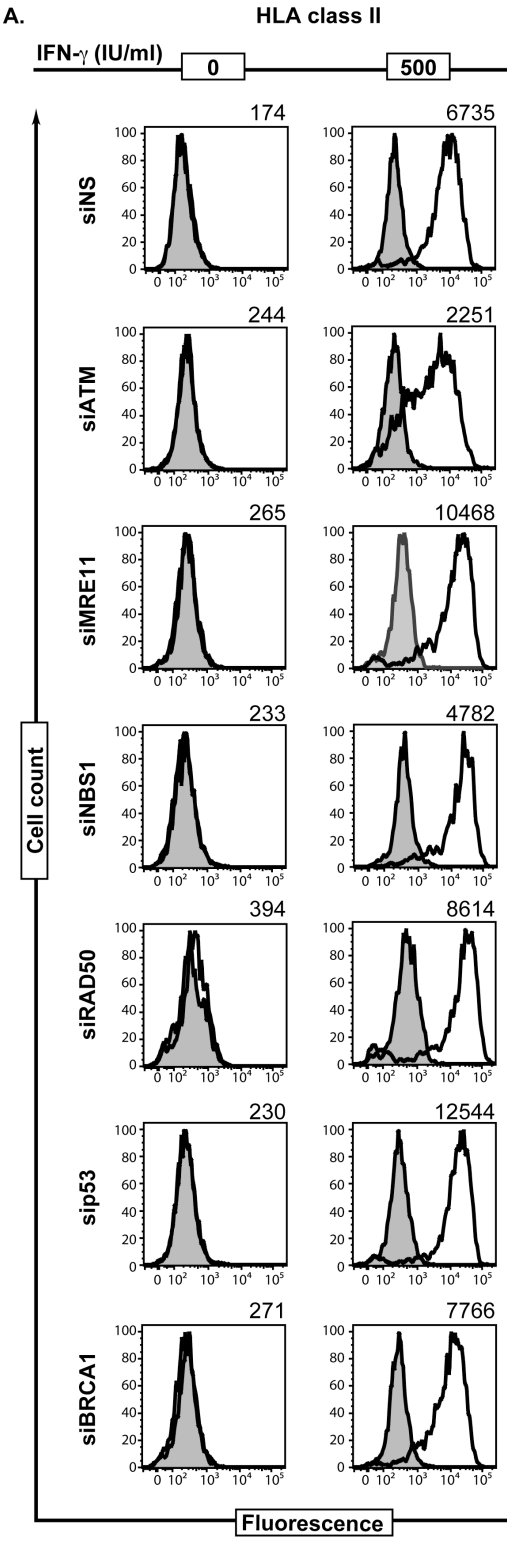


Figure 6.8. Expression of HLA Class II was reduced by ATM and NBS1 knockdown, but was increased by BRCA1 silencing even after prolonged exposure to IFN-γ for 72 hours. (Continued on page 230).

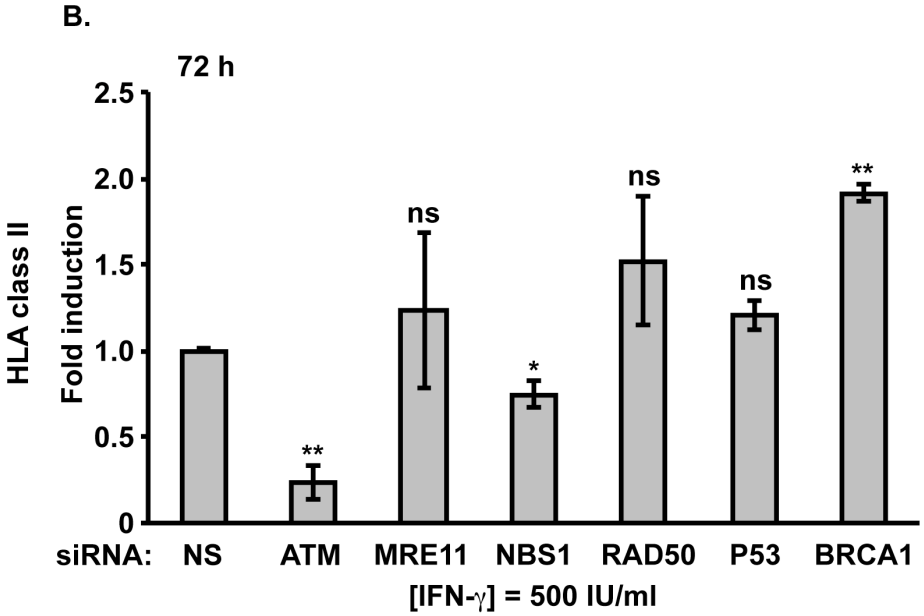


Figure 6.8. Expression of HLA Class II was reduced by ATM and NBS1 knockdown, but was increased by BRCA1 silencing even after prolonged exposure to IFN-γ for 72 hours. U2OS cells were transfected with 20 nM of siRNAs against ATM, MRE11, NBS1, RAD50, p53 and BRCA1 or with a non-targeting sequence, using RNAiMAX. Forty-eight hours after transfection cells were stimulated with IFN-γ (500 IU/ml) for 72 hours. Cells were then stained with a PE-conjugated anti-HLA-DRα class II antibody and cell surface expression of these molecules was determined by flow cytometric analysis using a FACSCanto. Results were analysed using FlowJo. **A.** HLA-DRα class II expression is presented as the geometric mean of stained samples (numbers on the top right hand corner of each graph). The matched unstained controls and stained samples correspond to the shadowed and transparent peaks, respectively. One representative experiment of two independent experiments yielding similar results is shown. **B.** For each condition the value of the geometric mean of the untreated sample was subtracted to the value of the IFN-γ treated sample and then normalised by dividing the obtained value by the siNS non-targeting control. Mean ± SD values obtained using three independent experiments after normalisation are shown for HLA class II. Values are expressed as fold change relative to the non-specific control. Statistical significance was calculated using Student's t-test relative to the non-specific control [non-significant (ns) - $p>0.05$, * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$, **** - $p<0.0001$]. NS - non-specific.

6.5. Discussion

Having shown that ATM is important for the expression of a subset of IFN-γ-responsive ISGs we next investigated the possible involvement of known ATM molecular partners. We focused our attention in KAP1 and members of the HDAC family in addition to a number of proteins known to interact with ATM in the DDR.

Our results by western blotting have shown that KAP1 acted as a negative regulator of STAT1 serine 727 and tyrosine 701 phosphorylation in IFN-γ-treated cells, since silencing of KAP1 by RNAi increased these post-translational modifications after stimulation (**Fig. 6.1**). Goodarzy and co-workers (2008) have identified KAP1 as a repressor of chromatin relaxation in the context of DSBs repair and shown that ATM negatively regulates it, in order to achieve DNA decondensation necessary for the DDR machinery to operate. Taking into consideration that by silencing KAP1, the chromatin structure can adopt a more relaxed conformation we hypothesized that this would facilitate tyrosine 701-phosphorylated STAT1 binding to the DNA template and, consequently, increase the phosphorylation of this protein on its serine 727 residue, which is likely dependent on DNA association. While KAP1 silencing would affect the occurrence of serine 727 phosphorylation, it is likely the modest increase observed in tyrosine 701 phosphorylation may be a consequence not of increased phosphorylation *per se*, but rather caused by a decrease in dephosphorylation of this tyrosine residue. Since the binding of STAT1 to the chromatin can protect this protein from dephosphorylation mediated by phosphatases such as TC45 (Mertens *et al*., 2006; ten Hoeve *et al*., 2002), a higher accessibility of the DNA template could determine a reduction in the accessibility of tyrosine 701-phosphorylated STAT1 to the action of the phosphatase. Importantly, KAP1 silencing reverted the impairment seen on STAT1 phosphorylation after ATM knockdown. This result suggested that KAP1 and ATM may act in the same axis and that ATM may modulate KAP1 activity in order to relax the DNA structure upon stimulation by IFN-γ, thus facilitating STAT1 binding to chromatin.

KAP1 was also found to repress NF-κB transcriptional activity induced by TNF-α by interacting with STAT3 and modulating its phosphorylation (Kamitani *et al*., 2011). It is possible that KAP1 acts as a regulator of several signalling pathways, initiated by different cytokines, which share as a feature the use of certain STAT family members for the transcription of particular sets of genes.

KAP1 silencing increased both constitutive and IFN-γ-inducible expression of HLA class I and led to the inhibition of the modest impairment caused by siATM, upon

IFN-γ-induced levels of HLA class I expression (**Fig. 6.2**). KAP1 knockdown was also capable of substantially reverting, although not completely, the more pronounced decrease of HLA class II after ATM RNAi (**Fig. 6.2**). Two possible explanations can be considered. Even when a specific siRNA sequence is efficient at targeting the mRNA, the reduction at the protein level is never absolute. Small differences in the level of knockdown and in the ratio of expressed ATM and KAP1 proteins may account for an incomplete reversion of the siATM phenotype by KAP1 silencing. This is probably even more significant in the case of HLA class II expression, which is more dependent on ATM expression. Another option to consider is that ATM regulation of HLA class II expression may be mediated by other factors besides KAP1 and that this is perhaps not the case for HLA class I.

Simply reducing KAP1 protein levels by RNAi did not result in HLA class II expression, although it did increase the basal level of constitutively expressed HLA class I in comparison with untransfected or siNS-transfected cells. It thus seems that, while KAP1 is likely involved in negatively regulating HLA class I and II expression and its silencing is sufficient to increase gene expression when the transcriptional machinery has already been recruited, it is not capable of initiating transcription *per se* in the absence of a stimulus.

KAP1 repressive action could be mediated through interaction of this protein with STAT1 (**Fig. 6.3**), an interaction that has been previously reported in a different context. The association between the two proteins was detectable in the absence of stimulation and seemed to decrease approximately 10 minutes after IFN-γ treatment. A plausible mechanistic explanation would be that STAT1 and KAP1 interact constantly but briefly (in an on and off way) in the absence of stimulation, when STAT1 is unphosphorylated and attempting to bind to ISGs promoters. KAP1 would maintain the chromatin condensed and in a structure unfavourable for the stable binding of STAT1 when this protein is unphosphorylated, thus preventing transcription. Upon stimulation, 701-tyrosine phosphorylated STAT1 would be recruited to the chromatin and associate with KAP1 located at STAT1 binding sequences of IFN-γ responsive genes. KAP1 would then be released from the chromatin and from the complex with STAT1 and, as a consequence, the repressive effect exerted by KAP1 would be terminated, chromatin relaxed and

STAT1 allowed to stably binding ISGs promoters and initiate transcription. We hypothesize that the interaction between KAP1 and STAT1 could be impaired by the action of ATM after IFN-γ signalling. Indeed, ATM also physically interacted with STAT1, with or without stimulation.

Our data suggests that KAP1 may act more as a general repressor of IFN-γ-induced transcription. KAP1 knockdown led to an increase of both HLA class I and class II expression, and while it reverted completely HLA class I impairment, it only partially recovered HLA class II expression in siATM-transfected cells. Therefore, the differences observed regarding the expression of these two types of molecules at cell surface in the absence of ATM must depend on additional factors, besides the inability to remove the restrictions imposed by KAP1 on chromatin structure. HDAC1 and HDAC3 could be part of that missing link.

Several reports have provided evidence that could sustain a functional link between KAP1, HDACs, ATM and STAT1, involving chromatin remodelling and transcription regulation (Goodarzi *et al*., 2008; Kamitani *et al*., 2008; Kamitani *et al*., 2011; Kramer *et al*., 2006; Kramer *et al*., 2009; Li *et al*., 2007; Li *et al*., 2010; Zeng *et al*., 2008; Ziv *et al*., 2006). Our data supports a role for HDAC1 and for HDAD3 in the differential modulation of IFN-γ-mediated responses with regards to the inducibility of HLA class I and II expression (**Fig. 6.4**).

Silencing of HDAC1, HDAC2 and HDAC3, either individually or combined in double transfections, was either without effect on HLA class I induction or this effect was quite modest and variable. In contrast, silencing of HDAC1 partially mimicked the phenotype seen in siATM-transfected cells, and, in fact, this was completely recapitulated by HDAC3 knockdown. HDAC2 silencing *per se* did not alter significantly HLA class II expression, but rather efficiently counteracted HDAC1 knockdown effects, while the siATM phenotype was still dominant over that of siHDAC2. HDAC3 exerted a dominant positive role in the induction of HLA class II molecules since it overcame the repressive action of HDAC2 and silencing of HDAC1 or HDAC1 and ATM did dot result in further impairment of HLA class II expression over the one achieved by siHDAC3.

According to Goodarzi and colleagues (2008), DSBs repair within heterochromatin requires the HDAC1/2 repressive action on chromatin structure to be regulated by ATM and, therefore, HDAC family members have been described as downstream targets of ATM. We believe that the same may happen during IFN-γ signalling, and that ATM may be relevant for HLA class II expression because of its ability is to modulate the different HDACs in a concerted way. Our data seem to support this as HDACs expression modulated HLA class II but not HLA class I expression.

Of all the additional molecules involved in the DDR mechanisms that were screened here for their relevance in IFN-γ signalling, only RAD50, NBS1 and BRCA1 warrant follow up (**Fig. 6.5 to Fig. 6.8**). RAD50 silencing was shown to have a preferential impact on IFNγ-induced expression of HLA class I, decreasing it after prolonged treatment (72 hours) with a saturating concentration of the cytokine. To date, no other reports exist suggesting that this is the case. The only information regarding RAD50 involvement in cytokine signalling pertains to the fact that the 3' end of RAD50 gene, which locates closely to IL-13 and IL-4 genes and functions as a locus control region for these genes (Kaneko *et al*., 2007; Monticelli *et al*., 2005). Likewise, no information was found concerning a possible link between HLA class II expression and NBS1. However, considering that this molecule is involved in ATM recruitment and assembly at DSBs (Bakkenist and Kastan, 2003; Lavin and Kozlov, 2007), we postulate that NBS1 may be involved in maintaining ATM associated with the DNA at the promoters of particular ISGs. When performing co-immunoprecipitation assays, we thus tried to detect NBS1 in association with ATM. We found no evidence for such occurrence, but this still does not mean that they do not interact and additional experiments are clearly required to settle this. Finally, BRCA1 was shown to regulate IRF-7 transcription together with STAT1 and STAT2 in response to IFN-γ (Buckley *et al*., 2007). BRCA1 was also found to activate transcription of a specific subset of genes in response to IFN-γ but not to type I IFNs, including OAS1 gene (Mullan *et al*., 2005). In addition, the Horvath group has also reported that BRCA1 cooperates with STAT1 by interacting with the C-terminus of this protein (Ouchi *et al*., 2000). BRCA1 was shown to differentially regulate transcription of certain IFN-γ-responsive ISGs dependently of STAT1 serine 727 transactivating

phosphorylation. BRCA1 promoted p21 transcription, but had no effect on IRF-1 expression (Ouchi *et al*., 2000). None of these reports mentioned BRCA1 to be involved in regulation of HLA expression. Our data, however, seems to suggest that this may be a general effect, since siBRCA1 increased both HLA class I and II expression.

In summary, our data supports a mechanistic view where ATM, STAT1 and KAP1 interact in a multi-protein complex and that these interactions are regulated by cytokine treatment. In addition, HDAC3 in particular may be a key molecule regulated by ATM in IFN-γ signalling, since silencing of this protein completely recapitulated the siATM phenotype.

CHAPTER 7

General discussion and future directions

7.1. Discussion and conclusions

IFN-γ is an important pro-inflammatory and immunomodulatory cytokine with pivotal roles in tumour immunosurveillance, elimination of pathogen infections and regulation of several autoimmune diseases (Berghmans *et al*., 2011; Brunda *et al*., 1995; Ikeda *et al*., 2002; Jankovic *et al*., 2007; Kakuta *et al*., 2002; Kelchtermans *et al*., 2005; Lunemann *et al*., 2011; Xu *et al*., 2009). This cytokine's functions are mainly mediated by JAK/STAT signalling pathways. However, the complexity associated with JAK/STAT activation by IFN-γ is clear, with many different molecular components controlling specific responses (Briscoe *et al*., 1996; Costa-Pereira *et al*., 2005). Indeed, transcription downstream of IFN-γ is highly and differently regulated for distinct ISGs (Benson and Ernst, 2009; Bhinge *et al*., 2007; Croce *et al*., 2003; Kovarik *et al*., 2001; Lang *et al*., 2012; Lee *et al*., 1999; Masternak *et al*., 2000; Morris *et al*., 2002; Smith *et al*., 2006; Ting and Trowsdale, 2002; Watling *et al*., 2008; Wormald *et al*., 2006; Zhang *et al*., 2010; Zika *et al*., 2003). Moreover, the JAK/STAT pathway is additionally modulated by other non-JAK, non-STAT signalling pathways, some of which remain as yet unidentified (Briscoe *et al*., 1996; Watling *et al*., 2008). For example, our group has shown that ATM, a kinase best known for its key role in DNA DSB repair, is involved in modulating specific arms of the IFN-γ response, such as Class II HLA induction and the antiviral state (Watling *et al*., 2008).

ATM also has roles as a modulator of chromatin structure, as it is able to induce chromatin relaxation to facilitate the repair of DSBs in highly condensed heterochromatic regions. This is thought to be mediated through direct regulation of KAP1-mediated chromatin repression, as well as of HDAC1/2 activity (Goodarzi *et al*., 2008). KAP1 and HDACs are important chromatin-modifying proteins, involved in the regulation of chromatin condensation during transcription and have been associated with STAT1 and/or IFN responses in several different contexts (Benson and Ernst, 2009; Chang *et al*., 2004; Guo *et al*., 2006; Hacker *et al*., 2009; Kamitani *et al*., 2008; Kamitani *et al*., 2011; Klampfer *et al*., 2004; Kong *et al*., 2009; Lang *et al*., 2012; Nusinzon and Horvath, 2003; Sakamoto *et al*., 2004; Snyder *et al*., 2005; Tsuruma *et al*., 2008; Xu and Price, 2011; Zeng *et al*., 2008; Zhang *et al*., 2010;

Zika *et al*., 2003). ATM itself has been reported to induce gene transcription upon recognition of acetylation changes promoted by HDAC inhibition, unravelling a functional link between ATM-mediated DDR and ATM-induced transcription (Ju and Muller, 2003; Lee, 2007).

Against this background, we hypothesised that ATM affected IFN-γ-induced transcription by controlling the accessibility of STAT1 to ISGs promoters. We further hypothesised that this happened through the regulation of chromatin-modifying factors and that, therefore, ATM acted as a chromatin-remodelling protein. Considering that ATM silencing severely impaired the IFN-γ antiviral response and the inducibility of HLA class II induction upon IFN-γ treatment, but had a minor impact on Class I HLA (Watling *et al*., 2008), we assumed that the requirement for ATM during IFN-γ-mediated transcription differed amongst distinct ISGs. The key objective of my PhD research was, therefore, to investigate the mechanism(s) behind ATM-dependent IFN-γ-induced responses. Apart from the great intellectual challenge that this posed, from a pragmatic point of view, this knowledge may actually prove useful in identifying therapeutic targets that may be used at a later stage for the development of immune-based treatments and/or safely modulate the IFN-γ signalling pathway in a variety of malignancies.

IFN-γ effects are essentially mediated by STAT1-induced transcription of ISGs. This is determined first by the phosphorylation of STAT1 on tyrosine 701 within the cytoplasm and subsequent conformational change of the STAT1 homodimers, leading to STAT1 nuclear localisation and binding to the DNA on GAS sequences within ISG promoters (Darnell, 1997; Fagerlund *et al*., 2002; McBride *et al*., 2002; Stark *et al*., 1998; Wenta *et al*., 2008; Zhong *et al*., 2005). A second post-translational modification is thought to occur while tyrosine-phosphorylated STAT1 is bound to DNA. This consists of serine 727 phosphorylation and is considered a transactivating event that leads to increased transcriptional activity, which is required for the expression of certain ISGs (Kovarik *et al*., 2001; Sadzak *et al*., 2008; Varinou *et al*., 2003; Wen *et al*., 1995; Zhu *et al*., 1997). ATM is a serine/threonine kinase and its functions have been so far associated with its enzymatic activity, including its roles within the context of DNA repair-induced

chromatin remodelling. We, therefore, investigated if the participation of ATM in IFN-γ responses was related to STAT1 phosphorylation.

Our results have shown that ATM acts as a positive regulator of serine phosphorylation, since ATM silencing resulted in a significant delay of this PTM (**Fig. 4.1**, **Fig. 4.2** and **Fig.4.3**). This was particularly evident in the nuclear compartment (**Fig. 4.2**) where this event takes place (Sadzak *et al*., 2008; Zhu *et al*., 1997) and where most ATM molecules reside (Gately *et al*., 1998). This delay, however, does not appear to be the result of impaired enzymatic activity of ATM since it STAT1 serine 727 phosphorylation was not affected upon chemical inhibition of ATM kinase activity (**Fig. 4.9**). The caveat here is that we did not conduct *in vitro* kinase assays and cannot, therefore, completely exclude the possibility that some residual ATM enzymatic activity was left after inhibition with the inhibitor KU55933. Curiously, STAT1 tyrosine 701 phosphorylation after ATM knockdown was also impaired (**Fig. 4.1**, **Fig. 4.2** and **Fig.4.3**). This reduction was visible in the nuclear compartment, indicating that these molecules may have had increased susceptibility to the action of nuclear phosphatases (**Fig. 4.2**). While serine 727 phosphorylation is thought to only occur when STAT1 phosphorylated on tyrosine 701 is bound to the DNA (Sadzak *et al*., 2008), this association at the level of ISG promoters also protects tyrosine phosphorylated STAT1 from the action of tyrosine phosphatases (Meyer *et al*., 2003). Therefore, our results suggest that ATM silencing delays STAT1 binding to the chromatin and, as a consequence, delays serine phosphorylation events and favours tyrosine dephosphorylation. These data are in agreement with the role ascribed to ATM in chromatin remodelling and relaxation.

ATM's role as a chromatin remodelling protein in the context of DNA repair was associated with its direct action over the chromatin repressor KAP1 (Goodarzi *et al*., 2008; Ziv *et al*., 2006). This involved KAP1 phosphorylation by ATM and subsequent release from heterochromatin, events which increased the opening and accessibility of the DNA template (Goodarzi *et al*., 2008). In addition, KAP1 has been previously reported to associate with several STAT proteins, including STAT1, and to negatively regulate STAT3 serine phosphorylation, although it was without effect on STAT3 tyrosine phosphorylation in response to IL-6 (Kamitani *et al*., 2008; Kamitani *et al*., 2011; Tsuruma *et al*., 2008). In contrast, other reports suggest that

in response to IFN-α, KAP1 did not have an effect on STAT1 serine or tyrosine phosphorylation (Kamitani *et al*., 2008), while no report has been made yet linking KAP1 to IFN-γ-induced STAT1 phosphorylation. It thus seems that the involvement of KAP1 in STAT-mediated responses is highly dependent on cellular context. Importantly, our results have shown that in U2OS cells KAP1 can act as a negative regulator of IFN-γ-induced serine 727 phosphorylation of STAT1, with KAP1 silencing increasing STAT1 serine 727 phosphorylation and counteracting the effect caused by ATM knockdown (**Fig. 6.1**). This observation suggests a functional opposing link between ATM and KAP1 in IFN-γ signalling, as it happens during heterochromatin DSB repair where ATM needs to remove KAP1 repressing action to ensure chromatin relaxation. We have also observed an increase in tyrosine 701 phosphorylation, which, given the role of KAP1 as a repressor of chromatin relaxation, may be due to an increase in the amount of DNA-bound STAT1 and/or increased stability and protection from nuclear phosphatases (**Fig. 6.1**). This is a very interesting observation and the functional importance of KAP1 should be further addressed in the context of STAT1 binding to ISG promoters and STAT1-driven transcription in response to IFN-γ.

Moreover, in agreement with previously published studies (Kamitani *et al*., 2008; Tsuruma *et al*., 2008), we have found that KAP1 interacts with STAT1 in the absence of stimulation (**Fig. 6.3**). It has been suggested that unphosphorylated STAT1 and STAT3 have higher affinity towards KAP1 and that KAP1 may sequester unphosphorylated STAT proteins and, in doing so, regulate serine phosphorylation (Kamitani *et al*., 2008). Differential affinities between KAP1 and phosphorylated and unphosphorylated STAT1 may explain, therefore, why throughout our studies this interaction decreased with increasing IFN-γ stimulation (**Fig. 6.3**).

In addition, to the best of our knowledge, no one has yet reported the association between ATM and STAT1, which we have observed both in resting cells and in cells treated with IFN-γ (**Fig. 5.8**). Although the STAT1α antibody used in the co-immunoprecipitation experiments immunoprecipitates both unphosphorylated and phosphorylated STAT1, tyrosine phosphorylation of STAT1 was only detected after

IFN-γ stimulation and ATM appears to associate with increasing amounts of tyrosine phosphorylated STAT1. Bearing that in mind, we suggest that ATM and unphosphorylated STAT1 form a complex in which replacement with phosphorylated STAT1 occurs as the amount of this form increases upon IFN-γ treatment. It is reasonable to envisage a scenario where the unphosphorylated STAT1-ATM complex is constantly binding to and dissociating from DNA-associated KAP1. STAT1 phosphorylation may stabilise STAT1-DNA interactions and this may depend on the ability of ATM to dislodge KAP1, or on decreased STAT1-KAP1 interaction affinity. This remains to be established and will require additional work.

Our results have established that in unstimulated cells ATM silencing consistently causes an increase in DNA-bound STAT1, presumably unphosphorylated, to the promoters of CIITA and HLA-DRα (**Fig. 5.5**). Unlike what was observed in cells transfected with non-targeting siRNA sequences, further STAT1 binding to these promoters was significantly impaired in response to IFN-γ. This was in stark contrast to what was observed with regards to the STAT1 occupancy of IRF-1 and TAP1 promoters (**Fig. 5.5**). These observations correlated well with the severe impairment of CIITA and HLA-DRα IFN-γ-mediated transcription seen after ATM silencing, which, reassuringly, was not observed for IRF-1 and TAP1 (**Fig. 4.7**). Together these data highlight the relevance of ATM for adequate recruitment and/or retention of STAT1 at the promoters of specific genes such as HLA-DRα, Ii and CIITA and warrant additional studies to fully establish the molecular mechanisms involved.

Consistent with the role of KAP1 as a novel negative regulator of STAT1 phosphorylation (**Fig. 6.1**), we have found that KAP1 represses both HLA class I and II expression (**Fig. 6.2**). In fact, KAP1 silencing resulted in increased expression of both these molecules and partially counteracted the siATM phenotype with regards to HLA class II, as shown by double knockdown of ATM and KAP1 (**Fig. 6.2**).

HDAC1 and HDAC2 have been acknowledged as negative regulators of chromatin relaxation, as indeed has KAP1 in the context of heterochromatic ATM-induced

DDR (Goodarzi *et al*., 2008). Additionally, KAP1 has been previously reported to mediate STAT3 interaction with HDAC3, and STAT3 deacetylation has been suggested to be involved in KAP1 repression of STAT3-induced transcription (Tsuruma *et al*., 2008). This is in agreement with HDACs function as repressors of chromatin decondensation and transcription. Moreover, several other authors have suggested that the mechanistic basis for KAP1-mediated repression of transcription relies in the direct interaction between this protein and HDACs (Satou *et al*., 2001; Schultz *et al*., 2001; Underhill *et al*., 2000). However, HDAC function has also been reported to be essential for IFN-γ-induced STAT1-mediated transcription (Guo *et al*., 2006; Klampfer *et al*., 2004; Nusinzon and Horvath, 2003). In light of these opposing roles attributed to HDACs regarding transcription, we assessed the importance of all three HDAC1, HDAC2 and HDAC3 in IFN-γ-induced expression of HLA class I and II, in the attempt to establish their importance for ATM-mediated regulation of IFN-γ responses. We have observed that none of these HDACs affected HLA class I expression (**Fig. 6.4**). Interestingly, we have found that HLA class II expression was positively regulated by HDAC1 and HDAC3, with HDAC1 and HDAC3 silencing partially or completely recapitulating the siATM phenotype, respectively (**Fig. 6.4**). Curiously, HDAC2 silencing had no effect on HLA class II expression *per se*. It counteracted the HDAC1 effect but it did not overcome the phenotypes induced by transfection with siATM or siHDAC3 (**Fig. 6.4**). On the face of it, these data seem at odds with that of others that has implicated HDAC2 in the deacetylation of CIITA and subsequent impairment of IFN-γ-induced expression of HLA class II (Kong *et al*., 2009). Assessing the role of those HDACs regarding STAT1 phosphorylation, as well as the ability of KAP1 to behave as a negative regulator of HDAC1 and HDAC3-modulated expression of HLA class II will be crucial to understand the functional and mechanistic associations between these molecules and the regulatory components underpinning these.

Our results are in agreement with data from other research groups (Guo et al., 2006; Nusinzon and Horvath, 2003) that suggest HDAC activity is required for IFN-γ signalling: for example, IP10 is positively regulated by HDAC1. However, these data are in contrast to several other reports, in particular those supporting the need for histone acetylation at the CIITA promoter for its transcription to occur

(Benson and Ernst, 2009). Described as the master regulator of HLA class II expression, CIITA appears to regulate the acetylation of the HLA-DRα promoter, an event essential for the transcription of HLA-DRα (Beresford and Boss, 2001). This also differs from what has been described regarding ATM-induced chromatin relaxation, with both HDAC1 and HDAC2 functioning as negative regulators of this process. Moreover, the need for histone deacetylation seems to have a certain specificity, since it was required for HLA class II expression (**Fig. 6.4**), which is also highly dependent on ATM, but not for HLA class I expression [**Fig. 4.4** and **Fig. 4.5** and (Watling *et al*., 2008)]. Additionally, our findings support a rather intricate and complex interaction between the three different HDACs analysed in this work regarding HLA class II expression. While both HDAC1 and HDAC3 acted as positive regulators of this IFN-γ response, HDAC2 opposed HDAC1 but not HDAC3 activity, which appeared to act as a dominant form. These data are in agreement with the well-known fact that the transcriptional regulation of HLA class II is mechanistically more complex than that of HLA class I.

HDACs involvement in transcription has been associated with their ability to remove acetyl groups from histones, a modification associated with chromatin remodelling. However, the deacetylase activity is not restricted to histones and it has been suggested that HDAC3 may be responsible for the deacetylation of STAT3 (Tsuruma *et al*., 2008). In addition, HDAC1 and HDAC3, but not HDAC2, have been found to interact with STAT1, which led to the suggestion that STAT1 deacetylation may be undertaken by these two enzymes. It was also suggested that the inhibition of HDACs and subsequent increase in acetylation have the ability to decrease STAT1 tyrosine phosphorylation and activity (Kramer *et al*., 2006). These results have been long disputed (Ana Costa-Pereira, personal communication) and recently formally refuted. Indeed, HDAC inhibition was convincingly shown to have no effect on either STAT1 acetylation, or STAT1 tyrosine phosphorylation; it solely affected STAT1 nuclear translocation and STAT1 binding to DNA (Antunes *et al*., 2011).

ATM acetylation by Tip60 was shown to correlate with increased activity (Jiang *et al*., 2006; Murr *et al*., 2006; Sun *et al*., 2005; Sun *et al*., 2007) and this raises the

possibility of another mechanism of interaction between ATM and the HDACs. The need for acetylation of ISG promoters has been frequently associated with IFN-γ-induced transcription and with the recruitment of RNA polymerase II into the transcriptional complex (Darnell, 1997) (Hiroi and Ohmori, 2003). In striking contradiction, histone deacetylation has also been reported as being essential for the recruitment of RNA polymerase to the promoters of selected ISGs in response to type I IFNs (Sakamoto *et al*., 2004; Snyder *et al*., 2005), which suggests that the same could happen with IFN-γ since both types of cytokines use STAT1 as a transcription factor. In the particular case of IFN-β, HDAC inhibition using trichostatin A (TSA) did not affect STAT1 and STAT2 phosphorylation; neither did it affect IFN-β-activated STAT1 homodimers binding to IRF-1 promoter, or STAT1 and STAT2 association with the ISG54 promoter (Sakamoto *et al*., 2004). Nonetheless, the lack of HDAC activity selectively impaired the recruitment of RNA polymerase II to the ISG54 promoter (Sakamoto *et al*., 2004). This profile of differential transcriptional regulation amongst ISGs that are activated by the same stimulus is thus similar to the one observed regarding ATM regulation of IFN-γ-induced transcription. The idea that transcriptional dependence on HDAC activity correlates with the need to recruit RNA polymerase II could explain why HDACs are dispensable for constitutively expressed ISGs, such as TAP1, since the polymerase is already bound to the promoters prior to cytokine stimulation (Sakamoto *et al*., 2004). It could also be the reason why transcription of IRF1 is not impaired as a consequence of HDACs inhibition (Sakamoto *et al*., 2004), since the RNA polymerase II binding to the promoter of this ISG is not actively repressed in resting cells as it happens for most of the ISGs (Wormald *et al*., 2006). We have detected an IFN-γ-inducible interaction between STAT1 and RNA polymerase II (**Fig. 5.9**), which is consistent with the fact that STAT1 is known to recruit this enzyme to DNA to initiate transcription (Bhinge *et al*., 2007) and both proteins interacting at the promoters of different ISGs (Hiroi and Ohmori, 2003; Snyder *et al*., 2005). Our results also suggest a possible interaction between ATM and RNA polymerase II and this requires further validation (**Fig. 5.9**).

Given our observations, as well as the studies referred above, we would like to further dissect the underlying mechanisms and interactions between ATM, KAP1

and the different HDACs in IFN-γ responses. We would thus like to determine if the selective transcriptional impairment of a subset of IFN-γ-responsive genes, such as CIITA, Ii and HLA-DRα, caused by ATM silencing (**Fig. 4.7**), is due to alterations in the recruitment of RNA polymerase II upon IFN-γ treatment. In addition, we would like to know if ATM, KAP1 or HDACs proteins can regulate the interaction between this RNA polymerase II and STAT1. We also hypothesise that while KAP1 may act as a general repressor of transcription (suggested by its negative regulation of both HLA class I and II expression stimulated by IFN-γ treatment) ATM together with HDAC1, HDAC2 and HDAC3 may provide the fine-tuning needed for the correct regulation of the more complex HLA class II cytokine-induced transcription.

We have also investigated if ATM involvement in IFN-γ responses correlated with the formation of DSBs. These are considered to trigger ATM activation and initiate the DDR. They can also occur as a consequence of cytokine-induced transcription (Guirouilh-Barbat *et al*., 2008; Williamson and Lees-Miller, 2011). Our results thus far do not support this notion (**Fig. 5.1** and **Fig. 5.2**). Other events that do not provoke DSBs but which alter the chromatin structure have been suggested to also activate ATM (Ju and Muller, 2003). Curiously, inhibition of HDAC activity has been shown to be one such event. Indeed, we have shown that HDAC1 and HDAC3 knockdown was detrimental for IFN-γ-induced expression of HLA class II, as was ATM silencing. It could be that changes in the acetylation pattern at the promoters of specific ISGs are needed for ATM to properly recognise these sites so that the repression exerted by KAP1 can be relieved and the chromatin relaxed. Alternatively, these acetylation changes may be required for the recruitment of RNA polymerase II.

Besides KAP1 and the HDACs, we have also assessed if other molecules, known to be involved in the ATM-mediated DDR, had roles in IFN-γ signalling. Of all the additional molecules tested only RAD50, BRCA1 and NBS1 appeared to play a role (**Fig. 6.5**, **Fig. 6.6**, **Fig. 6.7** and **Fig. 6.8)**. We have showed that BRCA1 acts as a negative regulator of IFN-γ-induced expression at cell surface of both HLA class I and II. This is in disagreement with previously published reports where

BRCA1 was described to activate transcription of IFN-γ-responsive genes (Buckley *et al*., 2007; Ouchi *et al*., 2000). The reasons behind these discrepancies are still unclear. RAD50 acted as a positive regulator of IFN-γ-induced HLA class I expression and, as far as we know, this is the first time that this has been reported. It would be interesting to follow up this finding and to clarify the mechanistic basis of RAD50's involvement in HLA class I cytokine-driven expression. NBS1 silencing reduced HLA class II expression in response to IFN-γ. This molecule is known to mediate ATM recruitment and assembly at the site of DSBs (Lavin and Kozlov, 2007) and, as such, we hypothesise that it may also be necessary for promoting the binding of ATM to the DNA upon cytokine treatment. Although we could not detected an interaction between ATM and NBS1 when immunoprecipitating ATM, this could simply be due to technical issues and, therefore, the possibility of such an interaction should still be further assessed.

Curiously, our data suggests that the role of ATM as a modulator of IFN-γ responses is structural rather than enzymatic (**Fig. 4.9** and **Fig. 4.11**). This is intriguing since its role in orchestrating the DDR is entirely dependent on ATM's serine/threonine kinase activity and phosphorylation of numerous substrates. Most importantly, even the reported ATM inhibition of KAP1 repression was shown to be mediated by phosphorylation of serine 824 (Goodarzi *et al*., 2008). However, when ATM enzymatic activity was inhibited with the chemical inhibitor KU-55933 at a concentration of 10 μM neither STAT1 phosphorylation, nor HLA class II expression were affected. The concentration of inhibitor used in these experiments is frequently used in the literature and we found it to efficiently abrogate ATM serine 1981 phosphorylation (associated with ATM activation), and to inhibit p53 ATM-induced serine 15 phosphorylation, a downstream target of ATM activated upon DSBs induced by etoposide treatment. However, we cannot exclude the possibility that a residual fraction of ATM was still enzymatically active and that this was sufficient to regulate IFN-γ-induced STAT1 phosphorylation and expression of HLA class II expression. Additionally, it has also been suggested that ATM may become catalytically active by a conformational change that does nor involve auto-phosphorylation on serine 1981 (Lavin and Kozlov, 2007). Therefore, the importance of ATM kinase activity for IFN-γ responses should be

further assessed to establish, beyond doubt, that this is not, in fact, required for the modulation of IFN-γ responses.

As previously mentioned, ATM can modulate chromatin remodelling and this involves direct control of KAP1 repressive action over chromatin structure (Goodarzi *et al*., 2008; Ziv *et al*., 2006). In this work we have established a link between these two proteins and provided evidence that suggests that they have opposing effects regarding STAT1 phosphorylation (**Fig. 6.1**) and HLA class I and II expression in response to IFN-γ (**Fig. 6.2**). HDAC2 was found to act as a repressor of HLA class II expression, controlling CIITA recruitment to target promoters (Kong *et al*., 2009). However, HDAC1 and more strikingly HDAC3 were identified during the course of this work as positive regulators of HLA class II expression stimulated by IFN-γ treatment. The involvement of these chromatin-modifying factors in IFN-γ responses, either counteracting ATM phenotype, such as KAP1 and HDAC2, or instead recapitulating it, as was the case of HDAC1 and HDAC3, supports our hypothesis that ATM regulation of specific IFN-γ responses at the transcriptional level likely involves its chromatin remodelling ability. We propose a mechanism whereby ATM and STAT interaction allows the binding of ATM to ISGs promoters to be accompanied by local directional chromatin relaxation. This may be caused by ATM-induced displacement, or prevention, of KAP1 repression, which may be facilitated by HDAC1 and HDAC3 activity. Accordingly, this hypothesis will be further ascertained in our laboratory. The ability of IFN-γ to promote chromatin decondensation and chromatin loops at the Class II HLA locus after 10 minutes of stimulation has been previously reported (Christova *et al*., 2007). It would be an irrefutable proof of ATM chromatin remodelling activity downstream of IFN-γ to show that these events are in fact dependent on ATM.

The key findings of my PhD research have been summarised in **Figure 7.1**, which also indicates mechanism(s) by which ATM can potentially regulate some IFN-γ-induced responses. Experimental approaches to be followed in the future in order to confirm and expand the suggested mechanism(s) are discussed in the following section of this chapter.

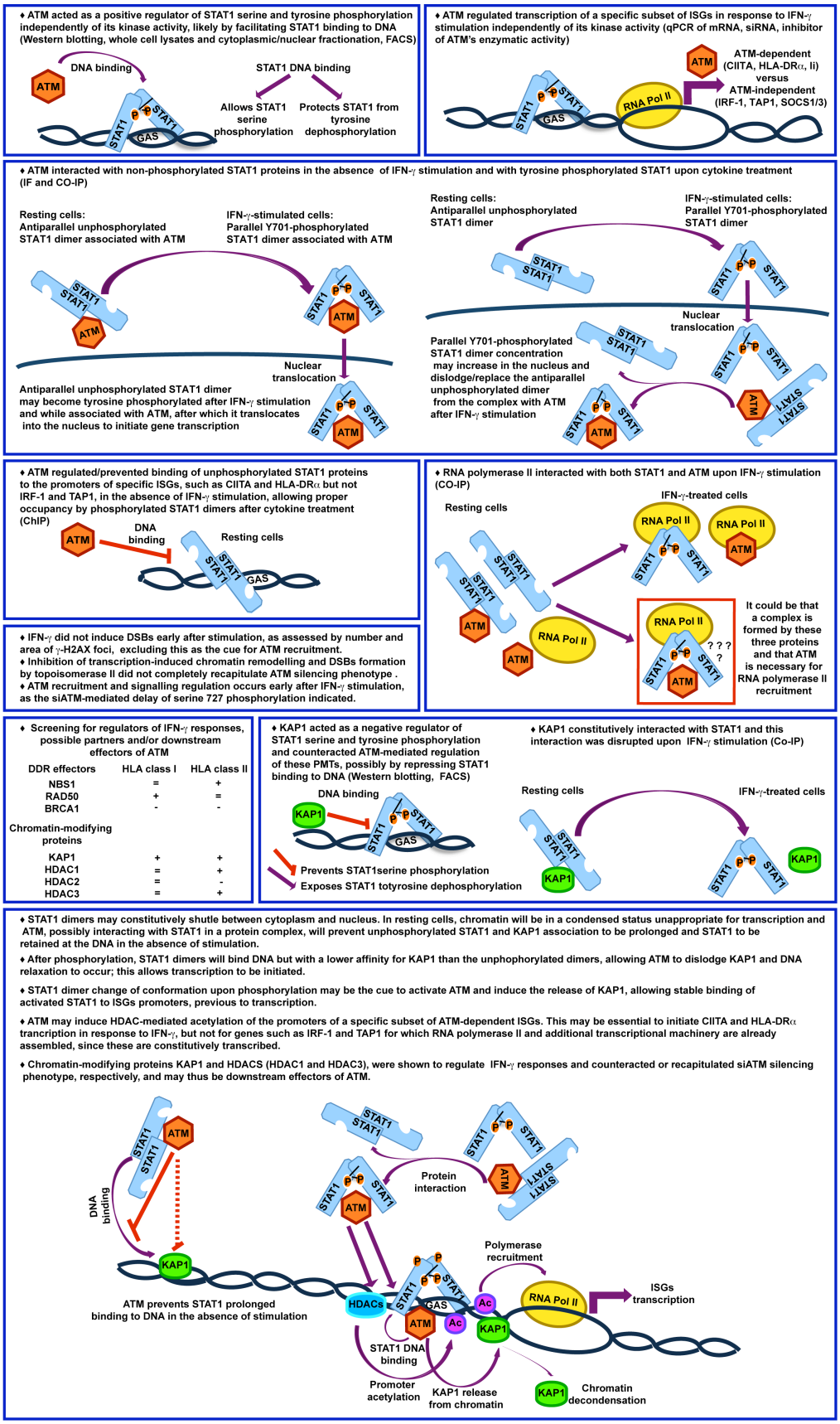


Figure 7.1. Summary of the main results and the possible mechanisms by which ATM may regulate IFN-γ-induced responses.

7.2. Future directions

During the course of my PhD studies many questions were answered but as often is the case with research, these all triggered additional questions that merit further attention.

How does the impairment of STAT1 serine 727 phosphorylation observed upon ATM silencing impair IFN-γ-induced ISG transcription? Can this inhibition be overcome by KAP1 knockdown?

In order to determine the relevance of serine 727 phosphorylation for transcription of IFN-γ-responsive genes, transfection of STAT1 wild-type and STAT1-serine 727 mutated inducible constructs (S727A) could be used. This approach should allow us to establish which ISGs are dependent on this PMT for transcription.

Does ATM interact with KAP1 or with NBS1?

In view of our data, it would be important to determine if ATM interacts with KAP1, which would provide support to the existence of a multimeric complex formed by ATM, KAP1 and, possibly, STAT1 since ATM and STAT1 were shown here to interact. If this was the case, IFN-γ-induced STAT1 phosphorylation could be the triggering event responsible for removing KAP1 repression in an ATM-dependent manner. It would also be relevant to identify where the interactions involving ATM, STAT1 and KAP1 take place. Presumably, if these molecules interact, they will do so in the nuclear compartment and this could be addressed wither by using confocal microscopy or by performing co-immunoprecipitations using nuclear and cytoplasmic extracts. We have shown that NBS1, a DDR effector and a close interacting partner of ATM, was a positive regulator of HLA class II induction. As such, it also possible that this molecule interacts with ATM and this could be explored by performing co-immunoprecipitation experiments. Furthermore, it would be important to determine if this molecule plays a role at the transcriptional level, which could be carried out by analysing the impact of silencing NBS1 using RNAi on ISG mRNA transcripts in response to IFN-γ.

Does KAP1 bind to ISG promoters? Does ATM or IFN-γ treatment determine KAP1 release?

No studies have been done so far regarding KAP1 localisation at γ-ISG promoters. Thus, ChIP assays could be used to establish if KAP1 localises at ISG promoters in resting cells and to assess whether stimulation with IFN-γ alters KAP1 occupancy at these promoters, concomitantly with increased occupancy by STAT1. Moreover, it would be interesting to analyse if KAP1 occupancy differs amongst different ISG promoters and if it increases in the absence of ATM for certain ISGs, such as CIITA and HLA-DRα. Such an increase in promoter occupancy by KAP1, known to associate with unphosphorylated STAT1 with higher affinity than with phosphorylated STAT1, could help explain the reason why ATM silencing provoked an increased in the amount of STAT1 bound to CIITA and HLA-DRα promoters in resting cells.

Recruitment of RNA polymerase II is a crucial event without which transcription cannot occur. There is evidence suggesting that this is highly regulated and that it differs amongst the ISGs, raising the possibility that ATM modulation of IFN-γ responses may be achieved at this level. It is also conceivable that it may involve the participation of KAP1 and/or HDACs. ChIP experiments could be used to examine the status of occupancy of different ISGs promoters by RNA polymerase II before and after IFN-γ treatment. Subsequently, one could determine if ATM, KAP1 or any of the HDACs is involved in RNA polymerase II recruitment, by additionally silencing these proteins.

What is the role of the different HDACs in IFN-γ-induced STAT1 phosphorylation, acetylation of ISG promoters and transcription? Do these events correlate with the expression of ATM and KAP1?

It would be of interest to assess the impact of silencing the different HDACs regarding STAT1 phosphorylation by western blotting and/or flow cytometric analysis. Confocal microscopy and fractionation experiments could then be used to establish how RNAi against these molecules impacts on the subcellular localisation of STAT1 in resting and cytokine-treated cells. This would allow us to determine if HDAC1 and HDAC3 act as positive regulators of STAT1 phosphorylations, as does

ATM. If the knockdown of KAP1 is able to overcome the effect of silencing HDAC1 and/or HDAC3, in what HLA class II expression is concerned, this would suggest that there is in fact a functional link between ATM, HDACs and KAP1 important for mediating IFN-γ responses. Following these studies, one could analyse the impact of inhibiting each of these HDACs on the transcription of several ATM-dependent and -independent ISGs. If a parallel between the phenotype of siHDAC1 or siHDAC3 and siATM could be drawn, then this would further support the notion that these molecules operate in the same pathway as positive regulators of IFN-γ specific responses, having HDAC2 as a negative regulator. Histone acetylation is a frequent, and important, aspect of chromatin remodelling. Therefore, the normal acetylation status of ISG promoters in response to IFN-γ should be evaluated by performing ChIP assays with antibodies specifically raised against acetylated histones 3 and 4. It would also be interesting to determine the identity of the HDAC responsible for the acetylation of each ISG by performing ChIP after silencing the different HDACs with RNAi. Moreover, assessing if ATM silencing would perturb the acetylation pattern of ISGs promoters could potentially establish a direct connection between ATM and HDAC activity in the regulation of IFN-γ responses and provide evidence for a role for the HDACs as downstream effectors of ATM.

Do HDAC1 and HDAC3 interact with STAT1 and/or ATM? Do these HDACs deacetylate STAT1 or ATM in response to IFN-γ?

Reciprocal co-immunoprecipitation experiments could be carried out to determine if HDAC1, HDAC2 or HDAC3 are able to interact with either STAT1 or ATM, and promote deacetylation of these proteins. This would also enable to unravel how these events are regulated by IFN-γ stimulation. Given the inexistence of specific antibodies against acetylated-STAT1 or acetylated-ATM, a pan antibody against acetylated-lysine residues will need to be used together with silencing of each individual HDAC to probe ATM and STAT1 immunoprecipitates.

Is DNA decondensation and the formation of chromatin loops at the Class II HLA locus in response to IFN-γ dependent on ATM?

IFN-γ treatment is known to rapidly induce giant chromatin loops on chromosome 6 where the HLA Class II genes are located. Accordingly, it would very pertinent

based on our data presented here to study if this fascinating phenomenon depends on ATM. This could be done through FISH and image analysis, using specific fluorescent probes for the HLA Class II locus and in collaboration with Professor Denise Sheer (Queen Mary, University of London, London, UK), a long-standing collaborator of Dr Ana Costa-Pereira.

Is phosphorylation of ATM and its enzymatic activity totally dispensable for IFN-γ responses?

To firmly establish that ATM enzymatic activity is not required for the modulation of IFN-γ signalling, one could resort to transfecting GFP-tagged ATM wild type and ATM kinase dead mutant constructs into ATM-deficient cells. Then, HLA class II expression in GFP-positive cells could be studied by flow cytometry. If the kinase activity is required then a defect will be picked up in the kinase dead-transfected cells. Moreover, it will be interesting to determine by immunoprecipitation with large amounts of protein (e.g. 3-4 mg) if it is phosphorylated on serine or threonine phosphorylation, or if it is acetylated in response to IFN-γ treatment.

In summary, our data assigns an important role for ATM as a regulator of IFN-γ responses at the transcriptional level. The underlying mechanism of action may involve both ATM physical interaction with STAT1 and STAT1 interaction with KAP1. We have found evidence that ATM modulation of IFN-γ-induced STAT1-mediated transcription, as well as STAT1 phosphorylation, depends on the suppression of KAP1-mediated repression, likely to happen through inhibition of chromatin relaxation. ATM-mediated regulation of IFN-γ responses also seems to depend on HDAC1 and HDAC3 activity, which may act as downstream effectors of ATM and differentially acetylate ISGs promoters. Ultimately, ATM regulation of these chromatin-modifying factors may determine the selective transcription of ISGs, with ATM being crucial at least for CIITA, Ii and HLA-DRα expression. This differential transcriptional regulation likely results in HLA class II expression being highly dependent on ATM, comparatively to other ISGs and to HLA class I expression.

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