

Molecular Mechanisms and Outcomes of Arsenic-Induced Histone Acetylation and MicroRNA Regulation in Cellular Transformation.

By

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Abstract

Chronic exposure to arsenic causes negative health outcomes, particularly malignant neoplasms of the skin, lung and bladder. Although epidemiological data has associated arsenic exposure to cancer, a clear molecular mechanism has remained elusive. This thesis studied the impact of As₂O₃ exposure on histone acetylation and microRNA expression at both tolerated and toxic levels *in vitro* to determine an epigenetic-based mechanism of carcinogenesis.

This thesis outlines a framework for identifying tolerated and toxic As₂O₃ exposures, as a prerequisite to epigenetic characterisation. Tolerated As₂O₃ exposure increased cellular survival, anchorage-independent colony formation, cell-cycle progression and proliferation in HEK293T cells. HEK293T and UROtsa cells treated with tolerated As₂O₃ exhibited global H3K9 hyperacetylation at 3 hours and global H3K9 hypoacetylation at 72 hours. This was mediated by an imbalance in the intracellular HDAC2 to PCAF mRNA expression ratio. Global H3K9 hypoacetylation occurred for both tolerated and toxic exposures, giving poor mechanistic differentiation between these separated cellular outcomes. Chromatin immunoprecipitation identified PCAF recruitment, E2F1 binding and H3K9 acetylation at the *FOS* proto-oncogenic promoter leading to an elevation in FOS mRNA levels at the tolerated concentration only. This thesis also reports As₂O₃ induced chromatin relaxation in HEK293T cells followed by a return to nominal levels for the tolerated concentration. This is in contrast to the toxic exposure, which leads to clear chromatin condensation and apoptosis. This thesis postulates that arsenic-induced global H3K9 hypoacetylation is caused by a miR-372-mediated attenuation mechanism targeting PCAF mRNA, as predicted through bioinformatic analysis.

In summary, tolerated As₂O₃ exposure resulted in measurable perturbations in both global and promoter-specific histone acetylation in addition to the aberrant expression of microRNAs, which led to cellular transformation over toxicity.

Declaration of Originality

I, Sunniyat Rahman hereby declare that the work submitted in this thesis is my own and entirely original. This thesis has not and will not be submitted for any other degree at any other institution. Where figures, graphics, data and materials have been provided from an external source, a citation has been made to acknowledge such a contribution. A full reference list is provided at the end of this thesis with regards to in-text citations.

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Dedication

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Chen, CN., Hajji, N., Pullamsetti, S. S., Cotroneo, E., Stenmark, K. R., **Rahman. S.**, Wharton, J., Wilkins, M. R. and Zhao, L. Histone deacetylation inhibition in pulmonary hypertension: immune modulation by valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA).
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Abbreviations

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
acetyl-CoA	acetyl coenzymeA
ACTR	nuclear receptor coactivator 3
ADP	adenosine diphosphate
AGO	argonaute protein
AKT	protein kinase B
Alu	arthrobacter luteus element
AS3MT	arsenic (+3 oxidation state) methyltransferase
ATSDR	Agency for Toxic Substances and Disease Registry
AURB	aurora kinase B
BAP1	BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)
BAX	BCL-2 associated X protein
BCL ^{-XL}	BCL-like 1
BCL2	b-cell CLL/lymphoma 2
BID	BH3 interacting domain death agonist
BMI1	polycomb group RING finger protein 4
BSA	bovine serum albumin
C1QTNF6	C1q and tumour necrosis factor related protein 6
CARM1	coactivator-associated arginine methyltransferase 1
CAsE-PE	chronic-arsenic-exposed human prostate epithelial cells
CBP	CREB-binding protein
CCND1	cyclin D1
CFOS	FBJ murine oestrosarcoma viral oncogene homolog
CGA	cologenic assay
ChIP	chromatin immunoprecipitation
ChIP-Seq	chromatin immunoprecipitation with massively parallel DNA sequencing
CHK1	checkpoint kinase 1
CKII	casein kinase 2
COX-2	prostaglandin-endoperoxide synthase 2
CpG	cytosine-guanine dinucleotide
CYP1A1	cytochrome p450, family 1, subfamily A, polypeptide 1
DAPK	death-associated protein kinase 1
DBC1	deleted in bladder cancer 1
DGCR8	DGCR8 microprocessor complex subunit, pasha
DMA	dimethylarsinic acid
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha

DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
DOT1	putative histone-lysine N-methyltransferase
DSB	DNA double strand breaks
E2F1	E2F1 transcription factor 1
ECL	enhanced chemiluminescence
Eh	redox potential
EPA	U.S Environmental Protection Agency
ERCC2	excision repair corss-complementation group 2
ERK	extracellular signal-regulated kinase
EuHMTase	euchromatic histone-lysine N-methyltransferase 1
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FAD	flavin adenine dinucleotide
FAM83A	family with sequence similarity 83, member a
FBS	foetal bovine serum
G9a	euchromatic histone-lysine N-methyltransferase 2
GAD1	glutamate decarboxylase 1
GNAT	Gcn5 N-acetyltransferase
GSH	glutathione
GSSG	glutathione disulphide
GTP	guanosine triphosphate
HaCaT	normal immortalised human keratinocyte
HASPIN	germ cell associated 2
HAT	histone acetyltransferase
HAT1	histone acetyltransferase 1
HBEC	normal human bronchial epithelial cells
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HEK293T	human embryonic kidney cells
HELF	human embryo lung fibroblast
HIF-1 α	hypoxia inducible factor
HL60	acute myelogenous leukaemia
HMT	histone methyltransferase
HOX	Homeobox
HSP	heat shock protein
IARC	International Agency for Research on Cancer
iAs	inorganic arsenic
IGH	immunoglobulin heavy locus
INS	insulin
JHDM1A	K(lysine)-specific demethylase 2A
JMJ	jumonji domain containing proteins
JMJD2A	K(lysine)-specific demethylase 4A
JMJD2C	K(lysine)-specific demethylase 4C
JMJD3	K(lysine)-specific demethylase 6B
JUN	jun proto-oncogene

K562	chronic myelogenous leukaemia
KLF4	kruppel-like factor 4
KRAS	kirsten rat sarcoma viral oncogene homolog
LCN2	lipocalin 2
LET-7C	microRNA let-7c
lincRNA	large intergenic non-coding RNA
LINE	long interspersed elements
lncRNA	long non-coding RNA
LSD1	K(lysine)-specific demethylase 1A
MBD	DNA methyl-binding protein
MBT	malignant brain tumour domain
MDM2	MDM2- proto-oncogene, E3 ubiquitin protein ligase
miR	microRNA
miR	microRNA
MLH1	mutL homolog 1
MLL	K(lysine)-specific methyltransferase
MMA	monomethylarsonic acid
MNase	micrococcal nuclease
mRNA	messenger RNA
MSK1	ribosomal protein S6 kinase
MSK2	ribosomal protein S6 kinase polypeptide 4
MST1	macrophage stimulating 1
MT-3	metallothionein 3
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MYC	v-myc avian myelocytomatosis viral oncogene homolog
MYST	K(lysine) acetyltransferase 8
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
ncRNA	non-coding RNA
NFKB	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NRF2	nuclear factor, erythroid 2-like 2
NSD1	nuclear receptor binding SET domain protein 1
P16	cyclin-dependent kinase inhibitor 2A
P300	E1A binding protein p300
P53	tumour protein p53
PARP	poly (ADP-ribose) polymerase-1
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCAF	K(lysine) acetyltransferase 2B
PCDH	protocadherin
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDX1	pancreatic and duodenal homeobox 1
PHD	plant homeodomain

piRNA	PIWI-interacting RNA
PKB	protein kinase B
PKC	protein kinase C
PKC α	protein kinase C, alpha
PLA2G2C	phospholipase A2, group IIC
PMRT1	protein arginine methyltransferase 1
PRMT5	protein arginine methyltransferase 5
PTM	post-translational modification
PUMA	BCL2 binding component 3
QOF	quality of life
qRT-PCR	quantitative real-time polymerase chain reaction
RING1A	ring finger protein 1
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNF168	ring finger protein 168, E3 ubiquitin protein ligase
RNF20	ring finger protein 20, E3 ubiquitin protein ligase
RNF40	ring finger protein 40, E3 ubiquitin protein ligase
RNF8	ring finger protein 8, E3 ubiquitin protein ligase
ROS	reactive oxygen species
RWPE	human prostate epithelial cells
SAH	s-adenosylhomocysteine
SAHA	suberilohydroxyamic acid
SAM	s-adenosylmethionine
SDS-PAGE	polyacrylamide gel electrophoresis
SET	<i>Drosophila</i> Su(var)3-9 and enhancer of zeste proteins
SET1A	SET domain containing 1A
SETDB1	SET domain, bifurcated 1
SIRT	sirtuin
SLC4A4	solute carrier family 4 (sodium bicarbonate cotransporter), member 4
SOC	super optimal broth
SOCS6	suppressor of cytokine signalling 6
SP1	SP1 transcription factor
SQSTM1	sequestome 1
SSB	single stranded DNA breaks
SUV39H1	suppressor of variegation 3-9 homolog 1
SUV420H1	suppressor of variegation 4-20 homolog 1
SUV420H2	suppressor of variegation 4-20 homolog 2
SUZ12	polycomb repressive complex 2 subunit
T-UCR	transcribed ultraconserved region
T24	human bladder carcinoma cells
TA	tubastatin
TIF2	transcriptional intermediary factor 2
TIP60	K(lysine) acetyltransferase 8
TK6	human immortalised lymphoblast

TMS1	target of methylation-associated silencing-1
TR	thioredoxin reductase
Trx	thioredoxin
TSA	trichostatin A
U-tAs	maternal urinary arsenic measurement
UHRF1	ubiquitin-like with phd and ringer finger domains 1
UROtsa	normal human urothelial cells
USP21	ubiquitin specific peptidase 21
USP22	ubiquitin specific peptidase 22
USP3	ubiquitin specific peptidase 3
UTR	untranslated region
WHO	World Health Organisation
WNT5A	wingless-type MMTV integration site family, member 5A
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulphophenyl)-2 <i>H</i> -Tetrazolium-5-Carboxanilide
ZNF	zinc finger
ZSCAN	zinc finger and scan domain containing 1

Introduction

1.1 – Chemistry and Behaviour of Arsenic in Brief.

Arsenic (As) is a grey metalloid element found at an abundance of $2-10 \times 10^{-4} \%$ in the Earth's crust (Grund et al. 2008). It is in group 15 and period 4 with an atomic number of 33 and an atomic mass of 74.92 (<http://www.rsc.org/periodic-table/element/33/arsenic>, accessed: 02.14). The electronic configuration of arsenic is $[\text{Ar}]3d^{10}4s^24p^3$, it remains solid at 25°C and sublimes at 616°C . Arsenic can form over 200 different minerals in the environment, the most prevalent of which is arsenopyrite (FeAsS) – an arsenic sulphide. This mineral form is usually oxidised by O_2 , Fe^{3+} and NO_3^- (Bhattacharya 2004). Arsenic can also occupy a number of oxidation states including -3, 0, +3 and +5. This variety in oxidative states allows for the formation of many different arsenic compounds in the environment (Table. 1).

+ **Table 1: Inorganic and organic arsenic species found in the environment.**
Adapted from: (Ng et al. 2001).

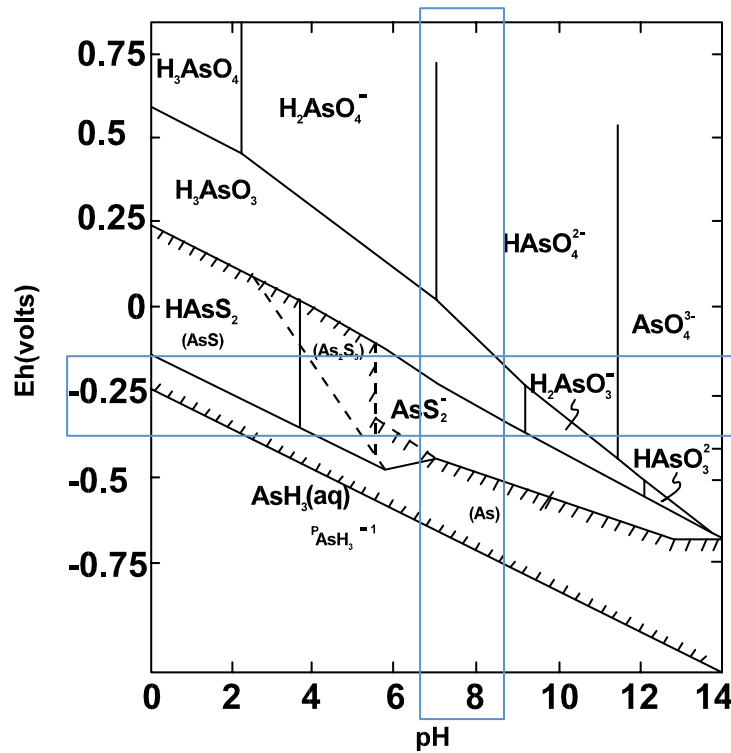
Name	Chemical Formula
<i>- Trivalent inorganic arsenicals -</i>	
arsenic (III) oxide	As_2O_3
arsenous acid	HAsO_2
arsenic (III) chloride	AsCl_3
arsenic (III) sulphide	As_2S_3

Table 1 cont.

Name	Chemical Formula
<i>- Pentavalent inorganic arsenicals -</i>	
arsenic (V) oxide	AsO ₅
arsenic acid	H ₃ AsO ₄
arsenenic acid	HAsO ₃
<i>- Organic arsenicals -</i>	
methylarsine	CH ₃ AsH ₂
dimethylarsine	(CH ₃) ₂ AsH
trimethylarsine	(CH ₃) ₃ As

Arsenic demonstrates great chemical persistence especially in groundwater, which has a pH range of 6.5 – 8.5. Compared to other metal contaminants such as Ni²⁺, Zn²⁺ and Pb²⁺, arsenicals are able to remain soluble across the whole groundwater pH range leading to the accumulation of arsenic species up to concentrations of many micrograms per litre (Smedley & Kinniburgh 2001). The combined redox potential (Eh) and pH properties of groundwater make it a favourable chemical environment for arsenic persistence.

This is partly due to arsenic speciation, which is dependent on Eh and pH of the environmental solvent (Fig. 1.1). In a study systematically measuring groundwater properties in Bangladesh, Anawar *et al.* showed that Eh and arsenic concentration are inversely correlated (Anawar et al. 2011). When dissolved in water, arsenic can form a number of different compounds including arsenate, arsenite, monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Ng et al. 2001).



+ **Figure 1.1: Arsenic speciation with respect to redox potential (Eh) and pH at 25°C and 1 atmosphere.**
The blue boxes represent the measured range of Eh and pH usually found in groundwater in Bangladesh. Adapted from Ferguson & Gavis, 1972, combined with data from Anawar *et al.* 2011.

The real world solvent environment is rarely homogenous; instead it is decorated with sedimentary material, clays, metallic compounds and organic matter. Arsenic can adsorb onto these materials at differing affinities and it does so with some dependency on phosphate concentration, the redox state of metal oxides, the speciation of arsenic itself, such as As(V) or As(III), the microbial biota and pH (Kent & Fox 2004; Z. Lin & Puls 2000). Due to these variable-dependent adsorption effects, these materials not only behave as natural attenuators of environmental arsenic but also simultaneously act as fragile arsenic sinks susceptible to mobilisation by chemical changes induced by human behaviours. In the Ganges delta for

example, the use of fertilisers in addition to the withdrawal of groundwater led to a localised adjustment in phosphate levels leading to the desorption of arsenic (Chowdhury et al. 1999).

1.2 – Sources of Human Exposure to Arsenic.

Naturally occurring arsenic is predominantly in the form of arsenopyrite (FeAsS). It is also often found mixed with other mineral deposits, especially those that have a high sulphide content, as arsenic can co-ordinate with sulphur ligands (Ng et al. 2001). Due to this, arsenic can make its way into groundwater especially in areas with high levels of sulphide mineralisation. Of the naturally occurring arsenic minerals, 60% exist as arsenates, 20% as sulphides and the remaining 20% are composed of arsenites, oxides, silicates and elemental arsenic (Mandal & Suzuki 2002).

Industrial and polluting sources of arsenic have been on the decline due to policy changes driven by environmental concerns. Very few countries actually produce arsenic compounds, with a limited number allowing the use of arsenic as a plant and wood preservative. Historically arsenic compounds were used in large quantities as pesticides (Ng et al. 2001). Arsenic trioxide is still produced by China and exported to the US in metallic form for industrial processes (Grund et al. 2008). The U.S Environmental Protection Agency (EPA) has allowed the use of organic arsenic compounds at strict thresholds – specifically a dosage that does not cause bladder cell cytotoxicity, as herbicides and pesticides, whilst banning its use in rights-of-way and recreational parks, with the promise of regular re-evaluations on their usage (Hughes et al. 2011).

The largest recorded human exposure to arsenic is in Bangladesh, where a 10-year survey between 2000 – 2010 estimated that 35 to 77 million people have been chronically exposed via contaminated drinking water (Flanagan et al. 2012). Of this population, 57 million people have been exposed to concentrations exceeding World Health Organisation (WHO) guidelines at 10

µg/L, with the remaining individuals being exposed to concentrations greater than Bangladesh's own internal guidelines of 50 µg/L (Anawar et al. 2011). Arsenic exposure is a global issue, as elevated groundwater arsenic concentrations have also been identified across the world in Argentina, Canada, Chile, China, Hungary, Japan, Mexico, New Zealand, Taiwan, Thailand and the United States (Moore et al. 2002; Kurtzio et al. 1999; Hopenhayn-Rich et al. 1998; C. J. Chen et al. 1985; Bhattacharya 2004). Furthermore, arsenic can enter the food chain through normal agricultural activities such as the growth of rice and vegetables (Mandal & Suzuki 2002). This is of particular concern as these products are often exported to locations without environmental arsenic contamination.

The mechanism through which arsenic makes its way into groundwater is still under debate. The oxidation of arsenopyrite is believed to be the most significant contributor of arsenic release into the environment. However, iron compounds with adsorbed arsenic susceptible to microbial metabolism, have also been implicated as these breakdown liberating arsenic into sedimentary material and groundwater (Nickson et al. 1999; McArthur et al. 2001). The heavy use of fertilisers is also believed to exacerbate the problem, as this encourages microbial growth and alters the local phosphate levels. The resulting change microbial and phosphate levels provisions the release of arsenic into the environment (Hartley et al. 2013).

1.3 – Epidemiological Evidence of Arsenic-Induced Negative Health Outcomes

Arsenic poisoning can occur over acute and chronic time scales with differing health outcomes. Historically arsenic has been used for a variety of purposes including medicinally in the late 1700s, pigments in toys and wallpapers, and as a intentional homicidal poison (Hughes et al. 2011). Whilst acute arsenic exposure is relatively rare today, cases of accidental consumption in the form of pesticides or through intentional ingestion for suicide have been reported (Ratnaike

2003). Small doses of arsenic trioxide can induce severe acute toxicological responses including vomiting, diarrhoea, abnormal cardiac function, muscular spasms and damage of the gastrointestinal tract (Grund et al. 2008). A lethal dose of arsenic in acute poisoning is 200 mg to 300 mg, although one case has been reported where a 43-year-old male survived following the consumption 54 g of arsenic trioxide due to intense medical intervention and surgery (Dueñas-Laita et al. 2005).

Chronic arsenic exposure leads to a broad range of deleterious health outcomes including cardiovascular disease, neuropathy, cognitive impairments, reproductive issues, in addition to cancers of the skin, bladder and lungs (Kapaj et al. 2006). An early report of cardiovascular impairment as a result of chronic arsenic exposure was the identification of a blackfoot disease endemic in Taiwan localised to regions with access to arsenic contaminated drinking water (Tseng 1977; Engel et al. 1994). The study by Tseng *et al.* used blackfoot disease as a proxy for skin cancer, and demonstrated that these two health outcomes were correlated. This also presented early evidence of the association between chronic arsenic exposure and carcinogenesis. Chronic arsenic exposure can have damaging effects in conjunction with lifestyle choices such as smoking. A 6-year study that examined smokers exposed to arsenic contaminated drinking water demonstrated an increased mortality rate from heart disease compared to a geographically local control group (Y. Chen et al. 2011). Arsenic exposure is also believed to exacerbate existing health issues, such as diabetes mellitus, where those suffering from this illness also exhibit elevated impairment of kidney function (J. P. Wang et al. 2009). This study also verified the effect in rats with diabetes.

There is growing evidence of neurological disorders that arise as result of long-term arsenic exposure, particularly negative effects on motor function, cognitive ability and psychiatric state. Chronic arsenic exposure has been associated with reduced juvenile intellectual function and poorer school performance in a dose-dependent manner (Wasserman et al. 2004; Ehrenstein et

al. 2007). Psychiatric issues have also been reported in adults diagnosed with arsenicosis including an elevation of depression, anxiety and suicidal attempts in comparison with a control group (Sen & Biswas 2011). Given that these neurological issues affect both children and adults, there was a standing hypothesis that chronic exposure over a lifetime has a severe impact on quality of life (QOF). One study examined QOF through the utility of a self-reporting questionnaire, covering multiple life metrics including mental health to demonstrate a lower QOF score and socio-economic status in arsenic exposed patients (Syed et al. 2012).

The International Agency for Research on Cancer (IARC) has classified arsenic as a human carcinogen, i.e. Group 1 (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans 2004). There is substantial evidence to support this classification, including early studies in areas with blackfoot disease where higher standard mortality rates and cumulative mortality rates from cancers of the bladder, kidney, skin, lung and liver were reported (C. J. Chen et al. 1985; C. J. Chen & C. J. Wang 1990). The prevalence of these malignancies increased in areas with higher arsenic contamination alluding to the indication of a dose-response relationship. Similar findings were also reported in a 5-year study in Córdoba – Argentina, which demonstrated an increase in kidney and lung cancer mortality with arsenic exposure through the examination of standard mortality rates for men and women (Hopenhayn-Rich et al. 1998). Interestingly there is a discrimination regarding the types of cancers that are induced by chronic arsenic exposures, where correlate more readily compared to others. Epidemiological evidence across the counties of Nevada – USA, with a low (<10 µg/L) to high (90 µg/L) arsenic contamination range demonstrated no significant correlation between arsenic exposure and childhood leukaemia (Moore et al. 2002).

Arsenic-induced illnesses and mortalities are significant health burden globally. Currently, arsenic is #1 of the Agency for Toxic Substances and Disease Registry (ATSDR) substance priority list, which allocates points weighing the public health hazard of a toxicant against locations in the

USA (Naujokas et al. 2013). Mortality hazard ratios for avoidable cancer, cardiovascular and infectious illnesses all increase with arsenic exposure, and arsenic related illnesses could cost the US economy \$13B USD through lost productivity over the next two decades (Argos et al. 2010; Flanagan et al. 2012). For the well-known exposure in Bangladesh the situation remains dire. An examination of 11,746 individuals over a 9-year period demonstrated that mortality rates from a number of illnesses combined into a hazard ratio correlated with arsenic exposure measured from urinary samples (Y. Chen et al. 2011). This probabilistic indicator projects that “all-cause mortality” is elevated with arsenic exposure and surprisingly 24% of the participants were exposed to concentrations less than the WHO guideline, suggesting that other countries believed to have “safe” levels of arsenic may still fall foul to the toxicant. Indeed a recent epidemiological study from Finland has confirmed such fears, where 144,627 people – 99% of which were exposed to arsenic concentrations less than the WHO guideline, demonstrated an elevation in bladder and kidney cancer incidence (Kurttio et al. 1999).

These studies are on the macroscopic and epidemiological scale. The need to understand the underlying mechanisms driving these arsenic-induced illnesses, particularly cancer is great. It is clear that both time and dose are important factors in arsenic-induced deleterious outcomes, but these studies demonstrate that there is a significant need to investigate chronic low-level arsenic exposures and the associated contributory biochemical mechanisms that lead to malignancies.

1.4 – Metabolism of Arsenic

Arsenic can accumulate in keratin-rich structures such as hair, nails, and muscle and to a lesser extent bone. The approximate half-life of arsenic is 4 days in humans, and around 40 – 60 % of the toxicant can remain in the aforementioned biological structures leading to toxic and carcinogenic effects (Bustaffa et al. 2014; Raab & Feldmann 2005). Arsenic metabolism in mammals is achieved through a series of methylation reactions. A number of tissues including the testes, kidneys, liver and lungs are able to readily methylate arsenic (Vahter 2001). Whilst there is considerable consensus regarding the fundamental role of methylation in arsenic metabolism, there is a continuing debate regarding the reaction scheme describing the metabolic process.

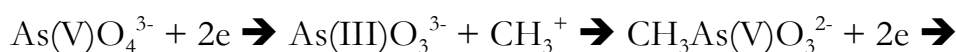
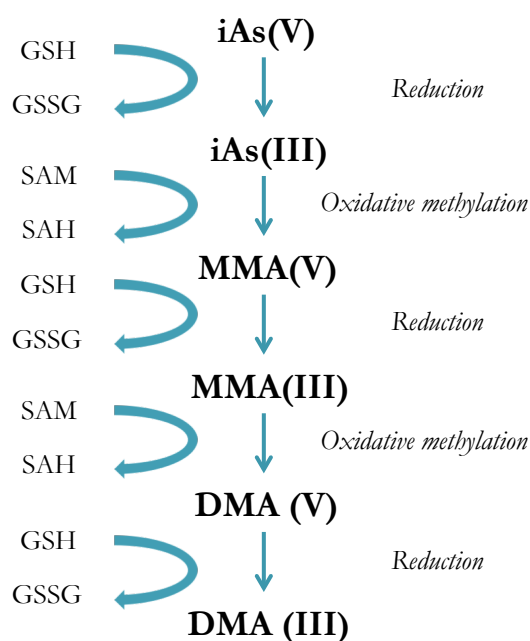
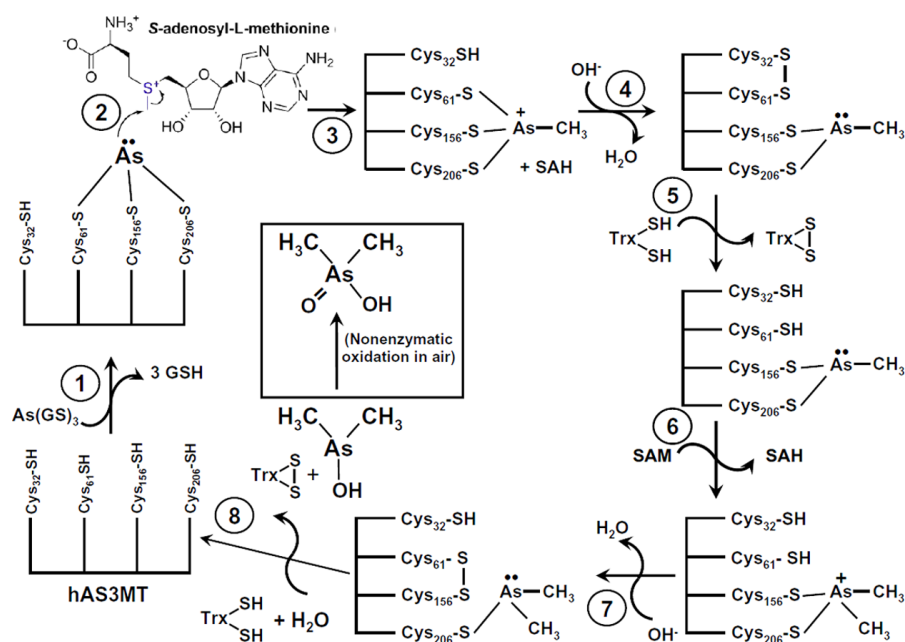


Figure 1.2: Classical reaction scheme describing arsenic metabolism in humans.
Chemical intermediates are: iAs = inorganic arsenic, MMA = monomethylarsonic acid, DMA = dimethylarsinic acid. Enzymes and co-factors are: GSH = glutathione, GSSG = glutathione disulphide, SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine. A written reaction scheme is also shown. Adapted from Styblo *et al.* 2002. and Lin *et al.* 2002.

The classical mechanism describes two chemical reactions that occur in tandem; the first being the reduction of pentavalent arsenicals to a trivalent state, the second being oxidative methylation to a pentavalent metabolite (Fig. 1.2) (Stýblo et al. 2002). This occurs a number of times in sequence to progress from inorganic arsenic (iAs) to monomethylarsonic acid (MMA) and finally dimethylarsinic acid (DMA). As these metabolites have been detected in urine, this classical reaction scheme is widely considered to be representative of the arsenic metabolic process (Hopenhaynrich et al. 1992; C Hopenhayn-Rich 1996; Le et al. 2000). In addition to this, *S*-adenosyl-methionine (SAM) is believed to be the methyl donor in the reactions, glutathione as the reducing agent and methyltransferase as the enzyme (Roy 2002). Experimental evidence suggests that glutathione is crucial for arsenic methylation, as hepatic depletion of this reducing agent results in significantly elevated toxicity in hamsters and reduced arsenic methylation (Hirata et al. 1988).

Although these reactions were postulated to be enzymatically catalysed, they were initially attributed to an uncharacterised methyltransferase. This was until Lin *et al.* isolated arsenic methyltransferase from the liver cytosol of rats which is encoded by the *AS3MT* gene, and specifically transfers methyl groups from SAM to trivalent arsenicals (S. Lin et al. 2002; Waters et al. 2004). Detailed biochemical analysis of this enzyme not only provided a greater understanding of the chemical processing of arsenic, but it also began to challenge the existing classical reaction scheme. There is a continuing debate regarding the number of conserved cysteine residues within the enzyme, with the most recent data suggesting that 4 residues, Cys32, Cys61, Cys156 and Cys206 are conserved (Dheeman et al. 2014). Furthermore, substitutions of these residues halt iAs methylation. Dheeman *et al.* challenge the classical reaction scheme on three fronts. The first is that there is minimal evidence to support the formation of pentavalent products by other SAM methyltransferases in nature, the second is that pentavalent intermediates are more likely formed in complex with the enzyme and thirdly that the reducing agent glutathione (GSH), is likely bound to inorganic arsenic prior to enzymatic processing.



+ **Figure 1.3: hAS3MT reaction scheme proposed by Dheeman et al (2014).**
 (1) Initial methylation is carried out via three thiol transfer reactions from $\text{As}(\text{GS})_3$ resulting in the binding of $\text{As}(\text{III})$ to hAS3MT. (2) The SAM methyl group is attacked by the arsenic lone pair, (3) resulting in the formation of a pentavalent MAs(V) intermediate. (4) MAs(V) is reduced further to MAs(III) whilst remaining enzyme giving rise to a Cys32-Cys61 disulphide bond. (5) Trx reduces the disulphide bond allowing for another round of methylation. (6) The next methylation event forms a pentavalent DMAs(V) intermediate (7) which is reduced further to DMAs(III) by Cys61. (8) This results in the formation of another disulphide bridge which is reduced by Trx allowing for the liberation of the enzyme, ready to start the process again. Graphic from Dheeman *et al.* 2014.

Recent findings appear to support the hAS3MT reaction scheme (Fig. 1.3). For example, $\text{As}(\text{GS})_3$ and $\text{MAs}(\text{GS})_2$ bind at a faster rate to the enzyme than $\text{As}(\text{III})$ alone, which supports the notion that glutathionylated arsenicals are preferred substrates for the hAS3MT (Marapakala et al. 2012). Prior research has also shown that the endogenous enzyme can use other reductants such as thioredoxin (Trx), thioredoxin reductase (TR) and nicotinamide adenine dinucleotide phosphate (NADPH), rather than GSH alone (Waters et al. 2004). Whilst redundancy exists in the form of reducing agents, GSH is still considered fundamental for arsenic metabolism.

Discussions remain regarding arsenic metabolism and whether it should be considered a true detoxification or bioactivation process. Initially it was believed that arsenic methylation was a

detoxification process and that toxicity arose due to the presence of a threshold methylation capacity, which states that after a certain point inorganic arsenic can no longer be methylated leading to toxic effects. However this hypothesis was disproved by measuring the concentration ratios of $[iAs] / [iAs] + [MMA] + [DMA]$ in humans, which found that 20 – 25 % of iAs remains unmethylated irrespective of the magnitude of exposure (Hopenhaynrich et al. 1992). A follow up experiment measured urinary iAs levels and demonstrated that it too was unchanged against a gradient of increasing arsenic exposures (C Hopenhayn-Rich 1996).

Evidence for bioactivation resides in the form of *in vitro* toxicity measurements between arsenate, arsenite, MMA(V), MMA(III) and DMA(V). The cytotoxic response of Chang human hepatocytes was assessed by examination of lactate dehydrogenase leakage, intracellular potassium and mitochondrial metabolism of a tetrazolium salt XTT (Petrack et al. 2000). From this study it was revealed that MMA(III) is more toxic than arsenite and arsenate and that the pentavalent forms of MMA and DMA are both equally toxic. Another comparative study investigating the toxicity of arsenicals and methylated intermediates demonstrated that arsenic methylation did not protect the cells from acute toxicity, rather the methylation process itself significantly contributed to the toxicity of arsenic (Styblo et al. 2000). These studies demonstrate the challenge in defining arsenic metabolism as a detoxification process over bioactivation.

The factors affecting the toxicity of arsenic have diverged into new areas according to recent research. There is now evidence to suggest that microbial metabolism of arsenic in the gut may also alter the speciation of arsenic (Alava et al. 2014). In the study by Alava *et al.* quantifiable differences in the generation of hazardous arsenic species, particularly monomethyl arsonite and monomethylmonothio arsonate were observed between high fat and protein rich Western diets compared to fibre rich Asian diets. Hence the authors suggested that dietary background might contribute to arsenic toxicity. Furthermore, sulphate-reducing bacteria have been implicated in

the thiolation of methylated arsenicals, with toxicologists suggesting that the activity of such bacteria should be considered in future toxicokinetic analyses (D C Rubin et al. 2014).

Metabolic efficiency is also believed to play a role in arsenic toxicity, particularly with regards to the *AS3MT* gene. Three polymorphisms of *AS3MT* were associated to a statistically significant change in urinary MMA (Antonelli et al. 2014). Single nucleotide polymorphisms near the *AS3MT* gene also caused measurable differences in the efficacy of arsenic metabolism with negative health outcomes including the formation of skin lesions (Pierce et al. 2013; Schläwicke Engström et al. 2009).

1.5 – Possible Mechanisms of Arsenic-induced Toxicity and Carcinogenicity

There are a number of reported mechanisms that lead to toxic and carcinogenic outcomes following arsenic exposure. Some of these are in relation to the chemical reactivity of arsenic, such as its ability to interact with sulphurs and phosphates. Other mechanisms are dependent on cellular responses, such as the generation of reactive oxygen species and the activation of signal cascades (Table. 2).

+ **Table 2: A summary of proposed mechanisms through which arsenic induces cytotoxicity and/or carcinogenesis.**
Adapted from: (Hughes et al. 2011) (Bustaffa et al. 2014)

	Mechanism	Outcome
Arsenic exposure →	Interaction with sulphur	Formation of stable As-S complexes
	Interaction with phosphate	Formation of unstable arsenoesters
	ROS	Increase in H ₂ O ₂ , O ₂ ⁻ , ROOH, OH ⁻ and NO
	Genotoxicity	Chromosomal aberration, genomic instability
	Altered DNA repair	Inhibition of DNA ligase
	Signal transduction	Activation of mitogen activated protein kinase pathway
	Altered DNA methylation	Altered patterns of DNA methylation/ genomic imprinting.

Table 2 cont...

Histone tail modifications	Impacts on gene expression
miRNA	Impacts on gene expression

Poor arsenic metabolism is a considerable hindrance in the cellular environment as arsenite is predicted to inhibit the activity of approximately 200 enzymes through the interaction with sulphhydryl groups (Abernathy et al. 1999). Due to chemical similarities, arsenate can substitute phosphate in biochemical reactions leading to the formation of unstable arsenoesters. Furthermore, human erythrocytes exposed to 0.001 – 10 mM As(V) for 5 hours lead to the depletion of ATP which leading to cytotoxicity, plasma membrane deformation and haemolysis (Winski & Carter 1998).

Arsenic exposure generates reactive oxygen species and oxidative stress within the cellular environment. This is achieved through the conversion of DMA(V) to dimethylarsine, which leads to the formation of a number of free radicals including the dimethylarsenic peroxy radical, dimethylarsenic radical, superoxide radicals and hydroxyl radicals (Kitchin & Ahmad 2003). Opposing outcomes have been postulated as a result of arsenic-induced ROS generation, including apoptosis and cellular transformation. Arsenic-induced ROS generation leads to mitochondrial membrane permeability, cytochrome c release and the activation of downstream caspases, resulting in apoptosis induction and cell death (Banerjee et al. 2008). In contrast, ROS has also been shown to activate redox sensitive transcription factors, in addition to NF- κ B, AP-1, JUN, p38 and MAPK signalling cascades leading to increased cellular proliferation and the possibility for carcinogenesis (Flora 2011).

Arsenic is an effective clastogen as it induces genotoxicity via chromosomal instability. Genomic integrity is compromised following arsenic exposure due to ineffective DNA repair, aberrant chromosomal segregation and lapsed cell cycle control (Bhattacharjee et al. 2013). The result is a significant increase in DNA damage, mitotic arrest and unfavourable epigenetic alterations – all

of which can contribute to carcinogenesis. A dose-response relationship between arsenic exposure and the formation of DNA-double strand breaks and protein-DNA adducts has been shown in human lung fibroblast MRC-5 cells exposed to increasing concentrations of sodium arsenite (Mourón et al. 2006). Chinese hamster cells exposed to 10 μ M sodium arsenite for 24 hours exhibit a chromosomal number less than 21, which is the modal number for the cell line (Sciandrello et al. 2002). This latter study is particularly significant, as it demonstrates the rapidity through which genetic instability can occur following arsenic exposure, as a 24-hour exposure can generate aneuploidy in cells. These changes also persisted after a 5-day recovery without the toxicant, suggesting that such changes are relatively irreversible. A number of *in vivo* studies have identified aberrations of DNA repair mechanisms such as nucleotide excision repair and base excision repair following exposure to environmental levels of arsenic, with measurable elevations in DNA damage (Andrew et al. 2006; Sampayo-Reyes et al. 2010). More research is needed to understand the severity of DNA damage induced by different arsenicals. A study by Klingerman *et al.* demonstrated that MMA(III)-induced DNA damage is mediated by ROS, leading to the formation of short-lived single-strand breaks, which are quickly repaired through base excision repair (Klingerman et al. 2010). This is in contrast to arsenic-induced aneuploidy and disrupted mitotic spindle effects, which remain and do not repair effectively. Arsenic-induced genotoxicity also arises from the inhibited function of zinc-finger dependent DNA repair enzymes such as PARP, that are involved in the detection of single strand breaks (Ding et al. 2008).

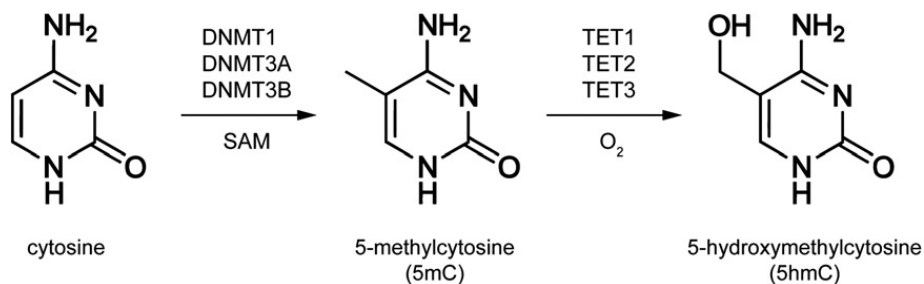
It is likely that arsenic-induced carcinogenesis is multifactorial given the variety of perturbations measured within the cellular environment. Recent publications have identified a number of aberrant epigenetic mechanisms at play following arsenic exposure, some of which overlap with existing mechanisms. For example, the identification that arsenic-induced DNA hypomethylation exacerbates the problem of chromosomal instability and the recent finding that low-level arsenic exposure increases the expression of microRNAs that attenuate the expression of a number antioxidant genes (Sciandrello et al. 2004; Ren et al. 2015).

1.6 – Epigenetic Mechanisms

The contemporary definition of epigenetics is the study of stable and potentially heritable changes in gene expression that do not arise from DNA sequence changes, whilst an epigenetic event is described as a structural modification of chromatin at a defined chromosomal region to register, signal or perpetuate an altered activity state (Y.-H. Jiang et al. 2004; Bird 2007). These epigenetic processes are communicated through three main epigenetic mechanisms including DNA methylation, histone post-translational modifications and microRNAs (miRs). Not all epigenetic events impinge on chromatin, rather non-coding RNAs such as microRNAs (miRs), can also influence gene expression through post-transcriptional control mechanisms (Ameres & Zamore 2013).

1.6.1 – DNA methylation

One of the most extensively studied epigenetic mechanisms is DNA methylation. It involves the methylation of cytosine nucleotides to create 5-methylcytosine (5mC), leading to a methyl group protrusion into the major groove of DNA (Virani et al. 2012). Whilst this chemical modification is believed to disrupt the binding of transcription factors, it is also thought to fulfil the inverse function by permitting the binding of additional proteins to DNA (Prokhortchouk & Defossez 2008). Reversible methylation of cytosine usually occurs at cytosine-guanine dinucleotides (CpGs), where the “p” represents a phosphodiester bond (Lim & Maher 2011). The mammalian genome is predominantly methylated, with unmethylated CpGs occurring in groups or islands at gene promoters to act as a platform for transcriptional control.



+ **Figure 1.4: DNA methylation on the cytosine nucleotide.**
Cytosine can be methylated by DNA methyltransferases DNMT1, DNMT3A and DNMT3B with the S-adenosylmethionine (SAM) methyl donor leading to the formation of 5-methylcytosine (5mC). TET proteins catalyse the formation of 5-hydroxymethylcytosine through the utility of oxygen. Adapted from Dahl *et al.* 2011.

DNA methyltransferases catalyse the addition of a methyl group to cytosine and are classified into two functional groups (Fig. 1.4). The first are the *de novo* DNA methyltransferases DNMT3A and DNMT3B that are involved in the methylation of fully unmethylated or “naked” DNA. The second group is occupied by DNMT1, which is considered a maintenance DNA methyltransferase as it only methylates hemi-methylated DNA, a partially methylated DNA form generated by DNA replication (Okano et al. 1999). The contemporary model suggests that the grouping of the enzymes between *de novo* and maintenance is oversimplified, rather specific DNA methylation events are dependent on the localised activity of DNA methyltransferases, DNA demethylases, DNA replication, and histone modifications; suggesting that a network of chromatin marks synergistically control DNA methylation (Jeltsch & Jurkowska 2014).

DNA methylation is of considerable biological significance with well-characterised roles in female X-chromosome inactivation, genomic imprinting of specific alleles, the transcriptional repression of transposons and pre-transcriptional regulation (Dahl et al. 2011). In addition to traditional DNA methylation in the form of 5mC, cytosines can be modified further to form 5-hydroxymethylcytosines (5hmC). This secondary form of DNA methylation is catalysed by translocation dioxygenases (TET), which oxidise 5mCs with molecular oxygen to form 5hmC.

The 5hmC modification is not catalytically added or recognised by DNA methyltransferases and is thought to play a role in mammalian development, with the loss of 5hmC and mutations in TET proteins also being implicated in cancer (Pfeifer et al. 2013). DNA methyl-binding (MBD) proteins such as the MBD family, Kaiso protein family and the ubiquitin-like with PHD and ring finger domain 1 (UHRF1) family of proteins are able to recognise DNA methylation, allow for further structural alterations, the recruitment of histone modifying enzymes and the assembly of repressive complexes at methylated CpGs (Gibney & Nolan 2010; Bogdanović & Veenstra 2009).

1.6.2 – Histone post-translational modifications

Cells package a vast amount of DNA into the nucleus. This is achieved through the organisational advantage of a three-dimensional structure created by wrapping linear DNA around histone proteins. A total of 146 base pairs are looped around an octomer of histones forming the basic unit of chromatin called a nucleosome. Each octomer is composed of two copies of four core histone variants including H2A, H2B, H3 and H4 (Virani et al. 2012). Nucleosomes are linked together with a short 10 to 80 base pair DNA length. A higher order of compaction is also achieved via the twisting of nucleosomes leading to the formation of the “30 nm fiber” in which six nucleosomes per turn create a solenoid structure (Y. Zhang & Reinberg 2001). This overall architecture is referred to as chromatin.

Chromatin is a dynamic structure with the potential to be remodelled and dynamically reorganised for the purpose of carefully regulated gene expression (Felsenfeld & Groudine 2003). The crux of this regulatory potential relies on the large variety of post-translational modifications (PTMs) that can occur on histones, including acetylation, lysine/arginine methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, deimination and proline

isomerisation (Kouzarides 2007). These modifications are usually enzymatically catalysed with functional consequences on transcription, DNA repair, replication and condensation (Table. 3.).

+ Table 3: Histone modifying enzymes and associated target residues and functional annotations.
Adapted from: (Kouzarides 2007), (Virani et al. 2012), (Lachner et al. 2003), (Ropero 2007), (Glozak & Seto 2007), (Drogaris et al. 2012), (Baek 2011)

Enzymes	Target Residues	Annotations
<i>Lysine acetyltransferases</i>		
HAT1	H4K5/K12.	Histone deposition, transcriptional activation
CBP/P300	H3K14/K18. H4K5/K8. H2AK5. H2BK12/K15.	Transcriptional activation
PCAF	H3K9/K14/K18.	Transcriptional activation
TIP60	H4K5/K8/K12/K16. H3K14	Transcriptional activation, histone deposition
<i>Lysine deacetylases</i>		
HDAC1-11	Global H3/H4 tail acetylation	-
SIRT1	H3K9, H4K16	-
SIRT2	H4K16	-
<i>Lysine methyltransferases</i>		
SUV39H1, H2	H3K9	Pericentric heterochromatin
G9a	H3K9	Transcriptional repression, imprinting
MLL1, 2, 3, 4, 5	H3K4	Transcriptional activation
SET1A, 1B	H3K4	Transcriptional activation, telomeric silencing, trithorax activation
SETDB1/ESET	H3K9	Transcriptional repression
EuHMTase/GLP	H3K9	Transcriptional repression
NSD1	H3K36	Transcriptional activation
DOT1	H3K79	DNA damage repair
SUV420H1/H2	H4K20	
EZH2	H3K27	Implicated in the progression of human prostate cancer and transcriptional repression.
<i>Arginine methyltransferases</i>		
CARM1	H3R2/R17/R26	Transcriptional activation
PRMT4	H4R3	Transcriptional activation
PRMT5	H3R8. H4R3.	-
<i>Lysine demethylases</i>		
LSD1/BHC110	H3K4/K9	Transcriptional repression
JHDM1A/B	H3K36	-
JHDM2A/B	H3K9	-

Table 3 cont...

JMJD2A/JMDM3A	H3K9, H3K36	-
JMJD2C/GASC1	H3K9, H3K36	Transcriptional activation
JMJD2D	H3K9	-
JMJD2C	H3K9	-
JMJD3	H3K27	Transcriptional activation
<i>Serine/threonine kinases</i>		
HASPIN	H3T3	Chromatin condensation
MSK1,2	H3S10, H3S28	Transcriptional activation
PKC α/β	H3T6	Transcriptional activation
PKB/Akt	H3S10	Transcriptional activation
CKII	H4S1	-
MST1	H2BS14	-
<i>Ubiquitinases</i>		
BMI/RING1A	H2AK119	-
RNF20/RNF40	H2BK120	-

Histone post-translational modifications are recognised by domains within specific proteins that form intimate interactions with chromatin. These “chromatin readers” include bromodomains, which can detect histone acetylation events, PHD, chromo, MBT and Tudor domains that can recognise methylation patterns and 14-3-3 proteins that identify phosphorylated histones (Mellor et al. 2008; Kouzarides 2007). The post-transcriptional potential of histone tails spawned the “histone code hypothesis” which states that multiple histone marks can be collectively read to help respond to signal pathways that impinge on chromatin (Strahl & Allis 2000). An additional component of this hypothesis is that histone PTMs are able to work co-operatively. One such example is the case of histone acetylation and phosphorylation, where the former involves the neutralisation of a positive charge and the latter introduces a negative charge leading to an overall effect of chromatin decondensation through electrostatic repulsion. The “histone code hypothesis” was developed further through the proposal of binary switches and modification cassettes that remain embedded within histone PTMs (Fischle et al. 2003). Fischle *et al.* state that modification cassettes composed of histone PTMs separated only by a few amino acids may exist as recognition marks for other proteins to influence gene expression.

Histone PTMs are added and removed with the assistance of histone modifying enzymes. Histone acetyltransferases (HATs) are grouped into different families depending on their catalytic domains. HATs enzymatically transfer an acetyl group from acetyl-coenzyme A to the ϵ -amino group of specific lysines within the N-terminal region of histones. The first HATs to be discovered were the Gcn5 N-acetyltransferases (GNATs) GCN5, PCAF and HAT1. MYST family HATs include hMOF and TIP60. The remaining families include the orphaned p300/CBP CREB-binding protein and nuclear receptor co-activators, steroid receptor co-activator 1 (SRC1), nuclear receptor co-activator 3 (ACTR), transcriptional intermediary factor 2 (TIF2), all of which have intrinsic HAT activity (K. Lee 2007; Sterner & Berger 2000).

Histone deacetylases (HDACs) are grouped into four classes spanning two families, the classical and the silent information regulator 2 (Sir2)-related “sirtuin” protein families. Within the classical family there are the following enzymes: class I, HDAC1, 2, 3, and 8; class II, HDAC4, 5, 6, 7, 9, and 10; class IV, HDAC11. All class I, II and IV enzymes require Zn^{2+} for catalytic activity. Within the sirtuin family reside the class III enzymes, which include SIRT1 – 7, which unlike the classical family, require the cofactor NAD^+ for enzymatic activity (X.-J. Yang & Seto 2007; Grozinger & Schreiber 2002; Delcuve et al. 2012). The catalytic mechanism of histone deacetylation is reliant on a charge-relay system facilitated by a Zn^{2+} ion residing within the bottom of the substrate binding pocket (de Ruijter et al. 2003). This charge relay system has been targeted by HDAC inhibitors (HDACi) such as trichostatin A (TSA) and suberanilohydroxyamic acid (SAHA) to displace the Zn^{2+} ion leading to the disruption of the relay and collapse of the catalytic activity (Finnin et al. 1999). HDACs are often recruited in complexes for co-repression and chromatin remodelling, with some interaction with DNA methylation machinery. For example, the recruitment of HDAC complexes to specific DNA loci by MBD proteins that recognise methylated DNA (de Ruijter et al. 2003).

Histone methyltransferases (HMTs) target lysine residues within histone tails for mono-, di- and tri- methylation in addition to arginine residues for monomethylation or symmetric/asymmetric dimethylation. There are three enzyme families, the PMRT1 family, SET-domain-containing family and the non-SET-domain family (Martin & Y. Zhang 2005). Compared to histone acetylation, methylation events are not only considered more stable, but the variety of methylation opportunities is believed to facilitate additional control options such as short and longer chromatin imprint durations (Lachner et al. 2003). The most common lysine targets for methylation are histone H3 Lys 4, -9, -27, -79 and histone H4 Lys20.

There are two families of histone demethylases each with distinct mechanisms of actions. The LSD demethylases utilise a flavin adenine dinucleotide (FAD)-dependent amine oxidation reaction to demethylate with substrate activity limited to mono and dimethylated lysines only (Bannister & Kouzarides 2005). The second family are the JMJC (jumonji C; jumonji = cruciform in Japanese) demethylases which a catalytic JMJC domain to allow for the demethylation of a number of lysines within the histone H3 and H4 termini. A total of 30 JMJD proteins have been identified, 18 of which have demethylase activity not only towards histone proteins but also non-histone proteins such as p53, DNMT1, E2F1 and NF- κ B (Kooistra & Helin 2012).

Although dedicated enzyme families do not exist for histone-specific phosphorylation, activated signalling cascades can lead to phosphorylation events by other serine/threonine kinases on specific residues within histone tails (Baek 2011). These phosphorylation events can occur on a number of sites including histone H3T3/T6/S10/T11/S28/T45. Phosphorylation events on these residues have been implicated in chromatin condensation, transcriptional activation/repression and apoptosis (Baek 2011; Metzger et al. 2008). Serine/threonine kinases phosphorylate histone tails with some dependence on the phosphorylation status of other residues. One such example is H3S10 phosphorylation catalysed by Aurora kinase B (AurB), which then allows for the targeted phosphorylation of H3T11/T6 by CHK1 and PKC

respectively (Liokatis et al. 2012). This interdependence in the form of histone PTM “crosstalk” allows for regulatory control in the absence of specific enzymatic families focused entirely on histone phosphorylation, with the combined advantage of tethering modifications to signalling cascades that conduct complex biological functions such as mitosis and meiosis (Nowak & Corces 2004).

Histone ubiquitination occurs on histones H2A and H2B at positions K119 and K120 respectively. It is catalysed by a number of histone ubiquitin ligases including the polycomb protein RING1B, which monoubiquitinates H2A with the assistance of other RING domain proteins, RING1A and BMI1 (J. Cao & Yan 2012). Monoubiquitination of H2B is catalysed by hBRE1/RNF20 and RNF40. First discovered in yeast, the human BRE1 homolog is a E2 ubiquitin ligase capable of altering the global H2B ubiquitination level at K120, and allowing for the activation of genes in concert with transcription factors (J. Kim et al. 2005). In addition to monoubiquitination, H2A and H2AX can be polyubiquitinated by RNF8 and RNF168 at K68 to mark the sites of DNA damage (Stewart et al. 2009). Histone ubiquitination is reversed by deubiquitinating enzymes, some of which have specific activity for H2A including 2A-DUB, USP21 and BAP1, whilst USP3 and USP22 can deubiquitinate H2B in addition to H2A (J. Cao & Yan 2012).

1.6.3 – microRNAs

MicroRNAs (miRs) are a class of small non-coding RNAs (ncRNAs) with implications in human disease – particularly cancer. There are many other ncRNAs in addition to miRs, such as transcribed ultraconserved regions (T-UCRs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lincRNAs) and long non-coding RNAs (lncRNAs) all of which have a variety

of cellular functions and when deregulated can lead to a number of diseases including cancer, Alzheimer's disease and Prader-Willi syndrome (Esteller 2011).

MicroRNAs are relatively well characterised as small RNA molecules of approximately 22 nucleotides in length with the primary function of post-transcriptional gene regulation via direct interaction with messenger RNA molecules. These interactions usually occurring in the 3'UTR lead to a number of outcomes including endonucleolytic cleavage at the mRNA:miRNA duplex binding site, translational repression by blocking of translational initiation or deadenylation, and mRNA turnover effects by decapping followed by 5' to 3' decay (Ameres & Zamore 2013). Due to these functions miRs are generally considered negative gene expression regulators or attenuators of their targets.

Biogenesis of miRs begins in the nucleus, where miR transcripts are generated by RNA polymerase II and undergo normal capping and polyadenylation (Fig. 1.5). These transcripts can originate from separate transcriptional units or from the introns of protein coding genes, which can give rise to clusters or single miRs (Carthew & Sontheimer 2009). The transcripts form double stranded RNAs called pri-miRNA, which are much larger than mature miRs. These pri-miRNAs are subjected to enzymatic processing by Drosha, an RNase III enzyme and DGCR8, a double stranded RNA binding protein also known as Pasha, leading to the formation of pre-miRNAs of approximately 60 nucleotides in length. Drosha can also excise multiple pre-miRNAs from the double stranded pri-miRNA molecule, allowing for the processing of polycistronic miRNAs, whilst DGCR8 works with Drosha to improve processing by increasing affinity for the substrate and cleavage accuracy. (Y. Lee et al. 2003). Once the pre-miRNA is formed, a nuclear transport receptor called Exportin 5 detects the stem and the ends of the molecule and exports it to the cytoplasm.

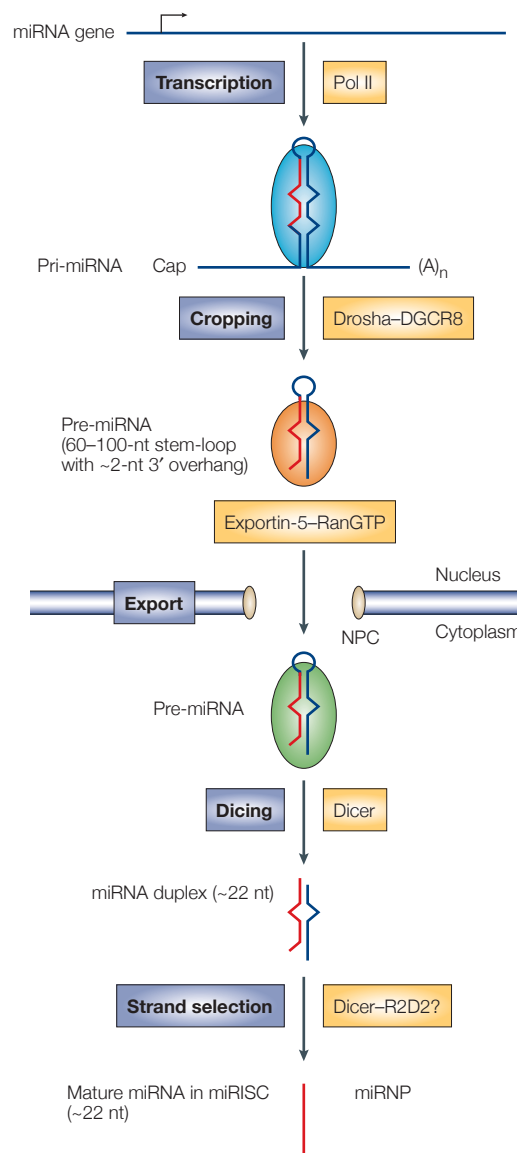


Figure 1.5: microRNA biogenesis
 A model outlining microRNA biogenesis, which begins with a microRNA transcript processed initially in the nucleus by Drosha-DGCR8 before export to the cytoplasm for further enzymatic processing by Dicer and accessory proteins. This then leads to the formation of a RNA-induced silencing complex (RISC) for targeting mRNA attenuation. Graphic from Kim. 2005.

Pre-miRNAs are processed further in the cytoplasmic space by Dicer, another RNase III enzyme leading to the formation of an miRNA:miRNA duplex of 21 to 22 nucleotides in length (V. N. Kim 2005). Other proteins bind to Dicer to assist in its processing of pre-miRNAs, such as loquacious (Loqs) in fruitflies or TRBP in mammals, allowing for adjustment of cleavage points with the molecule (Chendrimada et al. 2005). Once enzymatic processing has ceased, the miRNA:miRNA duplex is loaded into an argonaute (AGO) protein, leading to the formation of an RNA-induced silencing complex (RISC). RISC can then bind to mRNAs leading to attenuation of the message as a form of post-transcriptional gene regulation.

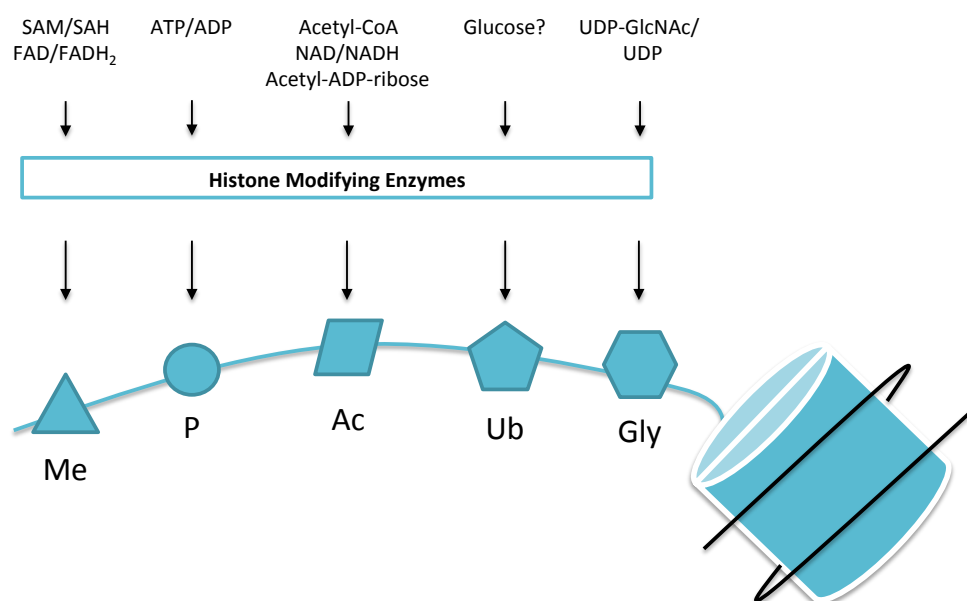
MicroRNAs are believed to play significant roles in cancer, with some microRNAs being classified as tumour suppressors or oncogenes and broadly referred to as oncomirs (Esquela-Kerscher & Slack 2006). The models proposed by Esquela-Kercher & Slack, state that miRs can attenuate the message of oncogenes, thereby acting as tumour suppressors, or aberrantly inhibit the translation of tumour suppressor mRNAs, thereby acting as oncogenes.

1.7 – Epigenetics: A Suitable and Challenging Approach to Interrogate Arsenic-induced Carcinogenesis

The examination of arsenic-induced cellular transformation through the scope of epigenetic mechanisms has gained considerable traction, with an increasing level of research overlap between the disciplines of mechanistic toxicology, molecular epidemiology and epigenetics. Some of the rationale for this is described below.

Cellular metabolic states are believed to influence chromatin structure. This is due to the behaviour of histone modifying enzymes that are reliant on key metabolites as part of catalytic processes. For example, a global reduction in nuclear acetyl-CoA leads to the reduction of

histone acetylation, whereas the reduction of NAD⁺ results in the inhibition of histone deacetylation (Katada et al. 2012; Wellen et al. 2009; Nakahata et al. 2009). As a result, histone-modifying enzymes represent a platform through which metabolic status can have an impact on histone PTMs (Fig. 1.6).



+ **Figure 1.6: Chromatin modifying enzymes as a sensor of cellular metabolism.** Histone post-translational modifications are represented schematically. Histone-modifying enzymes utilise a wide range of cellular metabolites, hence any significant disruptions to the levels of such metabolites may have consequences on the post-translational status of histone tails. Adapted from Katada *et al.*, 2012.

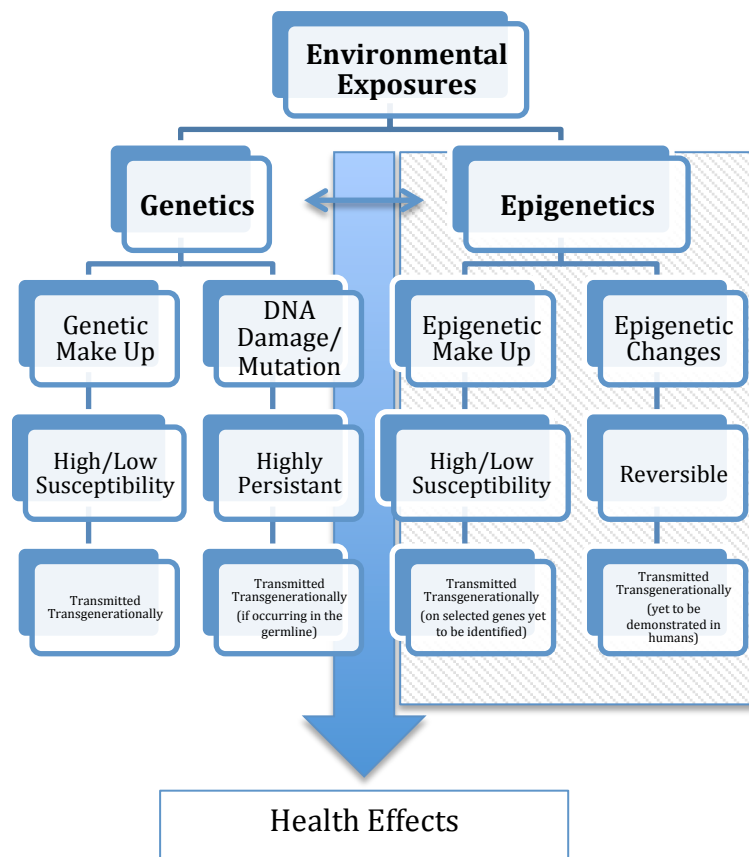
The varied kinetics of epigenetic marks across histone termini allow for both rapid changes as well as slow sticky post-translational modifications which respond to environmental stimuli. Some of these remain long enough to allow for the propagation of gene expression patterns to the next generation (Barth & Imhof 2010). These fast and slow marks allow for the codification of information from environmental exposures, such as toxicants, which may exist as a response with either benign or deleterious consequences (Watson & Goodman 2002). Environmental factors can not only influence gene expression, but also accumulate epigenetic changes

throughout a lifetime exposure from the early intra-uterine environment onwards. This accumulation of epigenetic marks then leads to an aberrant phenotype (Barros & Offenbacher 2009).

Another consideration is the fact that low-level chronic arsenic exposure can lead to cancer phenotypes. Cancer itself can occur from the deregulation of epigenetic processes. Aberrant DNA methylation is a classical contributor, as tumour cells have broadly hypomethylated genomes in addition to locus-specific hypo/hypermethylation events on genes that regulate the cell cycle, tumour cell invasion, DNA repair, chromatin remodelling, transcription and apoptosis (Robertson 2005). Unlike DNA methylation, where the range of epigenetic modifications are limited, histone modifications represent an opportunity for a significant variety of post-translational modifications and can work co-operatively in groups or antagonistically to have impacts on gene expression and thus cellular outcome (Fischle et al. 2003). Furthermore, the aberrant expression of histone modifying enzymes such as HDACs have been reported in gastric, prostate, breast and colon cancers (Ropero 2007).

Some toxicologists and those examining environmentally derived diseases have tried to consider how an environmental exposure or stimulus is communicated to a health outcome. Epigenetics has been implicated in such models, which broadly attempt to simplify the combination of effects that lead to complex diseases such as cancer and mental health disorders. One proposal called the “mixed epigenetic and genetic and mixed *de novo* and inherited” (MEGDI) model attempts to integrate variations of both *de novo* and inherited epigenetic and genetic varieties into an overall summation that leads to a complex disease outcome (Y.-H. Jiang et al. 2004). A relatively recent study attempted to address this issue in a similar way by presenting a dichotomous mechanistic approach between environmental exposure and health outcomes by nominating genetics and importantly epigenetics as the link between the two (Fig. 1.7) (Bollati & Baccarelli 2010). Some researchers have clarified this model by suggesting that epigenetic processes consisting of

chromatin and associated modifications, serves as an intermediary between the changing environment and the inherited genome (Szyf 2007). In the related field of toxicogenomics, some studies have already begun to routinely analyse epigenetic modifications as a result of environmental toxicant exposure (Reamon-Buettner et al. 2008).



+ **Figure 1.7: A model of genetic and epigenetic paths leading from environmental exposure to health outcomes.**
In this model, exposure to environmental toxicants leads to health effects through two pathways – a genetic pathway and an epigenetic pathway. Adapted from Bollati & Baccarelli, 2010.

There are however some challenges with using epigenetic approaches. For the most part, existing studies have predominantly focused on DNA methylation markers at a limited number of loci, without broader interrogations to characterise multiple interactions at multiple loci (Verma et al. 2014). Furthermore most cancer epidemiology epigenetic studies have utilised blood samples,

which in the context of arsenic-induced cellular transformation, may not be the most ideal biological specimen. With regards to the challenges, epigenetic mechanisms are fundamental for the maintenance of cellular identity, which means cells have different epigenetic backgrounds and responses following exposure to an environmental toxicant. Additionally, there is considerable impetus to understand the degree of causality between an epigenetic mark and disease, a requirement to understand the duration of exposures, the design of new tests to interrogate epigenetic inheritance and the appropriate separation of confounding factors (Marsit 2015).

1.8 – Arsenic-induced Perturbations of DNA methylation and Associated Outcomes.

Arsenic exposure leads to the disruption of nominal DNA methylation patterns with repercussions on gene regulation, chromatin stability, cancer development and progression. Both DNA hyper- and hypo- methylation events have been reported following arsenic exposure, with a number of proposed mechanisms implicating alterations in DNA methyltransferases activity and/or expression, oxidative stress and arsenic-induced metabolic depletion of the SAM methyl donor (Reichard & Puga 2010).

1.8.1 – Global DNA methylation levels are affected by arsenic exposure.

Global DNA hypomethylation following arsenic exposure has been reported in human colorectal, keratinocyte and prostate cells (Bustaffa et al. 2014). There is evidence to suggest long-term arsenic exposure depletes *S*-adenosylmethionine (SAM) through metabolic processing of the toxicant and additionally suppresses the expression of DNA methyltransferases *DNMT1* and *DNMT3A* resulting in global DNA hypomethylation (Reichard et al. 2007). Genome-wide changes in DNA methylation have also been demonstrated by digesting DNA from human

keratinocyte (HaCaT) and mouse testicular cells exposed to 1 ng/ml – 1000 ng/ml sodium arsenite with restriction enzymes sensitive to DNA methylation (Singh & DuMond 2007). The result is a DNA fingerprint that changes depending on the magnitude of the sodium arsenite dosage.

The picture is not entirely clear though as global DNA hypermethylation has also been reported following arsenic exposure. In a study of 320 adults, a dose-response relationship was identified correlating water, blood and urinary arsenic with global DNA methylation in peripheral blood mononuclear cells (PBMCs) (Niedzwiecki et al. 2013). Global DNA hypermethylation has also been measured within transposable genomic elements following arsenic exposure. In a study composed of 581 participants, a significant increase in DNA methylation was identified in *Alu* elements, which contribute to 11% of the genome (Lambrou et al. 2012; Deininger 2011). Paradoxically, global hypomethylation was also identified in long interspersed elements – LINE-1, especially in males increasing the likelihood of genomic instability and cancer due to the altered activity of these transposable elements (Lambrou et al. 2012).

These studies suggest that there are some challenges with investigating arsenic-induced global DNA methylation patterns. In addition to canonical DNA methylation, global DNA hydroxymethylation levels are adjusted following arsenic exposure. Furthermore, both DNA methylation measurements have been shown to be correlated with each other in blood samples taken from arsenic exposed individuals suggesting that one could be a proxy measurement of the other and vice-versa (Tellez-Plaza et al. 2014). Tellez-Plaza *et al.* were able to report differences in DNA methylation and hydroxymethylation in individuals with differing % measurements of urinary arsenic metabolites including DMA, MMA and iAs, effectively opening a debate about which measurement is the more effective biomarker. This discussion is particularly significant as differences in the % of arsenic metabolites have been attributed to genetic variations in genes encoding for methyltransferases *PRDM9*, *EHMT1* and *AS3MT* (Tellez-Plaza et al. 2013).

1.8.2 – Promoter-specific DNA methylation effects following arsenic exposure.

Whilst the notion of arsenic-induced global hypomethylation revolves around the suggestion that SAM is depleted after long-term arsenic exposure, there is significant evidence of promoter-specific DNA hypermethylation and hypomethylation occurring independently of global DNA methylation levels (Table. 4).

+ **Table 4: Gene promoter-specific DNA methylation aberrations following arsenic exposure.**

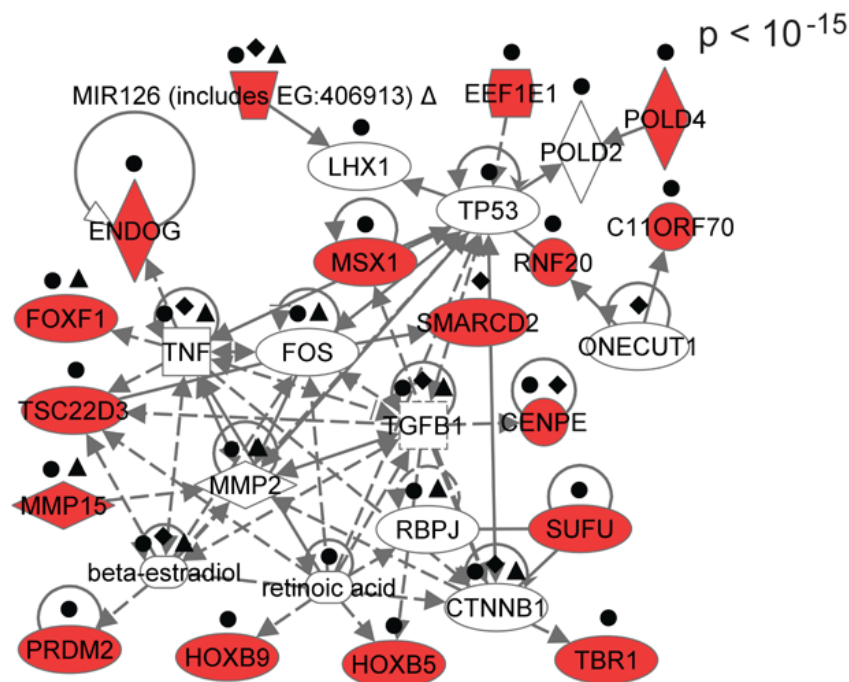
Direction of DNA methylation	Study Type	Arsenic Exposure	Gene	Annotations	Reference
↑ Hypermethylation	Molecular epidemiology	Drinking water (>10µg/l)	<i>DAPK, p16</i>	Downregulated, in arsenic induced skin lesions and cancer tissues.	(Banerjee et al. 2013)
	Molecular epidemiology	50 – 1000 µg/L arsenic in drinking water	<i>TP53, P16</i>	DNA hypermethylation of genes important in carcinogenesis.	(Chanda 2006)
	<i>In vitro</i>	100 ng/mL arsenic	<i>MLH1</i>	Silencing of MLH1 lead to increased cell growth, decreased apoptosis and cellular transformation.	(Treas et al. 2013)
	<i>In vitro</i>	5 µM sodium arsenite	<i>ZNF</i> genes	Silencing of <i>ZNF</i> genes in arsenite-induced cellular transformation	(Severson et al. 2013)
	<i>In vitro</i>	1 µM sodium arsenite	<i>LET-7C</i>	Leads to cellular transformation through disruption of Ras/NF-KB pathway.	(R. Jiang et al. 2014)
	<i>In vitro</i>	1 µM As(III) and 50 nM MMA(III)	<i>DBC1, FAM83A, ZSCAN, C1QTNF6</i>	Leads to cellular transformation.	(Jensen et al. 2008)

Table 4 cont...					
↓ Hypomethylation	Molecular epidemiology	Urinary: <7.2 µg/L (low) and > 14.0 µg/L (moderate)	<i>AS3MT</i>	Involved in arsenic metabolism.	(Gribble et al. 2014)
	Molecular epidemiology	Drinking water: 4.37 µg/L, Urine: 18.62 µg/L, Nail: 0.21 µg/g, Hair: 0.13 µg/g. (mean)	<i>ERCC2</i>	Involved in DNA repair.	(Paul et al. 2014)
	Molecular epidemiology	Urine: 1.4 ng/mL (mean)	<i>GAD1, INS, PDX1, SOCS6</i>	These genes are implicated in diabetes mellitus.	(Bailey et al. 2013).
	<i>In vitro</i>	0.08 µM sodium arsenite (low), 0.4 µM sodium arsenite (intermediate), 2 µM sodium arsenite (high)	<i>TP53</i>	Leads to disruption of a network of p53 controlled genes, leading to the differential expression and carcinogenesis.	(van Breda et al. 2014)
	<i>In vitro</i>	0.5 µM sodium arsenite	<i>LCN2</i>	Leads to cellular transformation.	(H.-H. Wang et al. 2014)

In the characterisation of promoter-specific DNA methylation patterns, the research agenda has remained focused on the promoters of genes that stimulate or control cellular transformation. In one such study, PBMCs were extracted for DNA methylation analysis from 72 individuals exposed to environmentally significant levels of arsenic. DNA hypermethylation was identified in the promoter regions of *DAPK* and *p16* genes, leading to a 3.4 fold and 2.2 fold decrease in expression respectively (Banerjee et al. 2013). In the same study, a similar reduction in the expression of these genes was identified in arsenic-induced skin lesions and cancer tissues taken from the study site participants. Low-dose sodium arsenite exposure also leads to cellular transformation of human urothelial cells. These cells exhibit DNA hypomethylation at the lipocalin *LCN2* promoter (H.-H. Wang et al. 2014). This occurs in concert with the binding of

the RelA and NF- κ B1 at the hypomethylated promoter, resulting in *LCN2* overexpression, anchorage-independent growth, resistance to serum deprivation and activation of NF- κ B signalling. Chronic arsenic exposure in mouse testicular leydig cells have also demonstrated the increased expression of genes associated with cellular proliferation such as *Pcna*, *CyclinD1* and *Dnmt1*, the latter gene encoding for DNA methyltransferase 1 (DuMond & Singh 2007).

DNA hypermethylation has also been identified at the promoter of the canonical tumour suppressor gene *TP53*, particularly in individuals exposed to arsenic drinking water concentrations of <50 μ g/l and 1000 μ g/l, suggesting that even a very low exposure can lead to aberrant DNA methylation (Chanda 2006). Chanda *et al.* also identified a subgroup of individuals exposed to the higher 100 μ g/l concentration of arsenic that exhibited DNA hypomethylation at the *TP53* gene locus, alluding to the presence of hyper-to-hypo methylation switch depending on the magnitude of arsenic exposure. Furthermore, DNA hypermethylation at the *TP53* locus was observed in people without malignancies giving some weight to its potential as an early biomarker. Treatment of human adenocarcinoma lung (A549) cells with low-level sodium arsenite also led to the differential methylation of a number of genes which were arranged in an interaction network impinging on *TP53* (van Breda *et al.* 2014). This type of “big-data” approach was also applied to 16 individuals diagnosed with arsenicosis exposed to arsenic through drinking water. In this study PBMCs were subjected to genome-wide methylation analysis followed by interaction mapping of genes that were differentially methylated (Smeester *et al.* 2011). This not only led to the identification of numerous genes that were hypermethylated and implicated in arsenic-induced diseases such as cancer, cardiovascular disease and diabetes, but also identified a complex of 17 tumour suppressors that were silenced by DNA methylation (Fig. 1.8).



+ **Figure 1.8: Inorganic arsenic-induced tumour suppressorome.**
Red symbols represent the products of hypermethylated genes; clear symbols represent other proteins that associate with these hypermethylated products to form an interaction network. Taken from: (Smeester *et al.*, 2011)

Similar studies have examined DNA methylation at the promoter-specific level allowing for the identification of large numbers of genes with differential DNA methylation patterns. Human uroepithelial (SV-HUC-1) cells exposed to 0.5 μM iAs, 0.05 μM – 0.2 μM MMA(III) and 0.2 μM – 0.5 μM DMA(III) led to the identification of 114 genes with differential DNA methylation patterns (Su *et al.* 2006). This methylation profile across the 114 genes was also similar to the transformed variant of the cell line, suggesting its importance in cellular transformation.

Impaired DNA repair mechanisms are also believed to play a role in arsenic-induced carcinogenesis. In a sample of 157 individuals, water, urine, nail, hair and blood samples were taken to assess the extent of arsenic exposure (Paul *et al.* 2014). Those with the highest arsenic exposures, as determined by dermatological lesions, exhibited DNA hypomethylation at the excision repair cross-complementation group 2 – *ERCC2* promoter. This was validated as a

dose-dependent effect in HepG2 and HEK293T cells, leading to a two-fold increase in *ERCC2* expression with an increase in micronuclei frequency, a proxy for genotoxic damage. Further assays were able to demonstrate that the DNA proofreading and repair complex, TFIID is regulated by *ERCC2*, which if overexpressed, suppresses the initiation of DNA repair at sites of DNA damage. This is a particularly significant finding, as it provides a mechanism for low-level arsenic-induced genotoxicity as a result of arsenic exposure, even though arsenic is itself considered a poor inducer of DNA double strand breaks at short 24 hour exposures *in vitro* (Mei et al. 2003).

Variations in DNA methylation have also been connected to arsenic metabolism. Genetic variation of the *AS3MT* gene leads to differential methylation on a number of other target genes including *C10ORF26*, *USMG5* and *TRIM8*, which suggests that *AS3MT* haplotypes can not only predict methylation outcomes on other genes, but also that these genes may have an impact on arsenic metabolism (Engström et al. 2013). Furthermore, promoter methylation at the *AS3MT* locus in 48 participants was shown to vary with urinary arsenic levels. Those with higher arsenic exposures, exhibited significantly higher levels of hypomethylation at the *AS3MT* promoter allowing for the increased expression of the gene (Gribble et al. 2014). This suggests the presence of an epigenetic throttle for arsenic metabolism, depending on the extent of exposure.

Arsenic exposure has undesirable effects on DNA methylation in concert with the steroid hormone oestrogen. In a unique study that examined combined arsenic and oestrogen exposure, DNA hypermethylation was observed at the *MLH1* promoter at a greater level than the individual treatments between the toxicant and hormone (Treas et al. 2013). This study was conducted after initial experiments that examined global DNA methylation and histone acetylation patterns both of which are altered following combined treatment (Treas et al. 2012). The identification of DNA hypermethylation at the DNA mismatch repair gene *MLH1* promoter was particularly significant as it diminished an MLH1-mediated apoptotic response

after arsenic exposure in human prostrate epithelial cells and allowed for cellular transformation. This study provides an insight into aberrant DNA methylation patterns that may be exacerbated by individuals on hormonal therapies whilst exposed to arsenic.

Other promoter-specific DNA methylation studies are not as easy to categorise or are not specifically relevant to carcinogenesis. In a molecular epidemiology study of 400 individuals exposed to arsenic contaminated drinking water, aberrant DNA methylation patterns were identified in white blood cells at the following loci: *PLA2G2C*, *SQSTM1*, *SLC4A4*, and *IGH* (Argos et al. 2014). In this study, the authors state that these genomic loci should be examined further as they may inform of novel pathways. Another genome-wide study conducted on a population from an arsenic contaminated region of Mexico, identified promoter-specific hypomethylation in circulating peripheral blood leukocytes at *GAD1*, *INS*, *PDX*, and *SOC56* (Bailey et al. 2013). Some of these individuals exhibited symptoms related to diabetes.

1.8.3 – DNA methylation effects following *in utero* arsenic exposure.

DNA methylation is fundamental to the programming of functional genomic states, which is under timely regulation for mammalian development. As a result of this, a number of studies have focused on characterising the effects of *in utero* arsenic exposure upon DNA methylation to determine if such exposures predispose new-borns to specific illnesses.

In a study by Pilsner *et al.* global DNA methylation patterns were examined from cord blood taken alongside maternal blood and maternal urinary arsenic measurements. An increase in maternal urinary arsenic concentration was associated with an increase in DNA methylation (Pilsner et al. 2012). These increases in methylation were measured by a luminometric methylation assay and were found specifically in *Alu* and LINE-1 repetitive genomic regions.

Prenatal arsenic exposure has led to the identification of gender-specific effects, with adverse DNA methylation in cord blood affecting boys in particular (Broberg et al. 2014). In boys, 74% of CpG islands were hypomethylated compared to 41% in girls. Long-term arsenic exposure *in utero* also alters the distribution of DNA methylation at 18% of the 385,249 CpG loci examined in lymphocytes, monocytes and granulocytes (Koestler et al. 2013).

Whilst these reported DNA aberrations are considered “global”, another study examined the effect of *in utero* arsenic exposure on the methylation status of genes involved in the infectious disease response (Rager, Yosim, et al. 2014). Through the cross-comparison of two genomic data sets, an infectious disease gene (IDG) database and an arsenic exposure responsive gene (ERG) database, a total of 82 genes were identified with differential DNA methylation patterns following arsenic exposure in genes specifically involved in regulating a response against bacterial, viral and parasitic infections. These patterns were observed in PBMCs from pregnant women and compared against urinary and nail arsenic measurements.

Some mothers take dietary supplements during pregnancy in arsenic contaminated areas. To examine the combinatorial effect of folate supplements and arsenic exposure, pregnant mice were exposed to inorganic arsenic and folate simultaneously. The combined exposure led to significant methylation changes of 2,931 genes within tissue samples taken from foetuses, including those associated with neurodevelopment, cancer, cell cycle and signalling (Tsang et al. 2012). Furthermore, combined folate and iAs exposure reduced foetal weight, compared to single exposures suggesting the presence of compromised foetal development which is itself a risk factor for early-onset disease. This is a particularly significant finding as arsenic exposure and low birth weight have been previously examined in epidemiological studies, but this is the first study which demonstrates additional dietary supplements believed to be benign having an adverse effect (Guan et al. 2012; Hopenhayn et al. 2003; Huyck et al. 2007).

1.9 – Arsenic-induced Changes of Histone Post-translational Modifications.

Unlike DNA methylation, a lot less is understood about the effects of arsenic on higher order chromatin structure. Some studies have been able to identify changes on histone PTMs following arsenic exposure including aberrations in acetylation, methylation and phosphorylation.

1.9.1 – Arsenic-induced effects on global histone post-translational modifications.

One of the first examples of aberrant histone acetylation and methylation following arsenite treatment came from an experiment using cultures of *Drosophila melanogaster* (common fruit fly). Treatment with 50 μ M sodium arsenite for 4 hours led to deacetylation and blocked methylation of the core histones H3 and H4 (Arrigo 1983). Although the author did not speculate on a mechanism for this effect, this study was significant as a “proof of concept” – that arsenic exposure does indeed have an effect on histone acetylation and methylation. In contrast, sodium arsenite treatment of hepatocarcinoma (HepG2) cells led to a significant increase in global histone acetylation (Ramirez et al. 2007). In this study global hyperacetylation was attributed to a HDAC inhibitory effect of sodium arsenite as similar changes were observed in cells treated with TSA – a HDAC inhibitor. Although in this study a comparison was made with TSA to imply the inhibitory effects of arsenic exposure, a more focused study was unable to reproduce a similar result. Ge *et al.* demonstrated global hypoacetylation in UROtsa cells treated with iAs and MMA(III), whilst a prolonged 12-week exposure led to increased expression and activity of HDACs (Ge et al. 2013). This global histone hypoacetylation led to malignant transformation after a 4-week arsenic exposure, which could be reversed by treatment with the HDACi SAHA.

Other studies have been able to identify specific changes in histones by examining specific residues with clinically relevant environmental exposures. Global histone acetylation and

methylation changes have been identified in circulating PBMCs. In a sample of 40 participants exposed to arsenic contaminated water in Bangladesh, total urinary arsenic concentrations were correlated with H3K9me2 and inversely correlated to H3K9ac (Chervona et al. 2012). Water arsenic concentrations were positively correlated with H3K4me3 and H3K27me3 among females, but negatively in males, leading to the suggestion that differential effects can occur depending on the gender of individuals exposed. Another study examined *in utero* exposure of arsenic by exposing mice to 100 µg/L arsenic in drinking water 1 week prior to conception (Cronican et al. 2013). This study allowed for the identification of global H3K9 hypoacetylation in the cortices and hippocampi of newborn pups, suggesting that *in utero* exposure can alter global histone acetylation and with the potential for cognitive impairment (Cronican et al. 2013). Understanding the significance of the direction of these changes on specific residues is still a challenge. In a similar study, opposite effects were observed by examining blood leukocytes from steel workers exposed to inhalable arsenic compounds. This led to an increase in H3K4me2 and H3K9ac proportional to the number of years of employment (Cantone et al. 2011).

Additional observations have been reported through the use of relevant *in vitro* models. Quantitative mass spectrometry revealed a decrease in H4K16 acetylation following 1 nM – 10 µM As(III) treatment for 24-hours in the UROtsa cell line (Chu et al. 2011). This study also presented evidence to suggest that the modifications are much more pronounced in the globular regions of histone H3, such as K18 and K23, over tail regions such as K9 and K14. A reduction in histone acetylation at histone H4K5, K8, K12 and K16 was also reported. The strength of this study is the variety of lysines examined and the different histone environments that these lysines represent. Although this study did not examine the toxicity outcome of the short 24-hour exposure, the reduction in H4K16 acetylation observed concurs with prior research. In the study by Jo *et al.* H4K16 was not only reduced, but it was identified as the substrate of MYST. Furthermore, silencing of this enzyme induced sensitivity to As(III) and MMA(III) suggesting

that H4K16 acetylation is required for treatment resistance (Jo et al. 2009). This would suggest that H4K16 hypoacetylation is an effect that ultimately leads to toxicity.

Exposure to tolerated As₂O₃ in BALB/c 3T3 cells not only led to cellular transformation and tumour generation in nude mice, but also a time-dependent increase in H3K27 trimethylation mediated by polycomb proteins BM1 and SUZ12 (H.-G. Kim et al. 2012). SiRNAs against messages for these enzymes suppressed arsenic trioxide induced cellular transformation. Arsenite exposure leads to an increase in histone H3S10 phosphorylation in a time and dose-dependent manner in mouse epidermal JB6 CI41 cells (Z. He et al. 2003). He *et al.* also demonstrated through the use of mutations and inhibitors that H3S10 phosphorylation is mediated by AKT1, extracellular signal regulated kinase 2 and P90 ribosomal S6 kinase 2.

1.9.2 – Arsenic-induced histone post-translational modification effects at promoter loci.

There is a distinct lack of information regarding promoter specific histone PTM effects following arsenic exposure. Treatment of CAsE-PE cells with 5 µM sodium arsenite for 37 weeks led to no significant change in DNA methyltransferase expression, and no significant changes in DNA methylation at the *KRAS* gene, and yet an increase in *KRAS* expression was observed (Benbrahim-Tallaa et al. 2005). This study demonstrated that other modifications on chromatin at specific promoter loci might influence gene expression, independent of DNA methylation.

Prior to this, histone H3 phosphoacetylation (Ser(P)-10/Ac-Lys-14) had been reported at the *CFOS* loci, but the exposure period was only for 60 minutes with a 400 µM arsenite exposure with no clear information on the cellular outcome (J. Li et al. 2003). Recent studies have used longer durations with lower concentrations. For example, promoter-specific acetylation changes in histone H3 were identified in cells exposed to As(III) and MMA(III) for 52 weeks leading to

malignant transformation. Hypoacetylation of histone H3 at the *DBC1*, *FAM38A*, *ZSCAN12* and *C1QTNF* gene loci was correlated with a reduction in expression (Jensen et al. 2008). Additionally these genes were found to be targets of increased DNA methylation, especially in the cells lines malignantly transformed by exposure. Promoter-specific histone acetylation changes can show increasing levels of complexity as highlighted by a study of histone modifications at the *MT-3* promoter in UROtsa cells transformed by exposure to As(III). Transformed cells exhibit an increase in histone H4 acetylation and histone H3K4 methylation, both transcriptionally active marks, but also an increase in histone H3K9 and H3K27 methylation, transcriptionally repressive marks. The investigators propose the idea that chromatin is in a bivalent state at the *MT-3* promoter, ready to switch between an expressive or repressive state. In full urothelial cancer, *MT-3* is present but absent in the normal cells (Somji et al. 2011). H3K9 hypoacetylation was also identified in the promoter regions of genes encoding for Krüppel associated box (KRAB) transcription factors following *in utero* arsenic exposure (Cronican et al. 2013). Inorganic arsenic induced histone H3K18 acetylation reduction co-occurs with a reduction in histone H4R17 methylation, this is achieved by disrupting the recruitment of co-activator associated arginine methyltransferase CARM1, leading to transcriptional repression of GR-regulated genes (Barr et al. 2009). This suggests that arsenic exposure may affect steroid hormone receptors via aberrations in transcriptional control.

1.9.3 – Arsenic-induced interference of histone modifying enzymes and proteins that regulate chromatin structure.

Arsenic exposure also interferes with the function of histone modifying enzymes and proteins that bind intimately to chromatin to regulate gene expression. HaCaT cells exposed to 1 – 200 μ M sodium arsenite exhibit perinuclear localisation of HDAC6 within 30 minutes of exposure along with physical interactions with ribosomes (Kappeler et al. 2012). This finding suggests that

HDAC6 is involved in responding to short-term arsenic-induced stress by regulating *de novo* protein translation. Kappeler *et al.* also demonstrated that the transcription factor NRF2, which exhibits elevated expression following oxidative stress, was similarly elevated following arsenite exposure, but treatment with Tubastatin A (TA) – a HDAC6 specific inhibitor, led to a reduction in NRF2 protein expression but not mRNA expression. This added evidence that HDAC6 is affecting *de novo* protein translation of NRF2. In addition to an inhibited stress response, arsenic exposure also leads to negative impacts on DNA repair. A recent study examined the function of RING finger E3 ubiquitin-ligases, which assist DNA repair mechanisms by ubiquitination of histone H2BK120. Cells treated with arsenite led to toxicant binding at the RING finger domain and impaired H2B ubiquitination (F. Zhang et al. 2014). This targeted interaction occurred at cysteine residues within the ubiquitin-ligases, leading to compromised DNA repair and a potentially carcinogenic outcome. DNA instability can also arise from an alteration in histone metabolism. Arsenic exposure has been shown to increase polyadenylation of the canonical histone H3.1 mRNA leading to altered histone mRNA metabolism (Brocato et al. 2014). The authors postulate that this is due to the downregulation of the stem-loop binding protein (SLBP) which binds to the 3' end of the histone mRNA to alter its metabolism. Excess canonical histone expression leads to the elevation in sensitivity to DNA damage and genomic instability, both of which are favourable for carcinogenesis.

1.10 – Disruptions in MicroRNA expression Following Arsenic Exposure and Implications.

Both DNA methylation and the post-translational modifications of histones are considered pre-transcriptional regulatory mechanisms. In contrast, microRNAs represent a significant opportunity for post-transcriptional control. A recent study has identified significant changes in gene expression following 1 μ M sodium arsenite exposure, due to post-transcriptional regulatory

mechanisms affecting mRNA stability (Qiu et al. 2014). The differential expression of microRNAs has been reported in a number of contexts following arsenic exposure including carcinogenesis and cytotoxicity (Table. 5).

+ **Table 5: Differential expression of microRNAs following arsenic exposure in carcinogenic and treatment contexts.**

Context	Toxicant	microRNA	Direction	Model	Reference
Carcinogenesis	Sodium arsenite	miR-22	↑	Human immortalised lymphoblast (TK6)	(Elamin et al. 2011) (Marsit et al. 2006)
		miR-34a	↑		
		miR-221	↑		
		miR-222	↑		
		miR-200b	↓	p53-knocked down human bronchial epithelial cells (P53 low HBECS)	(Z. Wang et al. 2011) (Z. Wang et al. 2014)
		miR-222	↑	Human immortalised lymphoblast (TK6)	(Ling et al. 2012)
		miR-21	↑	Human embryo lung fibroblast (HELFI)	
Cytotoxic treatment	Sodium arsenite	miR-181b	↓	Fertilised white leghorn chick eggs	(Cui et al. 2012)
		miR-9	↓		
	Arsenic trioxide	miR-222	↑	Human bladder carcinoma cells (T24)	(Y. Cao et al. 2010)

Human immortalised lymphoblast (TK6) cells exposed to 2 µM sodium arsenite led to the differential expression of a number of miRs including miR-22, -34a, and -221 (Marsit et al. 2006). This study also examined the effect of metabolic stress in the form of folate-deficiency by treating cells with sodium arsenite in folate-deficient RPMI growth medium. Under this circumstance miR-222 was increased, an effect that was also verified with an *in vivo* study. This

was a particularly significant finding as miR-222 has been implicated in cellular differential pathways of various blood cells. By using a similar treatment of 2.5 μ M sodium arsenite, p53-knockdown-human bronchial epithelial cells (HBECS) became malignantly transformed (Z. Wang et al. 2011). This effect also correlated with the significantly reduced expression of miR-200b. Overexpression of miR-200b via a stable transfection was able to counteract the transformative effect of sodium arsenite. A follow up study demonstrated that miR-200b attenuated PKC α which in turn diminishes Rho GTPase RAC1 activation (Z. Wang et al. 2014). As a result cytoskeletal reorganisation and cell migration is impeded, both of which are contrary to cellular transformation behaviours.

The differential expression of miRs following arsenic exposure has been implicated in pathways previously shown to have roles in arsenic-induced carcinogenesis, for example intracellular ROS and cell cycle control. In a study using human embryo lung fibroblasts (HELFL) treated with 1 μ M sodium arsenite, NF- κ B-mediated transcriptional transactivation of the miR-21 promoter was identified (Ling et al. 2012). Furthermore, the activation of NF- κ B itself was dependent on the activation of ROS sensitive extracellular signal-regulated kinase (ERK). Arsenic exposure impinges on cell cycle control via the deregulated expression of miR-2909 (Sharma et al. 2013). Sharma *et al.* found a regulatory network in which miR-2909 regulates cyclin D1 via KLF4 and SP1, both of which have inhibitory effects on cyclin D1. At a tolerated exposure miR-2909 is overexpressed, which leads to the attenuation of the negative regulators of cyclin D1 and deregulated cell cycle progression.

Pre-natal exposure to arsenic also has an impact on the expression of a number of miRs. New born cord blood samples were collected and maternal urinary arsenic (U-tAs) measurements were conducted allowing for the identification of 12 miRs including miR-126, -16, -17, -96, which demonstrated increased expression in positive correlation with U-tAs (Rager, Bailey, et al. 2014).

Pathway analysis was able to show that these miRs were involved in signalling pathways with consequences in cancer, diabetes and the regulation of innate and adaptive immune responses. Aberrant miR expression has been identified in fertilised chicken eggs injected with 100 nM sodium arsenite. By using a microarray the expression of a number of miRs were evaluated, including miR-181b and miR-9 both of which were significantly decreased (Cui et al. 2012).

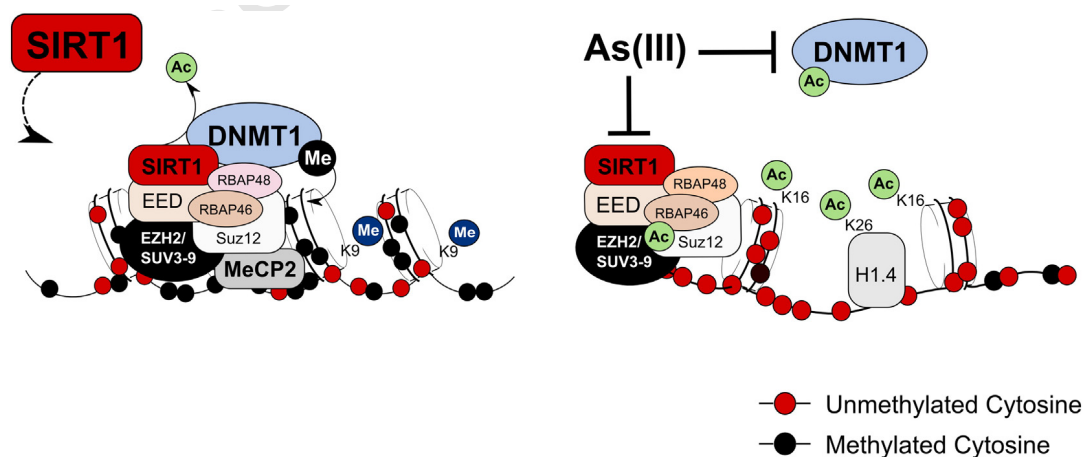
1.11 – Combined Epigenetic Effects Following Arsenic Exposure.

Arsenic exposure leads to the simultaneous disruption of DNA methylation, histone PTMs and miR expression with deleterious outcomes. Combinatorial effects are sometimes referred to as crosstalk events, where multiple epigenetic events interact with some independence. However this notion is still not a solidified idea or principle. Winter *et al.* has attempted to describe epigenetic crosstalk events as those that either positively or negatively regulate the impact of a subsequent epigenetic event, such as histone post-translational modification leading to the repulsion or enhancement of proteins at the inter- or intra- nucleosomal level (Winter & Fischle 2010). Similar discussions have focused on the interdependence of histone PTMs (N. Lee & Y. Zhang 2008; J.-S. Lee et al. 2010). Whilst these reviews focus on a much more molecular interpretation of crosstalk, there is benefit in considering crosstalk across the epigenetic landscape such as DNA methylation events altering the expression of miRs, or histone PTMs affecting the extent of methylation at specific promoters and indeed such examples exist in the context of arsenic-induced cellular transformation.

Human prostate epithelial (RWPE-1) cells exposed to 5 μ M sodium arsenite for 33 weeks became transformed with elevated H3K27me3 in PCDH, HOXC and HOXD gene family clusters and DNA hypermethylation at the same loci (Severson et al. 2012). Chronic-arsenic-exposed human prostate epithelial cells (CASe-PE) also exhibited DNA hypermethylation at

genes marked with H3K27me3 (Severson et al. 2013). Severson *et al.* demonstrated that normal immortalised cells have elevations in H3K4me3 at the 5' end of the genes encoding for C2H2 zinc finger (ZNF) proteins and H3K9me3 at the 3' end. However, this changes in malignantly transformed cells, where H3K4me3 methylation is switched to H3K9me3 at the 5' end. This leads to DNA hypermethylation and the downregulated expression of ZNF genes. Proteins encoded by ZNF genes make up one of the largest families of sequence specific DNA binding transcription factors, suggesting that these mechanisms may have significant implications for transcriptional co-activation. A number of genes believed to be related to arsenic-induced cellular transformation including *DBC1*, *FAM83A*, *ZSCAN* and *C1QTNF6* are hypermethylated along with a decrease in histone H3 pan-acetylation resulting in tight chromatin condensation and silencing (Jensen et al. 2008).

Tolerated arsenic exposure depletes microRNA *let-7c* through aberrant levels of DNA methylation. This miR is involved in the regulation of the Ras/NF- κ B signalling pathway, and its depletion leads to cellular transformation of HaCaT cells and the acquisition of cancer stem-cell like properties (R. Jiang et al. 2014). The methyltransferase inhibitor 5-aza-2'-deoxycytidine can rescue *let-7c* levels suggesting that aberrant DNA methylation is fundamental to this observation.



+ **Figure 1.9: Arsenic-induced alterations on DNA methylation and histone acetylation on the *pri-miR-34a* promoter.**
Arsenic inhibits the activity of SIRT1 leading to the disruption of histone acetylation and DNA methylation patterns. This results in the impedance of polycomb repressive group-mediated gene repression. Taken from: (Herbert *et al.*, 2014)

Human keratinocytes exposed to 0.5 μ M arsenite increases H4K16 acetylation, with chromatin remodelling at the *pri-miR-34a* promoter (Herbert *et al.* 2014). This occurs in concert with an increase in H4K16 acetylation at the *SIRT1* promoter (Fig. 1.9). Furthermore, DNMT1 is not deacetylated which has an inhibitory effect leading to a significant DNA hypomethylation at the *pri-miR-34a* promoter. This is good example of all crosstalk between histone PTMs, DNA methylation and miRs following arsenic exposure.

1.12 – Arsenic Exposure in a Therapeutic Context and Associated Epigenetic Effects.

Another impetus to investigate the carcinogenic mechanisms of arsenic is the fact that human exposure to arsenic is not limited to tolerated environmental exposures, rather human exposures also occur in a therapeutic context as arsenic is an effective cytotoxic anti-cancer therapy (Soignet *et al.* 1998). This paradoxical effect of arsenicals as a potent cancer treatment and carcinogenic

compound is still under intense investigation.

In a therapeutic context arsenic leads to epigenetic disruptions with cytotoxic endpoints. In the treatment of chronic myeloid leukaemia (K562) cells, 2 μM As_2O_3 significantly restored target methylation-associated silencing-1 - *TMS1* expression by reversing DNA hypermethylation at the promoter leading to apoptosis via the downregulation of BCL-2/BAX expression (H. Li et al. 2014). Rather than an actual reversal of DNA methylation, there is some evidence to suggest that arsenic inhibits both DNMT1 and DNMT3B protein expression in the mouse mammary cancer (EMT6) cell line (Q. Li et al. 2009).

Aberrations in histone PTMs have also been identified in a therapeutic context. Acute promyelocytic leukemia (NB4) cells exposed to As_2O_3 led to the induction of *CASPASE-10* (J. Li et al. 2002). Inhibition of CASPASE-10 with a specific inhibitor was able to attenuate As_2O_3 -induced apoptosis. Further investigation of the histone PTMs at the *CASPASE-10* locus identified an increase in H3S10 phosphorylation and H3K14 acetylation via a crosstalk mechanism. An increase in the gene-activating mark of H3K4me3 was observed in human lung carcinoma (A549) cells after treatment with 0.1 – 1 μM arsenite (X. Zhou et al. 2009). Interestingly this mark remained and was inherited through a number of cellular divisions over a period of 7 days after toxicant removal. A prior study with the same A549 cell line also identified an increase in H3K9me2 and a decrease in H3K27me3, both of which are important in gene silencing following a very low 0.1 μM sodium arsenite exposure (X. Zhou et al. 2008). Arsenic trioxide has also been shown to disrupt the serine/threonine kinase TOPK mediated phosphorylation of H2AX, which leads to apoptosis (Tatyana A Zykova & Dong 2006). Conversely the same study demonstrated that H2AX phosphorylation is required for tolerance against arsenic trioxide exposure.

Differential impacts on histone modifying enzymes following arsenic trioxide exposure have also

been identified in the treatment of multiple myeloma cells (Qu et al. 2012). Exposure to relatively low levels of As_2O_3 from 0.5 μM to 2.5 μM affected cellular survival, but only higher concentrations of 4 μM As_2O_3 and above led to an inhibition of HDAC activity in a dose and time-dependent manner, leading to an increase in α -tubulin acetylation, HSP90 acetylation and cytotoxicity.

Arsenic trioxide is believed to have some therapeutic benefit in the treatment of bladder cancer. Human bladder carcinoma (T24) cells exposed to As_2O_3 led to the differential expression of a number of miRs, including the significant reduction of miR-19a expression. Knockdown of miR-19a led to apoptosis and inhibited growth (Y. Cao et al. 2010). This research suggests that miR-19a may be an important therapeutic target in bladder cancer, which can be influenced by arsenic trioxide exposure. Arsenic trioxide treatment has also demonstrated some promise in attenuating the migration and invasiveness of human liver cancer (MHCC97H) cells via miR-491 (X. Wang et al. 2014). Wang *et al.* showed that DNA hypomethylation at the *miR-491* promoter permits an increase in its expression, and this miR targets NF- κ B leading to the blocking of mesenchymal to epithelial transition.

Hypothesis and Aims

The unified hypothesis of this thesis is stated below which has been explored through two closely related studies.

Hypothesis:

Long-term exposure to tolerated levels of As_2O_3 affects the relative activity and/or expression of HDACs, HATs, and microRNAs, leading to alterations in histone acetylation with impacts on gene expression favourable to cellular transformation.

The aims of each study are presented below and were generated to explore the hypothesis.

Study 1: Investigating mechanisms and outcomes of As_2O_3 -induced histone acetylation

1. Identify tolerated and toxic concentrations of As_2O_3 in HEK293T cells under short-term and longer-term exposures.
2. Determine if tolerated exposure leads to cellular physiological properties concomitant with cellular transformation.
3. Identify the differences in global histone acetylation on specific lysine residues as a result of As_2O_3 exposure at tolerated and toxic concentrations.

4. Identify the enzymes that are involved in modulating the acetylation status on specific lysines and evaluate their expression and/or activity under tolerated and toxic As₂O₃ concentrations.
5. Investigate the role of As₂O₃-induced histone acetylation and histone modifying enzymes in the transcriptional regulation and expression of proto-oncogenes, tumour suppressors and apoptotic genes.

Study 2: As₂O₃-induced microRNA aberrations and the consequences on histone acetylation.

1. Identify differentially expressed microRNAs under tolerated and toxic exposures to As₂O₃ in UROtsa cells and utilise bioinformatics to identify potential microRNA:HDAC/HAT interactions.
2. Determine microRNA and HDAC/HAT mRNA expression levels to identify inverse correlations between microRNA and HDAC/HAT expression.
3. Investigate for microRNA-mediated attenuation of HDAC/HAT mRNAs by using a 3'UTR luciferase reporter.
4. Use antisense-RNAs against candidate microRNAs to stop the attenuation mechanism and determine specificity.
5. Determine the impact of microRNA-mediated attenuation of HDAC/HAT mRNAs on histone acetylation.

The results for each study are separated into independent sections and then unified into a single discussion section.

Materials and Methods

3.1 – Cells, Culture Conditions and Harvesting

Two cell lines were selected as *in vitro* models to investigate the hypothesis of this study. The cells were of a relevant species i.e. human, exhibited a good toxicity profile after exposure to As₂O₃ and apart from minimal modifications to allow for continuous passaging, the cells exhibited normal morphological characteristics by growing adherently and as a monolayer.

The first model used was the human embryonic kidney (HEK) 293T cell line provided by Dr. Nabil Hajji (Imperial College London). HEK293T cells are a variant of HEK293 cells, created by Prof. Michele Calos (Stanford University) (Public Health England, http://www.hpacultures.org.uk/products/celllines/generalcell/detail.jsp?refId=12022001&collection=ecacc_gc, accessed 08.2013). These cells were immortalised by sheared human adenovirus type 5 DNA, decreasing the ability of the cells to fall into senescence and transfected with a gene encoding for the SV40 large T antigen (Kavsan et al. 2011). This allowed for extended exposure to As₂O₃ and subsequent experimental analysis.

The second model used was the normal human urothelium (UROtsa) cell line. This cell line was sourced from the United States with the assistance of Prof. Scott Garret (University of Dakota) during the latter stages of this study due to a lack of local depositories. UROtsa cells were considered for this study as the cell line was of an appropriate derivation; from the urothelial

lining of a 12-year-old female, immortalised by the SV40 large T antigen similarly to HEK293T and did not form colonies in soft agar or tumours after implantation into nude mice after 6-months (Petzoldt et al. 1995). UROtsa cells also exhibit morphological characteristics consistent with the normal human urothelium and are considered suitable for studying arsenic-induced epigenetic aberrations and carcinogenesis as the bladder is a target (Rossi et al. 2001; Eblin et al. 2008).

HEK293T and UROtsa cell lines were grown in T₁₇₅ flasks with Dulbecco's Modified Eagle's Medium (Sigma – D5671) completed with 10% Fetal Bovine Serum (Sigma – F7524), 1% L-Glutamine (Gibco – 25030) and 1% Penicillin-Streptomycin solution (Sigma – P4333). Both adherent cell lines were passaged twice a week by detachment from the cell culture surface with 1X Trypsin (SAFC Bioscience – 59418C) pre-warmed to 37°C followed by re-suspension in 20 mL of completed DMEM for seeding. Both cell lines were cultured at 37°C in a humid 5% CO₂ atmosphere.

Cells were harvested for downstream assays by collecting all the cell culture medium for each treatment into separate conical tubes. Cells were detached from the cell culture surface with 1X Trypsin (SAFC Bioscience – 59418C), which was inspected under a light microscope to ensure full detachment before collection. The remaining culture surface was washed with 1X PBS (Gibco – 14200-67) to ensure collection of all cells. Suspensions were centrifuged at 1,000 g for 5 minutes and the medium was aspirated. Cell pellets were washed in 1X PBS followed by centrifugation at 1,000 g for 5 minutes. After aspiration of the PBS, cell pellets were snap frozen in liquid nitrogen and stored frozen at -20°C. This harvesting method was used for all assays unless stated otherwise in the subsequent methods.

3.2 – Toxicants and Cell Treatments

Arsenic trioxide (As_2O_3 , Sigma – 311383) was prepared into 10 mM As_2O_3 stocks in a 1 M NaOH solvent. Autoclaved solutions were used and preparations were sterilised through a 0.4 μm filter and stored at -20°C . For cellular treatments the culture medium was first replaced with pre-warmed DMEM (Sigma – D5671) and then stock toxicant preparations were diluted in pre-warmed DMEM and added to live cell cultures to the desired concentration. Untreated controls were exposed to the vehicle alone. The cell culture flasks were then tilted gently to homogenise the toxicant solution with the medium.

3.3 – Protein Extraction

Harvested cells were allowed to thaw on ice. Cell pellets were re-suspended in 200 μL TGN buffer (1 M Tris-HCl, 2.5 M NaCl, glycerol, 0.5 M β -glycerphosphate, Tween 20 and Nonidet P40) completed with protease-inhibitor cocktail (Sigma – P8340) at a 1:1000 dilution. After 10 minutes on ice the suspension was mixed and lysed by the surfactants. After three repetitions of this process, the mixture was centrifuged at 16,000 g at 4°C for 10 minutes leaving the proteins in the supernatant. Proteins were then transferred to new micro centrifuge tubes.

3.4 – Histone Extraction

Harvested cells were incubated on ice for 15 minutes in lysis buffer (10 mM Tris pH = 6.5, 50 mM sodium bisulphate, 10 mM MgCl_2 , Sucrose 8.6%, 5mM sodium butyrate and 1% Triton X-100). The samples were then centrifuged at 10,000 g for 10 minutes at 4°C . The pellets were washed in ice-cold Tris-EDTA (10 mM Tris pH = 7.4, 13 mM EDTA) and centrifuged. The

cellular material was suspended in 50 μ L ice-cold miliQ H₂O. Samples were subsequently treated with 0.2 M H₂SO₄ (Sigma – 320501), homogenised by vortex and incubated on ice for 60 minutes. Samples were centrifuged again at 10,000 g for 60 minutes at 4°C. Histones were precipitated by incubating the supernatant with 1 mL acetone overnight at -20°C followed by centrifugation at 10,000 g for 10 minutes at 4°C. The supernatant was removed and the histones were air dried and suspended in 50 μ L miliQ H₂O.

3.5 – Bradford Assay of Protein

Protein concentrations were determined by using a Coomassie® Brilliant Blue dye-binding protein assay reagent (Bio-Rad – 500-0006) against a bovine serum albumin (BSA, Sigma – A9418) standard. BSA stocks of 2 mg/mL were diluted 1:10 before adding in increasing increments to the protein assay reagent at following final concentrations: 4×10^{-3} μ g/ μ L, 8×10^{-3} μ g/ μ L, 1.6×10^{-2} μ g/ μ L, 3.2×10^{-2} μ g/ μ L and 0.064×10^{-2} μ g/ μ L. Extracted proteins were measured by adding 1 μ L of each sample to 200 μ L of protein assay reagent in a 96-well plate. All measurements were taken in triplicate. Absorbance readings at 595 nm were measured on a GloMax® Microplate Reader (Promega). Protein concentrations of the samples were calculated by interpolation of a standard curve plot of known BSA concentrations against A₅₉₅.

3.6 – Immunoblotting of Histones and Other Proteins

Acetylation, methylation and phosphorylation post-translational modifications of core histones were determined by Western blotting. Proteins were separated by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Amersham Hybond ECL Nitrocellulose Membrane, GE Lifesciences). Membranes were blocked with 5% non-fat dry milk in PBS Tween 0.1% for 30 minutes and incubated with primary antibodies overnight at 4°C with

agitation. Membranes were washed with 0.1% PBS-Tween followed by incubation with peroxidase conjugated goat anti-rabbit IgG (1:8,000) (Sigma – A0545) and goat anti-mouse IgG (1:5,000) (GE Healthcare – NXA931) depending on the primary antibody host species.

Primary antibodies used to identify histone post-translational modifications were as follows: anti-acetyl Histone 3 (1:20,000) (Millipore), anti-acetyl Histone H3-Lys 9 (1:2,000) (Upstate, Millipore), anti-dimethyl Histone H3-Lys 9 (1:2,000) (Upstate, Millipore), anti-phospho Histone H3-Ser 10 (1:1,000) (Millipore), anti-acetyl Histone H4-Lys 12 (1:3,000) (Millipore) and anti-acetyl Histone H4-Lys 16 (1:10,000) (Millipore).

For intracellular protein analysis the following antibodies were used: anti-PARP (1:1000) (Cell Signalling), MDM2 (1:1000) (Millipore), p53 phosphorylated serine 15 (1:1000) (Cell Signalling), P53 (1:1000) (Cell Signalling), BID (1:1000) (Cell Signalling), CASPASE-3 (1:1000) (Cell Signalling) and β -ACTIN (1:1000) (Cell Signalling).

Membranes were visualised using ECL reagents composed of two solutions mixed 1:1 followed by exposure to ECL hyperfilm (Amersham, GE Healthcare). The two-component ECL solution was made of Solution A: 0.1 M Tris/HCl pH 8.5, (Sigma – T5941), 0.0091 M p-Coumaric acid (Sigma – C9008) and 0.00055M Luminol (Sigma – A8511) and Solution B: 0.1 M Tris/HCl pH 8.5 supplemented with 60 μ L H₂O₂ (Sigma – H0904). Densitometry analysis was conducted using the ImageJ software (National Institutes of Health, <http://rsb.info.nih.gov/ij/>, accessed: 08.2013).

3.7 – Real-Time Quantitative Reverse Transcription PCR

Total RNA was extracted from harvested cells by using the RNeasy Mini Kit (Qiagen – 74104) as per the manufacturer's protocol. The lysate was homogenised using the QIAshredder (Qiagen – 79654). All RNA samples were treated with amplification grade DNase I (Sigma - AMPD-1 Kit) using manufacturer instructions prior to spectrophotometric concentration determination (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific). Two-step quantitative real-time PCR was performed for HDACs and a number of other genes of interest, first by synthesis of cDNA using the ThermoScript RT-PCR System (Invitrogen) using a 5 µg input RNA amount for each sample; followed by quantitative real-time PCR using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen – 11733-038s) on the ABI 7500 Fast Real Time PCR System (Applied Biosystems). Quantification was by the standard curve method for relative quantification (Bookout et al. 2006).

The standard curve method for relative quantification is able to determine the expression level of a gene of interest (GOI) against a reference standard, also known as a housekeeping gene. Plates were prepared with serial dilutions of cDNA to plot a standard curve for one gene of interest and one housekeeping gene. Serial dilutions of the cDNA were prepared at the following dilutions: 1/5, 1/25, 1/125, 1/625, 1/3125 and 1/15,625. Using the Ct (cycle threshold) values generated from the serial dilutions it was possible to apply a linear regression analysis, to enable the interpolation of unknown quantities, which were diluted 1/125. Furthermore, this method allowed for a primer efficiency check by using the slope function from the linear regression. The equations used to plot the standard curve, quantify the expression of target mRNAs and check the efficiency using the exported Ct values from the PCR system are shown below:

Standard curve and quantification of GOI/Reference quantity:

$$y = mx + c$$

where y = Ct of unknown sample

m = slope

x = quantity of the unknown sample expressed as log (GOI/Reference quantity)

c = intercept

GOI/Reference quantity was calculated by interpolation of the above plot.

Normalised value = average GOI quantity / average reference quantity

Errors:

Standard deviation = normalised value $\times \sqrt{(CV \text{ of reference})^2 + (CV \text{ of GOI})^2}$

Coefficient of variation (CV) = standard deviation / average

Efficiency check:

$E = 10^{(-1/m)}$

Perfect doubling gives an efficiency coefficient (E) of 2

Primers for qRT-PCR were designed using a number of bioinformatics tools to ensure target specificity and to avoid primers prone to form secondary structures such as homodimers and heterodimers during the reaction. Coding sequences for the genes of interest were identified in the NCBI Nucleotide Database (National Centre for Biotechnology Information, U.S National Library of Medicine, <http://www.ncbi.nlm.nih.gov/nucore>, accessed 08.2013). Coding sequences for each gene of interest was used as the input variable for the Primer-BLAST tool (National Centre for Biotechnology Information, U.S National Library of Medicine, <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>, accessed 08.2013) and parameters were set to seek primers matching the following specifications: $T_m = 60^\circ\text{C} \pm 1^\circ\text{C}$, approximate primer lengths of 18 to 20 nucleotides, GC content of 40% to 60% and with a projected product size of 60 to 150 nucleotides (Udvardi et al. 2008). Primer pairs with no unintended targets were

analysed for secondary structures using the NetPrimer software (Premier Biosoft, <http://www.premierbiosoft.com/netprimer/>, accessed: 08.2013) and ordered as custom oligonucleotides (Invitrogen) (Table. 6). Primer pairs were subjected to melting curve analysis after each qRT-PCR reaction to check for primer dimers and the generation of products of multiple lengths signifying poor specificity. Although not a frequent occurrence due to the use of bioinformatic programs, primer pairs that exhibited symptoms of poor specificity were excluded and redesigned.

+ Table 6: Primer sequences used for qRT-PCR.

Primer pairs for each target gene of interest are detailed in this table with the associated NCBI reference number of each target, the co-ordinates of the coding sequences and the expected product length. The GC content and T_m for each primer pair are also stated.

Gene of Interest	NCBI Reference Sequence	Coding Sequence	Forward Primer 5'->3'	Reverse Primer 5'->3'	Notes:
HDAC1	NM_00496 4.2	64-1512	AGAATGCTG CCGCACGCAC C	CGAGATGCG CTTGTCAGG GTCG	Product Length: 108 bp, F-> T _m : 60.93 GC: 65%, R-> T _m : 60.18 GC: 63.64%
HDAC 2	NM_00152 7.3	288-1754	ATAGGCCCC ATAAAGCCAC TGCC	CACAGCTCCA GCAACTGAA CCG	Product Length: 206 bp, F-> T _m : 58.74 GC: 56.52%, R-> T _m : 58.37 GC: 59.09%
HDAC 3	NM_00388 3.3	67-1353	TGACGTGCC TGCAGACCTC C	ATTGGGTGC CTCTGGCCTG C	Product Length: 100 bp, F-> T _m : 58.72 GC: 65%, R-> T _m : 59.33 GC: 65%
HDAC 4	NM_00603 7.3	793-4047	TTCAACTCCG TGGCCGTGG C	GCCTGCTGG GTCCCGTTTC C	Product Length: 107 bp, F-> T _m : 59.90 GC: 65%, R-> T _m : 59.97 GC: 70%
HDAC 5	NM_00101 5053.1	333-3704	GAGGCCGAG ACTGTGAGC GC	TCCATGGGC TCCTCTGCCG G	Product Length: 107 bp, F-> T _m : 59.84 GC: 70%, R-> T _m : 60.32 GC: 70%
HDAC 6	NM_00604 4.2	94-3741	GATGGGCCT TGCCAGTGG CC	AGCAGGGGT GGTGGGTCT CC	Product Length: 120 bp, F-> T _m : 60.32 GC: 70%, R-> T _m : 59.82 GC: 70%
HDAC 7	NM_01540 1.3	196-3171	GGCACGTGC TCCTCTACGG C	CAACCCACCC ACAGGGCAG C	Product Length: 114bp, F-> T _m : 59.84 GC: 70%, R-> T _m : 60.18 GC: 70%
SIRT 1	NM_01223 8.4	54-2297	GACTGGACT CCAAGGCCA CGG	CAGGTGGAG GTATTGTTTC CGGC	Product Length: 118bp, F-> T _m : 59.12 GC: 66.67%, R-> T _m : 57.68 GC: 56.52

Table 6
cont...

SIRT 2	NM_01223 7.3	303-1472	CTTGTCGG AGGGAGCAC GC	CTCGTCCTTG GCAGGTGGC G	Product Length: 108bp, F-> Tm: 59.77 GC: 70%, R-> Tm: 60.04 GC: 70%
C-JUN	NM_00222 8.3	1044-2039	AAGCGCATG AGGAACCGC ATCG	TCACTTTTTC CTCCAGCCG GGC	Product Length: 82bp, F-> Tm: 60.43 GC: 59.09%, R-> Tm: 59.34 GC: 59.09%
C-FOS	NM_00525 2	206-1348	TCGGGCTTC AACGCAGAC TACG	AAGGAGTCT GCGGGTGAG TGGT	Product Length: 101bp, F-> Tm: 59.24 GC: 59.09%, R-> Tm: 59.53 GC: 59.09%
MDM2	NM_00239 2.5	307-1800	ATGGTGAGG AGCAGGCAA AT	AGGGTCTCT TGTTCCGAA GC	Product Length: 98bp, F-> Tm: 59.67 GC: 50%, R-> Tm: 59.68 GC: 55%
P300	NM_00142 9.3	396-7640	GACCCTCAGC TTTTAGGAAT CC	TGCCGTAGC AACACAGTG TCT	Product Length: 301bp, F-> Tm: 52.57 GC: 50%, R-> Tm: 56.38 GC: 52.38%
KAT2B (PCAF)	NM_00388 4.4	447-2945	TCAGCCTCTT CCAGAACCA GCCA	GACTGCTGC TCCCTGCGTT T	Product Length: 127bp, F-> Tm: 59.55 GC: 56.52%, R-> Tm: 57.55 GC: 60%
KAT8 (HMOF)	NM_18295 8.2/ NM_03218 8.2	19-1422/ 19-1395	ACCGCAAGA GCAACATCTC CGT	AGTCAGGAT GTAAAAGAC GAACGGC	Product Length: 146bp, F-> Tm: 58.84 GC: 54.55%, R-> Tm: 57.38 GC: 48.00%
CREB (CBP)	NM_00437 9.3/ NM_13444 2.3	252-1235/ 252-1277	ACATTAGCCC AGGTATCTAT GCCAG	CCTGAATGAC TCCATGGACT TGAAC	Product Length: 112, F-> Tm: 55.97 GC: 48%, R-> Tm: 55.65 GC: 48%
KAT5 (TIP60)	NM_00120 6833.1/ NM_18271 0.2/ NM_18270 9.2/ NM_00638 8.3	267-1751/ 267-1907/ 267-1652/ 267-1808	ATGAGCGGC TGGACCTAA AGAAGA	AGTCCTCATC AGTGCCCAA ACAAT	Product Length: 261, 417, 261, 417. F-> Tm: 57.67 GC: 50%, R-> Tm: 56.51 GC: 45.83%
α- TUBULI N	NM_00608 2.2	222-1577	GCCAAGCGT GCCTTTGTTC	CACACCAACC TCCTCATAAT CC	Product Length: 114bp, F-> Tm: 55.05 GC: 57.89%, R-> Tm: 52.58 GC: 50%
PPIA	NM_02113 0.3	84-581	CTGCACTGCC AAGACTGA	GCCATTCTTG GACCCAAA	Product Length: 109bp, F-> Tm: 57.21 GC: 55.56, R-> Tm: 57.14 GC: 55.56

For the microRNA qRT-PCR experiments, microRNA was extracted from total RNA by using a miRNeasy Micro Kit (Qiagen) as per the manufacturer's protocol. Two-step quantitative real-time PCR was performed, first by the synthesis of cDNA by utilising the miScript II RT Kit with

the HiFlex Buffer followed by quantitative real-time PCR. Data was quantified by utilisation of the Pfaffl method (Pfaffl 2001). Full list of microRNAs assayed on the miR PCR array can be found at the following address:

http://www.sabiosciences.com/mirna_pcr_product/HTML/MIHS-102Z.html, accessed: 03.2014. Proprietary primer pairs used to validate miR expression were purchased from Qiagen.

3.8 – Clonogenic Assay

Cells were seeded into six-well dishes at 500 cells per well and incubated overnight in DMEM (Sigma – D5671). After a medium change cells were then exposed to As₂O₃. Upon conclusion of the exposure period, cells were washed with PBS and fixed with 100% methanol for 10 minutes at -20°C. Cells were stained with a 5% Giemsa, 25% methanol and 70% PBS solution. Colonies were counted under a stereomicroscope using a hand counter. The survival fraction was calculated by taking into consideration the plating efficiency of each cell line. The equations used to quantify each component are stated below (Franken et al. 2006):

$$\text{Plating Efficiency} = \text{no. of colonies formed} / \text{no. of colonies seeded}$$

$$\text{Survival Fraction} = \text{no. of colonies formed after treatment} / \text{no. of cells seeded} \times \text{plating efficiency}$$

Average colony size was quantified using the ImageJ software (National Institutes of Health, <http://rsb.info.nih.gov/ij/>, accessed 08.2013). Plates were first scanned and processed within ImageJ to the parameters stated below:

Image Adjustments – Image Type: 8 bit, Threshold: Default + B&W

Analyse Particles – Size (pixel²): 20 – Infinity, Circularity: 0 – 1.0, Show: Ellipses

Using these parameters the ImageJ software (National Institutes of Health, <http://rsb.info.nih.gov/ij/>, accessed 08.2013) was able to provide an area measurement for each colony; from this an average was calculated for each treatment.

3.9 – Cell Proliferation Assay

Cells were seeded into a 96-well plate at 4×10^3 cells per well, with six wells per treatment and incubated overnight in 100 μ L DMEM (Sigma – D5671). Cells were then exposed to As₂O₃ for the desired exposure period. After conclusion of this exposure period, 10 μ L of the Cell Proliferation Reagent WST-1 (Roche) was added to each well and incubated for 4 hours at 37°C and 5% CO₂. WST-1 is converted to Formazan (A_{450}) by mitochondrial dehydrogenases, which directly correlates with the number of metabolically active cells in culture (Roche, https://cssportal.roche.com/LFR_PublicDocs/ras/11644807001_en_11.pdf, accessed: 08.2013). Medium was homogenised on a plate shaker for 1 minute before absorbance measurement at 450 nm with a reference wavelength of 600 nm in a spectrophotometer (FLUOstar OPTIMA, BMG Labtech). The proliferative index in arbitrary units was calculated using the following equation:

Assumption: Absorbance reading of Formazan is correlated to the number of metabolically active cells in culture.

$$\text{Proliferative Index} = A_{450} - A_{600}$$

Average proliferative indices were calculated for each treatment and plotted accordingly.

3.10 – Flow Cytometry Assays: Quantification of Mitochondrial ROS, Mitochondrial Membrane Potential ($\Delta\psi_m$), Cell-Cycle analysis and Caspase Activation

All flow cytometry-based assays started with cells cultured and treated with As_2O_3 in 6 well plates, using 3 wells per treatment to obtain triplicate measurements. Cells were harvested by removing the culture medium first into 15 mL conical tubes. Cells were detached with incubation with 1X Trypsin (SAFC Bioscience – 59418C) and also added to each respective 15 mL conical tube. Cells were then pelleted by centrifugation at 1,000 g for 8 minutes. After removal of the supernatant, cells were washed with 1X PBS (Gibco – 14200-67) and added to round bottom flow cytometry tubes (BD Bioscience) and spun at 2,000 g for 5 minutes. The PBS supernatant was aspirated and pellets were suspended in 1mL of PBS and placed into flow cytometry tubes. Different aspects of cellular physiology were examined using different dyes. Preparation and description of methods for each dye is stated below.

Mitochondrial ROS was identified in live cells using the MitoSOX Red (Invitrogen – M36008) fluorescent dye. Cells were harvested and washed with PBS and suspended into 250 μL of 2.5 mM Mitosox Red (Invitrogen) fluorescent dye dissolved in PBS. This suspension was allowed to incubate for 20 minutes under cell culture and light protected conditions. Cellular fluorescence for MitoSOX Red was measured using the FL2 channel on a flow cytometer (FACSCalibur, BD Biosciences).

Mitochondrial membrane potential ($\Delta\psi_m$) and apoptosis was assayed by incubating cells with a DiOC₆(3) (Invitrogen – D273) and propidium iodide (Sigma – 81845) co-stain. Cells were harvested and washed with PBS and suspended into 50 μM propidium iodide spiked with 2.5 μL of 80 μM DiOC₆(3) and incubated for 40 minutes under cell culture and light protected

conditions. Cellular fluorescence was measured using the FL1 channel for DiOC₆(3) and the FL3 channel for propidium iodide on a flow cytometer (FACSCalibur, BD Biosciences).

Cell cycle analysis was performed on fixed cells. At the end of the exposure period cells were prepared for fixation. Briefly, cells were harvested and washed with PBS then suspended in 1 mL of 70% ethanol added drop-wise during vortex. Tubes were stored at -20°C overnight to complete the fixation. Cells were pelleted by centrifugation at 1,000 g for 8 minutes and then stained in 300 µL of 50 µM propidium iodide (Sigma – 81845) solution prepared with PBS and allowed to incubate for 40 minutes at room temperature. Cell fluorescence was measured using the FL2 channel on a flow cytometer (FACSCalibur, BD Biosciences).

Activation of Caspases-3, -7 and -8 was measured with the use of a CaspaTag Caspase 3,7 In Situ Assay Kit (Millipore – APT423) and a CaspaTag Caspase 8 In Situ Assay Kit (Millipore – APT408). Both of these kits employ the use of carboxyfluorescein (FAM)-labelled peptide with an associated fluoromethylketone (FMK) caspase inhibitor. For Caspase-3 and -7 a FAM-DEVD-FMK inhibitor was used, and for Caspase -8 a FAM-LETD-FMK inhibitor was used. These inhibitors work by entering cells and covalently binding to active caspases, remaining bound within the cell whilst giving a fluorescent green signal which can be measured on a flow cytometer

(<http://www.celltechnology.com/fileuploader/download/download/?d=0&file=custom%2Fupload%2FFile-1388908941.pdf>, accessed: 07.2014,
http://www.emdmillipore.com/INTL/en/product/CaspaTag-Caspase-8-In-Situ-Assay-Kit%2C-Fluorescein,MM_NF-APT408#anchor_UG, accessed: 07.2014,
http://www.emdmillipore.com/INTL/en/product/CaspaTag-Caspase-3%2C7-In-Situ-Assay-Kit%2C-Fluorescein,MM_NF-APT423#anchor_UG, accessed: 07.2014). Each assay was completed as per manufacturer's instructions. Cells were harvested and centrifuged at 1,500 rpm for 5 minutes and re-suspended in 300 µL of PBS. A 10 µL volume of 30X FLICA reagent

containing the fluorochrome inhibitors was added for 1 hour at 37°C and 5% CO₂. Cells were protected from light with aluminium foil. Cells were washed twice with the provided buffers to remove any unbound fluorescent inhibitors and cells were re-suspended in 250 µL of wash buffer and analysed by flow cytometry. Fluorescein was measured on the FL1 channel. Caspase-negative cells were observed within the first log decade, whilst caspase-activated cells were scored as a separated peak to the right of the negative peak.

All samples were analysed on flow cytometer (FACSCalibur, BD Biosciences) and the raw data was analysed using Cyflogic Version 1.2.1 (Perttu Terho, Mika Korkeamäki, CyFlo Ltd).

3.11 – Transfections of Cells.

Transient transfections were achieved using filter-sterilised polyethylenimine (PEI) reagent (Polysciences, Inc Cat # 23966) made up to a concentration of 1 mg/mL in H₂O and adjusted to pH 6.8. Transfection reagent and plasmid complex was synthesised using a 1:4 DNA to PEI weight/volume ratio. The remaining volume was made up to 200 µL in non-FBS supplemented DMEM (Sigma – D5671). Cells were cultured up to 60% confluence and transfected through the drop-wise addition of the complex in 10 cm tissue culture dishes overnight in 5 mL of DMEM. Cells were rested the next day in fresh DMEM for 24-hours followed by treatment with arsenic trioxide for the desired exposure period after which cells were harvested for assays.

Plasmids used were HDAC1 (Addgene # 13820), HDAC3 (Addgene # 13819) both from Addgene. HDAC2 and HDAC4 generously provided by Dr. Ito Kazuhiro (Imperial College London). PCAF, HMOF, TIP60 and P300 kindly provided by Dr. Bertrand Joseph (Karolinska Institutet).

3.12 – 3'UTR Luciferase Reporter.

Two 3'UTR luciferase reporters were obtained via Active Motif from SwitchGear Genomics including a PCAF 3'UTR reporter and a control PPIA 3'UTR reporter. UROtsa cells were transfected using PEI in 24 well plates with 1 µg of plasmid for each well overnight. Cells were rested for 24 hours in fresh medium before arsenite treatment. Cells were harvested with trypsin, washed with PBS transferred in 100 µL aliquots into a white 96 well plate. A 100 µL cellular lysis and luciferase substrate reagent mixture (Promega – E2710) was added to each aliquot and incubated for 10 minutes prior to luminescence measurement in a GloMax 96 Microplate Luminometer. Readings were normalised against the PPIA control plasmid as per manufacturers guidelines

(http://switchgeargenomics.com/sites/default/files/pdf/LightSwitch_3UTRnorm.pdf, accessed: 03.2014).

3.13 Bacteriology – Plasmid Preparation

Plasmid preparations were used for all transfections. First, 50µL of highly competent *Escherichia coli* cells (Invitrogen – 10268-019) were thawed on ice and transformed by using 1 µL of the plasmid to be cloned. After 30 minutes of incubation on ice, the cells were subjected to heat shock for 25 seconds in a 42°C heat block and immediately placed on ice a further 2 minutes. Transformed cells were cultured in 900 µL SOC medium (Invitrogen – 15544-034) at 37°C with agitation for 90 minutes.

Cultures were spread evenly over LB Agar plates supplemented with the appropriate antibiotic for each plasmid to ensure the presence of an adequate selection pressure. The plates were incubated overnight in an incubator kept at 37°C. Single colonies were then selected and

incubated overnight with agitation in 5 mL of LB Broth supplemented with the appropriate antibiotic. Using 1 mL of this overnight culture 500 mL of LB Broth with antibiotics was inoculated for overnight culture with agitation at 37°C.

Plasmid purification was achieved by utilising the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen – K2100-06) as per the manufacturers guidelines. Briefly, cultures were first pelleted and then lysed. In this column based purification process, the lysis mixture was first precipitated and the supernatant was allowed to bind to the chemically equilibrated column. After a number of wash steps, the DNA was eluted from the column, subjected to an isopropanol and 70% ethanol wash. After centrifugation the pellet was air dried for 10 minutes and then the plasmid DNA was re-suspended in 200 µL TE Buffer (Invitrogen – 12090-015).

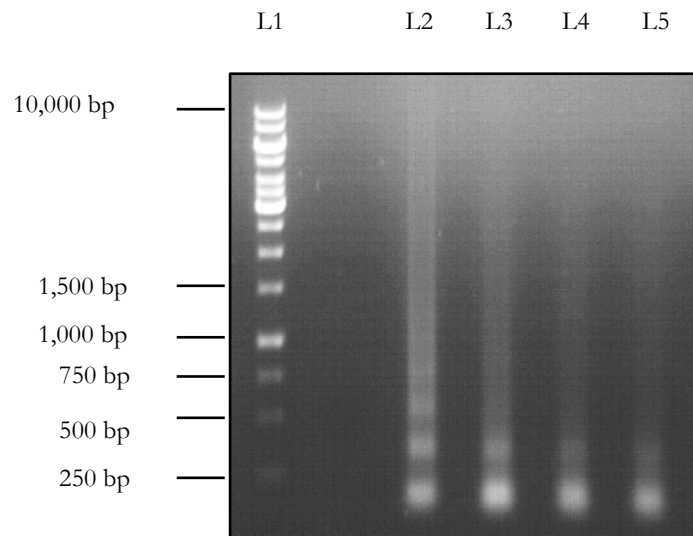
3.14 – Sensitivity of DNA to Micrococcal Nuclease (MNase)

Micrococcal nuclease induces DNA double-strand breaks between nucleosomal linker regions (Anon 2005). Due to this, the relative sensitivity of DNA to MNase digestion is a proxy measurement for chromatin relaxation. Cells were exposed to As₂O₃ for the desired exposure period. Harvested cells were then pelleted and lysed in 5 mL NP-40 lysis buffer on ice for 5 minutes. NP-40 lysis buffer is composed of: 10 mM Tris-HCl pH 7.4 (Sigma – T5941), 10 mM NaCl (Sigma – S3014), 3 mM MgCl₂ (Sigma – M8266), 0.5% Nonidet NP-40 (Sigma – 74385), 0.15 mM spermine (Sigma – S3256) and 0.5 mM spermidine (Sigma – S2626). Nuclei were pelleted by centrifugation at 1,000 g refrigerated to 4°C for 10 minutes. After removal of the supernatant the pellet was washed in 2.5 mL of MNase digestion buffer and pelleted again at 1,000 g and at 4°C. MNase digestion buffer is composed of: 10 mM Tris-HCl pH7.4, 15 mM NaCl, 60 mM KCl (Sigma – P9541), 0.15 mM spermine and 0.5 mM spermidine. The extracted nuclei were then suspended in 500 µL MNase digestion buffer containing 1mM CaCl₂ of which

100 μ L was taken forward for digestion. MNase (Sigma – N3755) of was added to each sample, specifically 0.025 units and incubated at 15-20°C for 5 minutes. The reaction was stopped by the addition of 80 μ L MNase digestion buffer, 20 μ L MNase stop buffer: 100 mM EDTA (Sigma – EDS) and 10 mM EGTA (Sigma – E3889) pH 7.5, 3.75 μ L proteinase K (Invitrogen – 4333793) and 20% SDS (Sigma – L3771) followed by overnight incubation in a 37°C water bath.

MNase digested DNA was extracted the next day by standard phenol/chloroform extraction methods followed by ethanol precipitation overnight at -20°C. Samples were centrifuged at 16,000 g at 4°C for 30 minutes. MNase digested DNA was dissolved in 50 μ L TE Buffer (Invitrogen – 12090-015). The concentration of the DNA was determined using a spectrophotometer (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific). For each sample 4 μ g was loaded into a 1% agarose gel supplemented with GelRed (Biotium – 41002) and visualised under a UV lamp. This method was adapted from (Anon 2005).

The number of units to be used in each reaction was standardised by using a range of MNase units for digestion and checking the digestion pattern (Fig. 3.1).



+ **Figure 3.1: MNase digestion profile of HEK293T nuclear lysates.**
HEK293T nuclear lysates were digested with increasing units (U) of MNase and run on a 1% agarose gel stained with GelRed for visualisation under a UV lamp. The first lane (L1) is the ladder. Each subsequent lane was incubated with 0.025 U, 0.5 U, 1 U and 2 U MNase respectively.

By observing the digestion pattern it was clear that the 0.025 units (L2) was suitable to use for the MNase sensitivity analysis as it did not fully digest the chromatin leaving only mononucleosomal fragments at approximately 150 bp. Clear separation was also observed between the mono-, di- and trinucleosomal fragments at 0.025 units. In contrast, using 2 units (L5) over digested the chromatin leaving only mononucleosomal fragments.

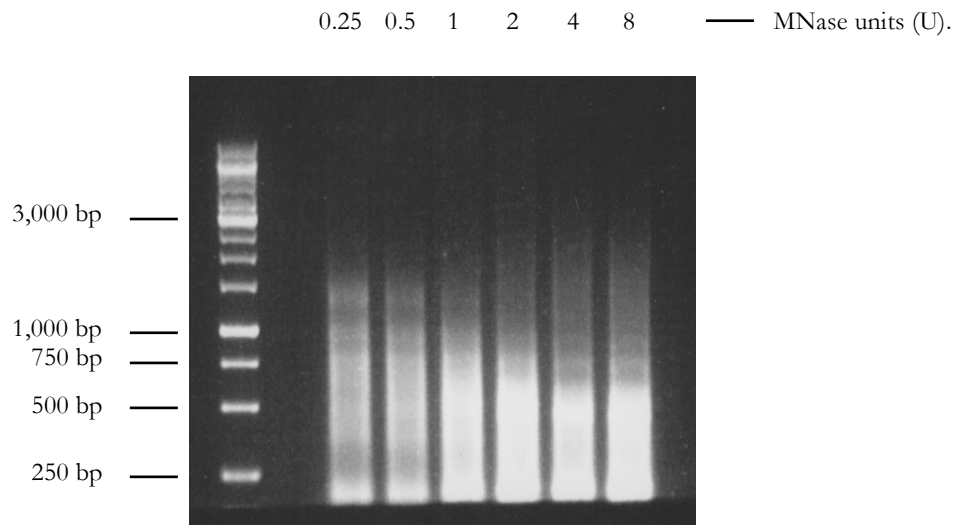
3.15 – Cell Growth in Soft Agar

Anchorage-independent growth was measured by suspending cells exposed to As_2O_3 in soft agar. Cells were first pre-treated for the desired duration with As_2O_3 . Plates were prepared using two layers of agar at differing densities. The base agar was made of 0.8% Noble Agar (Sigma –

A5431) dissolved in H₂O and DMEM (Sigma – D5671) 1:1 ratio solvent and allowed to solidify at 4°C for 20 minutes. The plate was then brought up to the ambient cell culture conditions i.e. 37°C and then the cells were seeded in falcon tubes with a 0.4% Noble Agar prepared by diluting the previous 0.8% Noble Agar mixture in DMEM at a 1:1 ratio and added to the base agar. A total of 2,500 cells were used per well of a 6 well plate. Cells were supplemented with DMEM to avoid nutrient depletion. Plates were stained with 0.5 mL 0.005% Crystal Violet (Sigma – V5265) and 20% methanol (Sigma – 322415) diluted in 1X PBS (Gibco – 14200-67) for 2 hours. Colonies were counted under a stereomicroscope with a hand counter.

3.16 – Chromatin Immunoprecipitation.

Cells were grown in 100 mm cell culture dishes, with 4 dishes for each treatment. At the end of the treatment duration cells were fixed for 10 minutes in 1% formaldehyde (Sigma – F8775) and quenched with 1M glycine (Sigma – G8898) for 5 minutes at room temperature. The formaldehyde/glycine mixture was aspirated followed by two washes of ice-cold 1X PBS (Gibco – 14200-67). Cells were detached by scraping and re-suspended in PBS. The cell suspension was centrifuged at 3,000 g for 5 minutes.



+ **Figure 3.2: MNase shearing profile of fixed HEK293T lysates for chromatin immunoprecipitation.**
HEK293T lysates were digested with increasing units (U) of MNase to optimise the shearing for chromatin immunoprecipitation and run on a 1% agarose gel stained with GelRed for visualisation under a UV lamp. The optimum digestion pattern to be identified needed to generate fragments between 1 kb and 200 bp in size. Digestion with 1 U of MNase provided a compatible shearing profile.

The remaining part of the experiment involved the utilisation of a Pierce Agarose ChIP Kit (Thermofisher – 26156, <http://www.piercenet.com/instructions/2162216.pdf>, accessed: 10.13). Briefly, cells were lysed and chromatin was sheared using 1 U of MNase per reaction to yield an optimum digestion pattern of 200 to 1,000 base pairs (Fig. 3.2). Chromatin digestion was stopped by the addition of a stop reagent and incubation on ice for 5 minutes. The nuclei were pelleted after centrifugation at 9000 g for 5 minutes. The nuclei were suspended in lysis buffer supplemented with protease/phosphatase inhibitors and incubated on ice for 15 minutes, with mixing for 15 seconds every 5 minutes by vortex. The mixture was centrifuged at 9000 g and the supernatant containing the digested chromatin was transferred to a new tube.

Immunoprecipitation was carried out by transferring 5 μ L of the digested chromatin to a separate tube as the 10% total input sample for one ChIP. The remaining 45 μ L was added to 450 μ L of 1X IP dilution buffer and incubated overnight at 4°C with a positive control antibody, anti-RNA polymerase II, a negative control normal rabbit IgG and an antibody against the target of interest. After incubation an A/G agarose resin was used to purify the antibody/chromatin complex over a 1-hour period at 4°C with rotation in a spin column. The complex was washed a number of times with an IP Wash Buffer before elution.

The elution process involved the addition of 150 μ L of 1X IP elution buffer to each spin column and incubation at 65°C for 40 minutes with agitation every 10 minutes. The total 10% input samples were supplemented with 150 μ L 1X IP elution buffer and samples in the columns were spun for 1 minute at 6,000 g and incubated with 6 μ L of 5 M NaCl and 2 μ L of 20 mg/mL Proteinase K for 1.5 hours at 65°C.

DNA was recovered using a column-based method by placing each eluted IP and total input sample into 750 μ L of a DNA binding buffer. This was then loaded into a DNA clean-up column. Through the utilisation of a number of wash steps, the purified DNA was eluted in a final volume of 50 μ L of DNA elution buffer for subsequent qRT-PCR analysis. The primers used for the qRT-PCR analysis are listed below (Table. 7)

+ Table 7: Primer sequences used for ChIP-qRT-PCR promoter analysis.
Primer pairs for each target promoter of interest and exon1 are detailed in this table with the associated NCBI reference number of each target, the co-ordinates of the amplification sequences and the expected product length. The GC content and Tm for each primer pair are also stated.

Target	Chromosome and NCBI reference.	Forward co-ordinates	Forward Primer 5'->3'	Reverse co-ordinates	Reverse Primer 5'->3'	Notes:
CFOS_P ROMOT ER	Chromosome: 14; NC_000014.8	757450 10 - 757450 29	GGTAA AAAGG CGCTC TCTGC	75745298 - 75745279	GATGA GGGGT TTCGG GGATG	Product Length: 289 bp, F-> Tm: 59.55 GC: 55%, R-> Tm: 60.18 GC: 60%
CMYC_P ROMOT ER	Chromosome: 8; NC_000008.11	127735 885 - 127735 904	CTAGA GCTAG AGTGC TCGGC	127736047 - 127736028	ATTATA AAGGG CCGGT GGGC	Product Length: 163 bp, F-> Tm: 59.41 GC: 60%, R-> Tm: 60.18 GC: 55%
PUMA_P ROMOT ER	Chromosome: 19; NC_000019.10	472329 15 - 472329 35	CAACA TTGGG AACTCC TCAGC	47233011 - 47232990	AAACA CCAGG AAGAA GGAAG GG	Product Length: 97 bp, F-> Tm: 59.18 GC: 52.38%, R-> Tm: 60.16 GC: 50%
BAX_PR OMOTE R	Chromosome: 19; NC_000019.10	489545 84 - 489546 03	GCTAA TTCCTT CTGCG CTGG	48954725 - 48954706	AGCAT CTCCC GATAA GTGCC	Product Length: 142 bp, F-> Tm: 59.34 GC: 55%, R-> Tm: 59.61 GC: 59.61
TP53_PR OMOTE R	Chromosome: 17; NC_000017.11	768767 6 - 768769 5	CCGAC CTGGT GCCGT AGATA	7687835 - 7687816	GATCC AGCTG AGAGC AAACG	Product Length: 160 bp, F-> Tm: 61.11 GC: 60%, R-> Tm: 58.99 GC: 58.99

ChIP-qPCR data analysis was achieved by using the percentage input method as described in the SA Bioscience tech sheet (<http://www.biomol.de/details/SA/chipqpcrmanual.pdf>, accessed: 07.11.13). In summary, ChIP-qPCR data was presented as “% input” by the following quantification process.

Normalisation:

$$\Delta Ct [\text{normalised ChIP}] = (Ct [\text{ChIP}] - (Ct [\text{input}] - \text{Log}_2 (\text{Input Dilution Factor})))$$

$$\text{Input dilution factor} = \text{fraction of the input chromatin saved}^{-1}$$

Normalised ChIP Ct values were averaged for replicate samples.

$$\% \text{ Input} = 2^{-\Delta Ct [\text{normalised ChIP}]}$$

A melting curve analysis was performed after each run to ensure the amplification of a single product.

3.17 – Immunocytochemistry

Immunocytochemistry was used to visualise the presence of specific proteins within cells. Cells were grown on pre-sterilised glass coverslips. Coverslips were sterilised using a microwave, where an 800 mL volume of H₂O was heated at the same time as the coverslips. Once the water started to boil the coverslips were considered sterilised. Cells were grown on the coverslips in 6 well plates under standard tissue culture conditions. Once cells were adherent, they were treated with the desired toxicant.

At the end of the treatment period, cells were washed twice with 1X PBS (Gibco – 14200-67) at room temperature. The PBS was then aspirated and 4% (v/v) paraformaldehyde (Sigma – P6148) was added to fix the cells for 10 minutes at 4°C. After fixation cells were rinsed three times with 1X PBS and allowed to stand in 1 mL/well of methanol (Sigma – 322415) for 10 minutes at -20°C. Cells were rinsed again two times with 1X PBS and then exposed to blocking/permeabilisation buffer composed of: 10 mM HEPES (Sigma – H3375), 0.3% Triton X-100 (Sigma – T8787), BSA (Sigma – A2153), pH 7.4 for 1 hour. Coverslips with adherent cells were incubated in a humid chamber by addition of 5 µL of the primary antibody with 600 µL of blocking/permeabilisation buffer. Each coverslip was placed cell-side down into the antibody and blocking/permeabilisation buffer mixture overnight at 4°C. Secondary antibody incubation

was achieved by addition of 3 μ L of secondary antibody to 600 μ L 1X PBS to create droplet. Each antibody was used depending on the host species in which the primary antibody was raised in combination with the requirements of the staining to ensure that there was no overlap between the excitation and emission wavelengths. The secondary antibodies used were: Alexa Fluor 633 Goat Anti-Mouse IgG (H+L) (Invitrogen – A21050) and Alexa Fluor 633 Goat Anti-Rabbit IgG (H+L) (Invitrogen – A21070).

The coverslips were then placed cell side down onto the droplet in the humid chamber and allowed to incubate for 30 minutes at room temperature. Coverslips were mounted on slides by using Vectashield Mounting Media (Vector Laboratories) to resist fading of the fluorescence. The gap between the coverslip and the glass slide was sealed with clear nail varnish. Slides were stored at 4°C.

Images of the slides were captured on a confocal microscope (Leica SP5/SP5II) and processed using the LAS AF (Leica, <http://www.leica-microsystems.com/products/microscope-software/life-sciences/las-af-advanced-fluorescence/>, accessed: 08.2013) and ImageJ software respectively (National Institutes of Health, <http://rsb.info.nih.gov/ij/>, accessed: 08.2013).

3.18 – Statistical Analysis

All statistical analysis was carried out using an unpaired two-tailed Student's T-Test. Data was analysed in Microsoft Excel for Mac 2011 Version 14.3.6 to generate a p-value after the comparison of two normally distributed data sets. Two groups were compared for each analysis, a control group versus treatment group. The null and alternative hypothesis used in all analyses are stated below:

$H_0 = \text{there is no difference between the treatment and the control.}$

$H_1 = \text{there is a difference between the treatment and the control.}$

Observations were considered statistically significant when the p value was below 0.05 i.e. at the 95% confidence level.

3.19 – Bioinformatics Analysis

A number of bioinformatic tools were used to support different components of this study. This information is detailed below (Table. 8).

+ **Table 8: Bioinformatic tools used in the project.**
Bioinformatic tools were used at various points within the project to develop accurate methodologies to assist in experimental design and help yield accurate data.

Utilisation	Software
Gene ontology.	NCBI Gene, http://www.ncbi.nlm.nih.gov/gene, accessed 04.10.2013. NCBI Nucleotide, http://www.ncbi.nlm.nih.gov/nucleotide, accessed 04.10.2013. Ensembl, http://www.ensembl.org/index.html, accessed 04.10.2013. Gene Cards, http://www.genecards.org, accessed 04.10.2013.
Primer design.	Primer-Blast, http://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed 04.10.2013.

Table 8 cont...

Primer secondary structure analysis.	Oligonucleotide Properties Calculator, http://www.basic.northwestern.edu/biotools/oligocalc.html , accessed 04.10.2013.
MicroRNA ontology.	miRBase , http://www.miRbase.org , accessed 04.10.2013.
MicroRNA/3'UTR target prediction & identification of validated targets.	miRWalk , http://www.umm.uni-heidelberg.de/apps/zmf/miRwalk/ , accessed 04.10.2013.

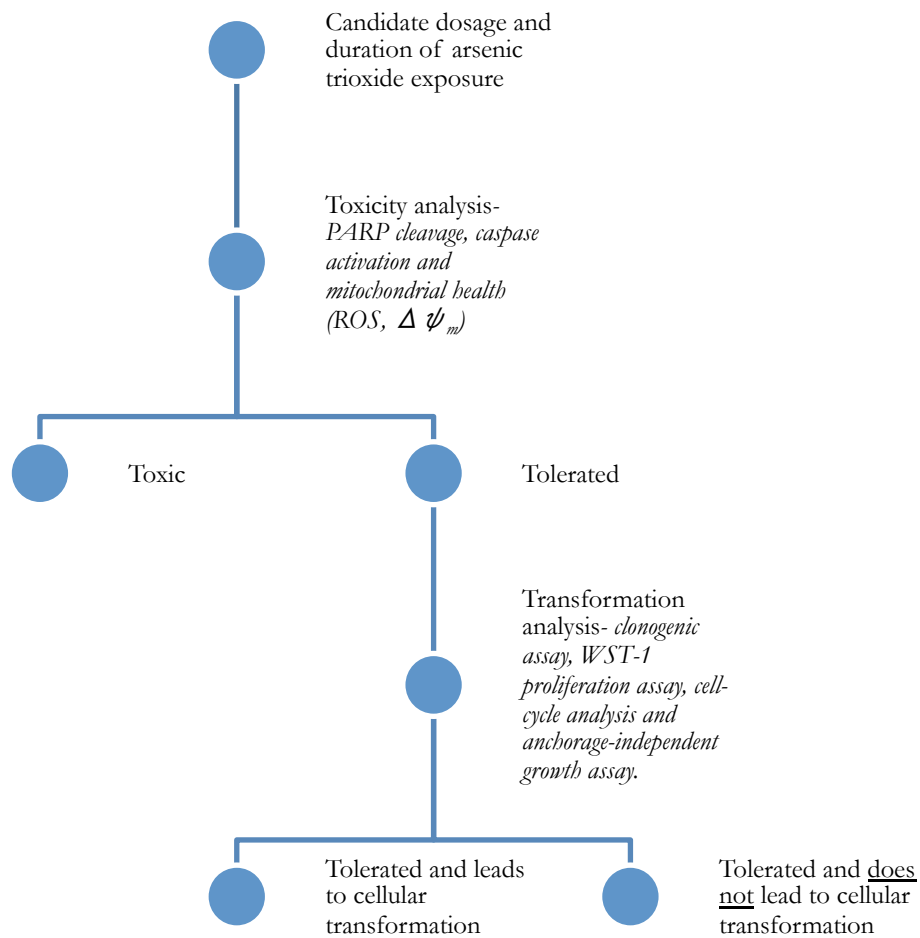
Results: Investigating Mechanisms and Outcomes of As₂O₃-induced Histone Acetylation

4.1 – Identification of Tolerated and Toxic Concentrations of Arsenic Trioxide (As₂O₃) to Human Embryonic Kidney (HEK293T) cells.

The first step of this course of research was to identify tolerated and toxic concentrations of arsenic trioxide (As₂O₃) to human embryonic kidney (HEK293T) cells. This would ensure that epigenetic and mechanistic information gathered would be anchored to an appropriate phenotypic outcome.

Two variables were investigated, the magnitude of the concentration and the duration of exposure. These were measured against a number of sensitive biochemical parameters that allowed for detailed evaluation of As₂O₃-induced toxicity. The first was poly(ADP-ribose) polymerase-1 (PARP) cleavage; a DNA repair enzyme that is rapidly targeted for proteolytic cleavage as an end-point of apoptotic cell death (Duriez & Shah 1997). The second was caspase activation including the initiator Caspase-8 and the effector Caspases-3 and -7. Examination of both initiator and effector caspases allowed for the explicit quantification of the intracellular

apoptotic signal (Taylor et al. 2008; Riedl & Y. Shi 2004). The third analysis interrogated mitochondrial health after As₂O₃ exposure with specific focus on the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) and the generation of mitochondrial reactive oxygen species, both of which are part of the apoptotic program (Christensen et al. 2013; Fleury et al. 2002). Collectively these assays ensured appropriate identification of toxic and tolerated exposures.



+ **Figure 4.1: Schematic detailing the tolerated and toxic concentration selection framework utilised in this study.**

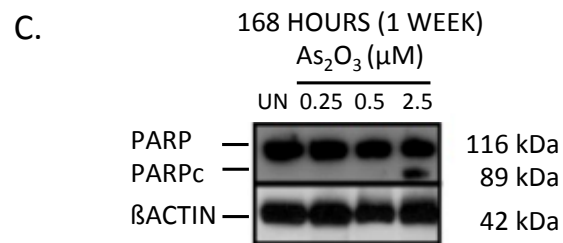
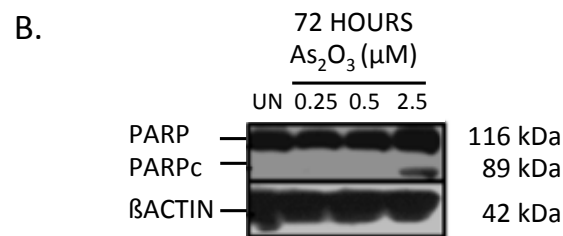
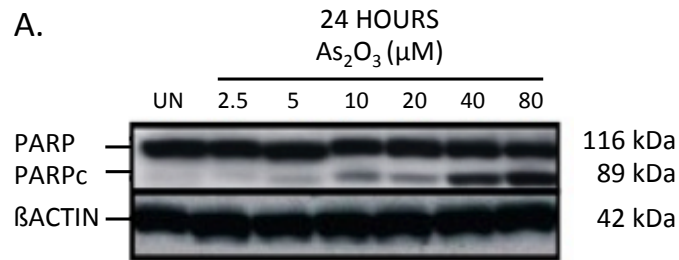
This framework provides an overview of the initial experiments carried out to select the concentrations and durations of As₂O₃ exposure.

Additional *in vitro* assays were utilised to examine cellular hallmarks of cancer, to ensure that a tolerated exposure was not just tolerated, but one that exhibited properties concomitant with cellular transformation. A clonogenic assay was used to quantify cellular survival and resistance to cell death. A WST-1 proliferation assay was used to measure cellular proliferation in response to sustained toxicant exposure in subconfluent cells. Flow cytometry based cell-cycle analysis was used to assess the cell-cycle distribution, particularly S-phase commitment and progression through the G2/M checkpoint both of which are un-regulated in cancer cells (Kastan & Bartek

2004). The soft-agar analysis allowed for the assessment of anchorage-independent growth, which is a strong indicator of malignant growth potential (Raptis & Vultur 2001). These cellular parameters including cell death resistance, sustained proliferation, elevated replicative potential and anchorage-independent growth are all considered as hallmarks of cancer (Hanahan & Weinberg 2011). The combined use of toxicity and *in vitro* cellular transformation assays together allowed for the identification of toxic and tolerated exposures to As₂O₃ (Fig. 4.1).

4.1.1 – HEK293T cells tolerate 0.25 µM and 0.5 µM As₂O₃ concentrations, but do not tolerate concentrations greater than 2.5µM As₂O₃.

To begin the toxicity analysis HEK293T cells were exposed to increasing concentrations of As₂O₃ and increasing time scales. For the initial 24-hour exposure, cells were treated with a range of concentrations from 2.5 µM – 80 µM As₂O₃ (Fig. 4.2, A). PARP cleavage was observed in concentrations from 5 µM – 80 µM As₂O₃ and these concentrations were considered toxic. Only the 2.5 µM As₂O₃ was tolerated with no PARP cleavage. This exposure and lower concentrations including 0.25 µM and 0.5 µM As₂O₃ were analysed at a longer 72-hour exposure to check for PARP cleavage (Fig. 4.2, B). In contrast to the 24-hour time-point, 2.5 µM As₂O₃ led to PARP cleavage after a 72-hour exposure. This suggests that As₂O₃-induced toxicity is both time and dose-dependent. Lower concentrations of 0.25 µM and 0.5 µM As₂O₃ did not lead to PARP cleavage, suggesting that these concentrations were tolerated. Further examination of the 0.25 µM and 0.5 µM As₂O₃ exposures were undertaken at a longer 168-hour (1-week) time-point (Fig. 4.2, C). No PARP cleavage was observed for 0.25 µM and 0.5 µM As₂O₃ exposures, whilst the 2.5 µM As₂O₃ exposure was toxic at this time-point. By using PARP cleavage analysis alone, 0.25 µM and 0.5 µM As₂O₃ was tolerated whilst the 2.5 µM As₂O₃ was toxic in exposures up to 1-week.

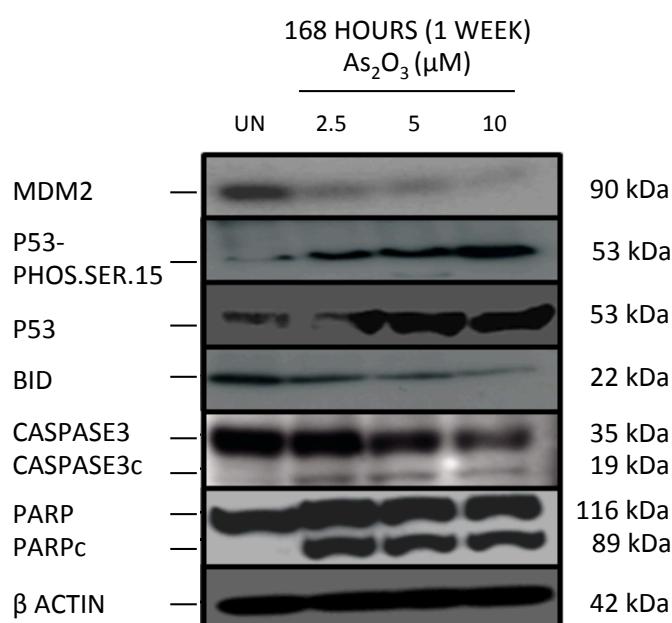


+ Figure 4.2: Immunoblotting for poly(ADP) ribose polymerase cleavage after a 24-hour, 72-hour and 168-hour exposure to As₂O₃ in HEK293T cells.

(A) HEK293T cells were exposed for 24 hours to 2.5 μM, 5 μM, 10 μM, 20 μM, 40 μM and 80 μM As₂O₃. (B) HEK293T cells were exposed for 72 hours to 0.25 μM, 0.5 μM and 2.5 μM As₂O₃. (C) HEK293T cells were exposed to 168 hours (1 week) to 0.25 μM, 0.5 μM and 2.5 μM As₂O₃. A total of 80 μg of protein was loaded in each well for immunoblotting.

4.1.2 – A minimum toxic exposure for HEK293T cells is 2.5 μM As_2O_3 .

Concentrations of 2.5 μM As_2O_3 and higher were examined further to identify a minimum toxic dosage. This would ensure that the toxic exposure selected would not lead to exaggerated artefacts such as necrotic cell death, protein degradation and DNA fragmentation. By utilising a minimum toxic concentration, rather than significantly higher toxic dosage it also provided adequate cellular material over longer exposures to complete downstream assays and allow for suitable comparisons against the tolerated dosage. To achieve this additional cytotoxicity analysis, the status of a number of proteins implicated in cell death was profiled in greater detail including MDM2 degradation, Caspase-3 cleavage, truncation of the pro-apoptotic factor BID, p53 stabilisation and activation by phosphorylation at serine 15 (Fig. 4.3) (Taylor et al. 2008; Chène 2003; Loughery et al. 2014).



+ **Figure 4.3: Immunoblotting of cytotoxic sensitive proteins including MDM2, P53, P53 serine 15 phosphorylation, BID, CASPASE-3 and PARP after a 1-week exposure to As_2O_3 .**

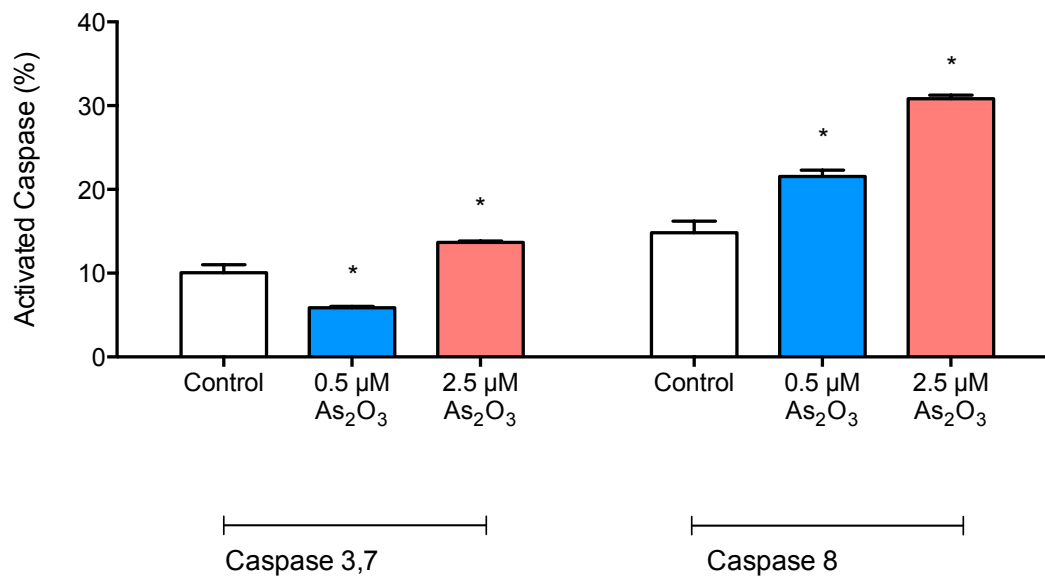
HEK293T cells were exposed to 2.5 μM , 5 μM and 10 μM As_2O_3 for 1-week. A total of 80 μg was of protein was loaded into each well for immunoblotting.

A 1-week exposure to 2.5 μM As_2O_3 led to MDM2 degradation – the negative regulator of P53. At higher concentrations of 5 μM and 10 μM As_2O_3 the levels of MDM were reduced further in a dose-dependent manner. Exposures from 2.5 μM to 10 μM As_2O_3 also led to the increased stabilisation of P53 and the pro-apoptotic phosphorylated serine 15 form. At concentrations of 2.5 μM As_2O_3 and higher, the full-length pro-apoptotic protein BID is cleaved. Apoptosis induction was clearly observed for all exposures as signified by cleavage and activation of the Caspase-3 and PARP cleavage. These blots collectively suggest that 2.5 μM As_2O_3 sits within the minimum cytotoxicity barrier, as higher dosages are significantly more toxic.

4.1.3 – Increased Caspase-3, -7 and -8 activation is observed after exposure to 2.5 μM As_2O_3 and reduced Caspase-3 and -7 activation after exposure to 0.5 μM As_2O_3 in HEK293T cells.

Further toxicity analysis was achieved by the detailed evaluation of caspase activation. Caspases are required for the transduction of apoptosis from early initiation stages to final execution with the cleavage of crucial cellular proteins (Riedl & Y. Shi 2004). Previous studies have been able to demonstrate caspase activation and apoptosis as a result of arsenic trioxide exposure in cancer cell lines, particularly Caspase-3, -7 and -9 activation in addition to the cleavage of PARP by Caspase-3 (X. Li et al. 2004; X. Li et al. 2003; Perkins et al. 2000). Similar caspase activation measurements were used in this study for detailed toxicity evaluation.

HEK293T cells were exposed to 0.5 μM and 2.5 μM As_2O_3 for 72 hours followed by flow-cytometry based analysis of caspase activation. This was achieved by the use of fluorescent inhibitors of the activated caspases, which bind only to active caspases and fluoresce. The fluorescence signal is proportional the amount of active caspases within the cell (see materials and methods section for additional details).



+ **Figure 4.4: Activated caspase -3, -7 and -8 assessment by flow-cytometry in HEK293T cells.**

HEK293T cells were exposed to 0.5 μM and 2.5 μM As₂O₃ for 72 hours. Caspase-3, -7 and -8 activation was measured. Activated caspase % was calculated by scoring the population of caspase-activated cells only. All bars shown are ± SD. * p< 0.05, N=3.

After a 72-hour treatment with 0.5 μM As₂O₃ there was a significant reduction in the activated form of Caspase-3 and -7 (Fig. 4.4). Although a significant increase in Caspase-8 activation was also observed at this dosage, no PARP cleavage was identified as shown previously. A significant increase in activated Caspase-3, -7 and -8 was observed with 2.5 μM As₂O₃. This data suggested that 2.5 μM As₂O₃ led to caspase activation, whilst the 0.5 μM As₂O₃ dosage led to only minimal activation of Caspase-8 and no activation of Caspase-3 and -7.

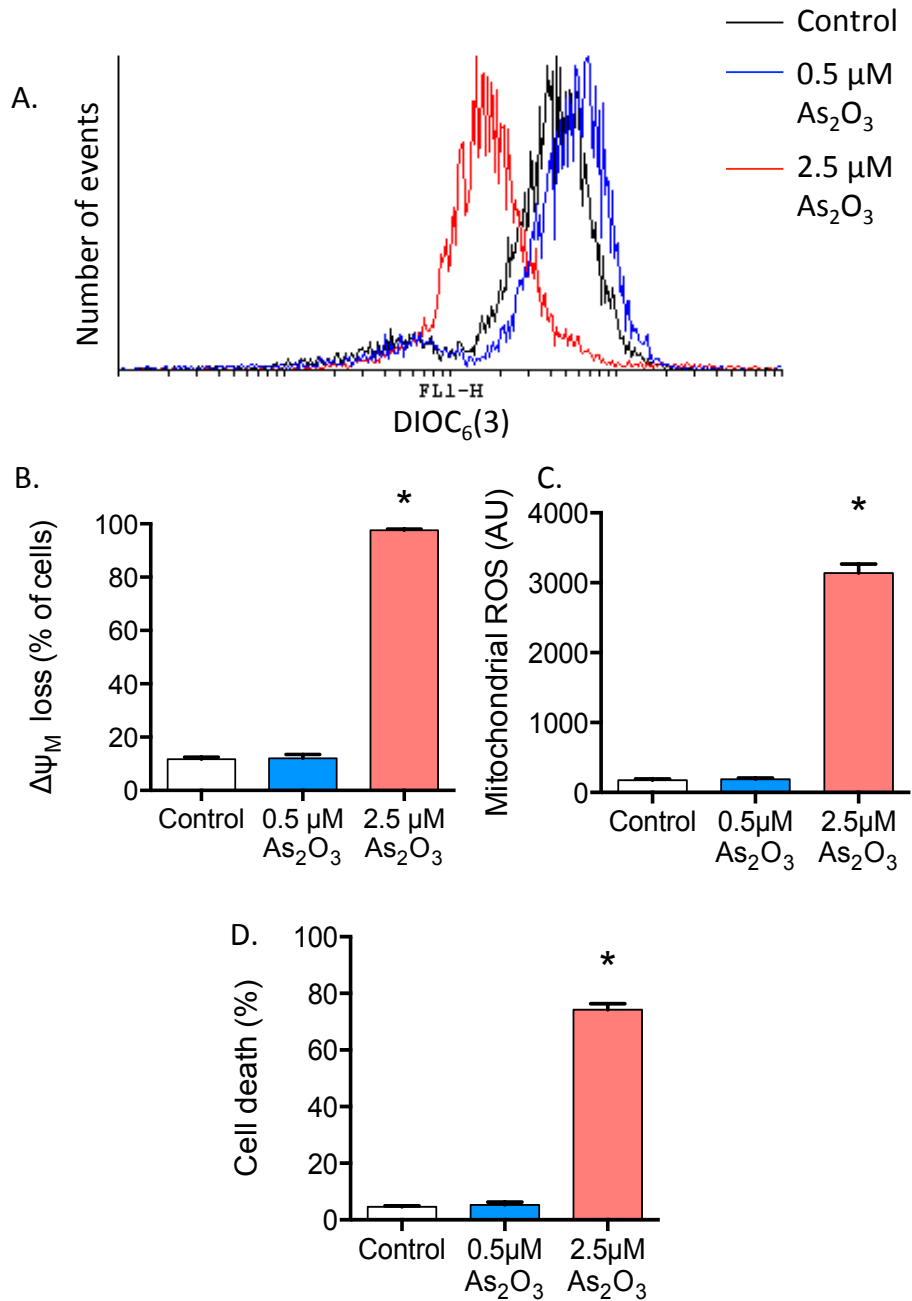
4.1.4 – No mitochondrial transmembrane potential ($\Delta\Psi_m$) loss or increase in mitochondrial superoxide generation is observed after exposure to 0.5 μM As_2O_3 for 72 hours, whilst exposure to 2.5 μM As_2O_3 leads to $\Delta\Psi_m$ hypo-polarisation and the generation of mitochondrial superoxide in HEK293T cells.

Additional interrogation of As_2O_3 -induced apoptosis was achieved by the assessment of two aspects of mitochondrial function. Mitochondria are able to produce adenosine 5' triphosphate (ATP) with the assistance of an electrical potential across the inner mitochondrial membrane. It has been demonstrated that the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$) is a hallmark event of apoptosis and it can be measured effectively via flow cytometry (Christensen et al. 2013). This was measured with lipophilic dye 3,3'-di-hexyloxacarbocyanine iodide – DiOC₆(3), which accumulates in healthy mitochondria with a large membrane potential, but is released when mitochondria experience membrane potential loss and cell death. This is observed as hypo-polarisation (Ozgen et al. 2000).

The generation of mitochondrial reactive oxygen species (ROS) was also assessed as it is involved in the initial and late steps of apoptosis. When apoptosis occurs the mitochondrial redox potential becomes more oxidised in contrast to periods of cellular survival during which anti-apoptotic factors such as BCL-2 and BCL-X_L adjust the redox potential to a more reduced state (Fleury et al. 2002). The measurement of mitochondrial superoxide with flow cytometry has been shown to be effective, precise and sensitive with the MitoSOX Red probe (Mukhopadhyay et al. 2007). Both the $\Delta\Psi_m$ and mitochondrial ROS were measured following As_2O_3 exposure.

Propidium iodide (PI) was also used with DiOC₆(3) in a co-labelling process. PI is a nuclear acid dye that cannot transverse the cellular membrane unless cells are undergoing apoptotic cell-death leading to the lack of membrane integrity and PI uptake (Ozgen et al. 2000). This co-labelling

process allowed for the identification of PI positive and PI negative cells, which represent apoptotic and viable cells respectively.



+ **Figure 4.5: Assessment of mitochondrial function after exposure to 0.5 μM and 2.5 μM As₂O₃ for 72 hours in HEK293T cells.**

HEK293T cells were exposed for 72 hours to 0.5 μM and 2.5 μM As₂O₃. (A) Three coloured overlay histogram showing the FL1 signal proportional to DiOC₆(3) uptake in the mitochondria for an untreated control, 0.5 μM As₂O₃ and 2.5 μM As₂O₃ exposure. (B) Percentage of cellular population in the hypo-polarised DiOC₆(3) range after treatment with 0.5 μM As₂O₃ and 2.5 μM As₂O₃ (C) Quantification of mitochondrial superoxide by flow cytometry. (D) Percentage of PI positive cells in late apoptosis and cell death. All bars shown are $\pm$ SD. * $p < 0.05$, $N = 3$.

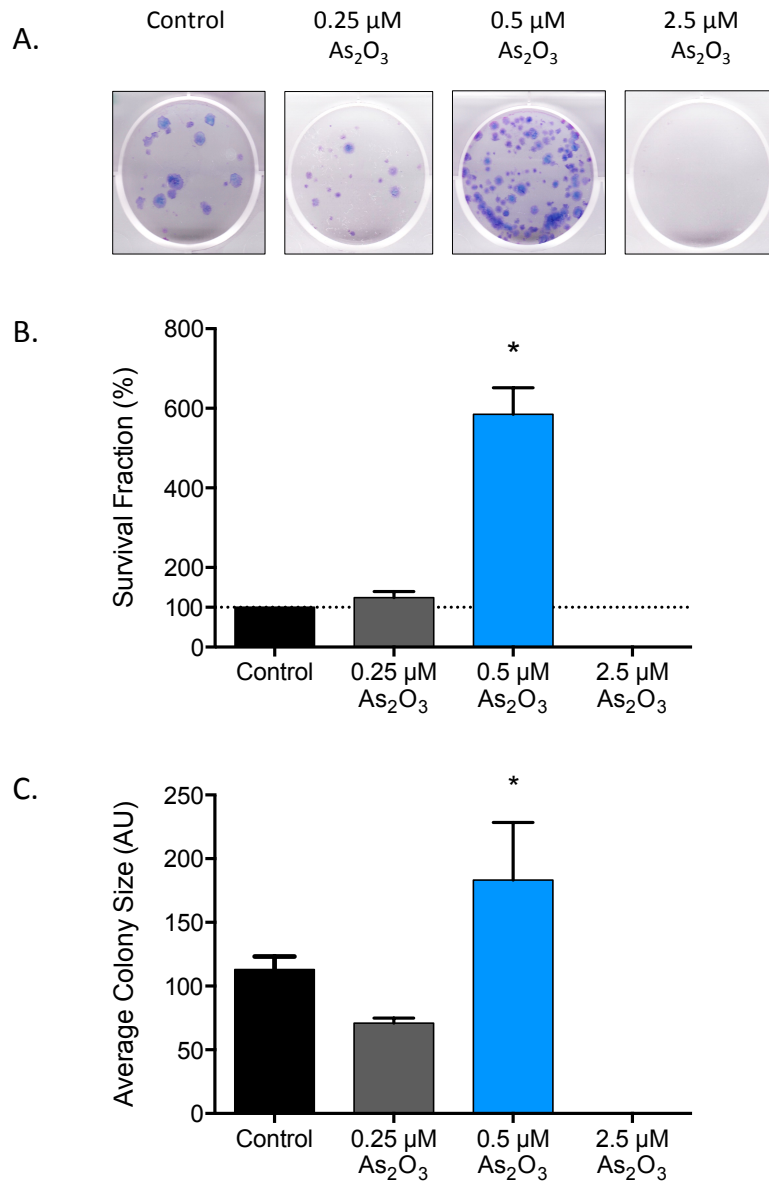
HEK293T cells were treated for 72 hours with 0.5 μM and 2.5 μM As_2O_3 prior to mitochondrial function assessment. Treatment with 0.5 μM As_2O_3 led to similar levels of mitochondrial transmembrane potential as the untreated control (Fig. 4.5, A, B). In contrast, treatment with 2.5 μM As_2O_3 led to significant hypo-polarisation in greater than 80% of cells. No changes were observed in mitochondrial superoxide levels after treatment with 0.5 μM As_2O_3 compared to the untreated control (Fig. 4.5, C). However, exposure to 2.5 μM As_2O_3 led to the generation of large amounts of mitochondrial superoxide, which was significantly higher than that of the untreated control. PI staining was able to show no discernable differences in cell death after treatment with 0.5 μM As_2O_3 , this was coupled with no significant changes in the mitochondrial transmembrane potential or mitochondrial superoxide. In contrast, 2.5 μM As_2O_3 initiated cell death in 70% of the cellular population, with mitochondrial transmembrane hypo-polarisation and an increase in mitochondrial superoxide. This mitochondrial function assessment suggested that 0.5 μM As_2O_3 was not toxic and disruptive to mitochondrial function unlike the 2.5 μM As_2O_3 . The latter concentration led to significant disruption of mitochondrial function and ultimately an increase in cell death.

The mitochondrial function analysis concluded the assays used to identify toxic dosages. By considering PARP cleavage, caspase activation and mitochondrial function all together it was clear the 2.5 μM As_2O_3 concentration was toxic and that concentrations <0.5 μM As_2O_3 were not toxic. Although the 0.5 μM As_2O_3 dosage was not toxic, additional assays were conducted to evaluate if this dosage was tolerated and one that would lead to cellular transformation.

4.1.5 – HEK293T cells tolerate both 0.25 μM and 0.5 μM As_2O_3 , however only the latter leads to increased cellular survival.

As PARP cleavage analysis led to the identification of two tolerated (0.25 μM and 0.5 μM) concentrations of As_2O_3 further assays were required to identify a dose that would lead to cellular transformation as per the aforementioned framework (Fig. 4.1).

To begin this analysis a clonogenic assay was used to assess the impact of sustained As_2O_3 exposure on cellular survival and colony size. This *in vitro* method allowed for the evaluation of cytotoxicity by examining the growth of single cells in colonies made of 50 cells and greater, and effectively tests the ability of the cellular population to undergo division and growth (Franken et al. 2006). HEK293T cells were exposed for 15-days to 0.25 μM , 0.5 μM and 2.5 μM As_2O_3 (Fig. 4.6).



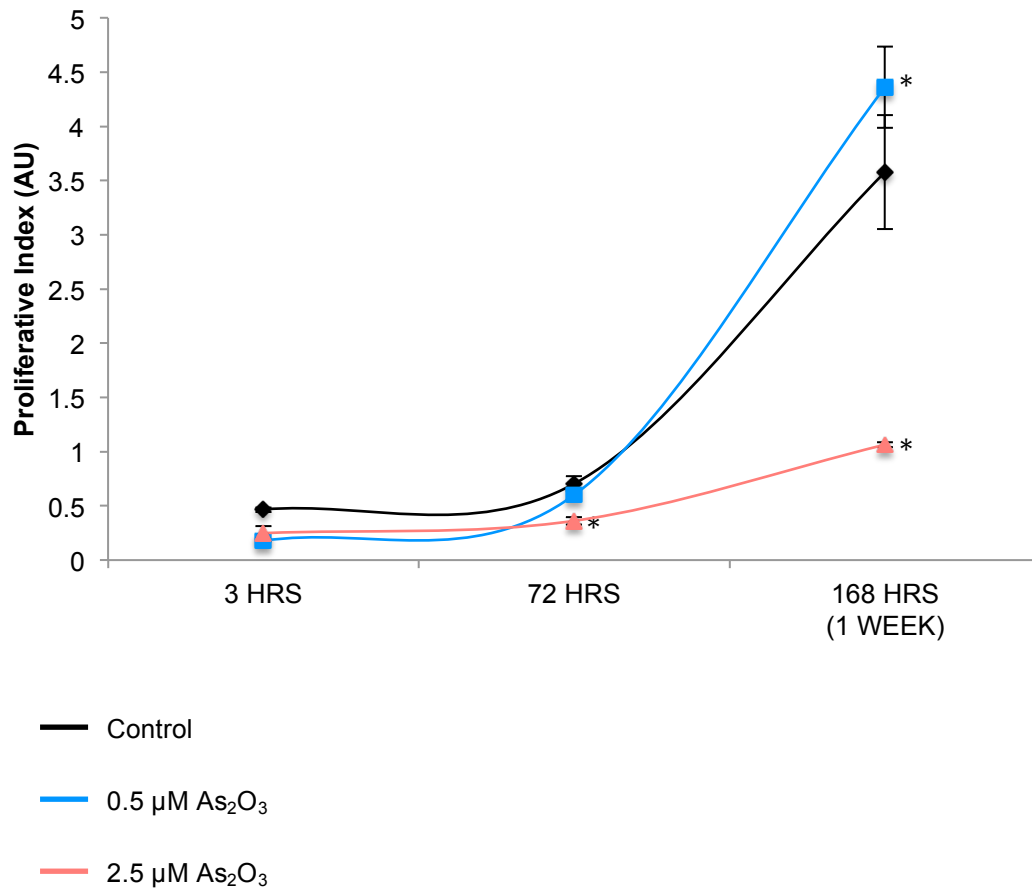
+ **Figure 4.6: Clonogenic assay of HEK293T cells exposed to As_2O_3 .** HEK293T cells were exposed for 15-days to 0.25 μM , 0.5 μM and 2.5 μM As_2O_3 . (A) Photographs of wells taken post-treatment after staining with 5% Giemsa, 25% methanol and 70% PBS solution. (B) Survival fraction after seeding 500 cells per well followed by As_2O_3 exposure. (C) Average colony size after seeding 500 cells per well followed by As_2O_3 exposure. Full calculation methods for both survival fraction and average colony size are provided in the methods section. All bars shown are $\pm$ SD. * $p < 0.05$, $N=3$.

Analysis by clonogenic assay in HEK293T cells identified increased cellular survival after a 0.5 μM As_2O_3 exposure for 15 days (Fig. 4.6, A, B). A lower dose exposure of 0.25 μM As_2O_3 , although tolerated was unable to stimulate increased cellular survival. Furthermore, exposure to 0.5 μM As_2O_3 not only generated more colonies but also larger colonies, suggesting the presence of rapid cellular proliferation compared to the untreated controls within the same exposure duration (Fig. 4.6, C).

4.1.6 – Exposure to 0.5 μM As_2O_3 leads to increased cellular proliferation in HEK293T cells.

Although elevated cellular survival was observed, an additional assay was required to examine the proliferative potential of the cells exposed to 0.5 μM As_2O_3 . This was achieved by using the WST-1 Cell Proliferation reagent (Roche) which has an advantage over other tetrazolium dyes such as MTT as the water soluble formazan product can be assayed colorimetrically without the requirement of another detergent solubilisation step and it has enhanced sensitivity (Buttke et al. 1993; Gieni et al. 1995).

HEK293T cells were exposed to 0.5 μM and 2.5 μM As_2O_3 for 3 hours, 72 hours and 1 week (Fig. 4.7). This allowed for the focused assessment of proliferation over the course of a full 1-week exposure.



+ **Figure 4.7: WST-1 proliferation assay was used to determine the proliferative index of HEK293T cells treated with As_2O_3 at 3 hours, 72 hours and 168 hours.** HEK293T cells were treated with 0.5 μM and 2.5 μM As_2O_3 . Full calculation methods for the proliferative index are to be found in the material and methods section. All bars shown are $\pm$ SD. * $p < 0.05$, $N = 6$.

No significant differences in the proliferative index were observed at the shorter time-points of 3 hours and 72 hours after treatment with 0.5 μM As_2O_3 . However after a 72-hour exposure the proliferative index was significantly reduced as a result of a 2.5 μM As_2O_3 . Between the 72-hour and 1-week time-point the rate of proliferation increased with the 0.5 μM As_2O_3 exposure making the final proliferative index measurement significantly higher than the control.

These results suggested that the 0.5 μM As_2O_3 concentration led to an increase in overall proliferation and rate after 72 hours. In contrast the 2.5 μM As_2O_3 led to a decrease in proliferation.

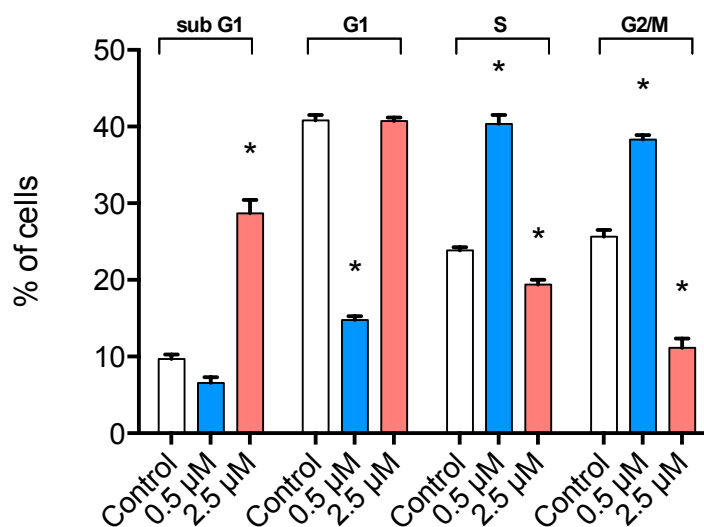
4.1.7 – Exposure to 0.5 μM As_2O_3 leads to increased progression through the G1/S and G2/M cell cycle checkpoints.

A cell cycle analysis was performed on HEK293T cells exposed to 0.5 μM and 2.5 μM As_2O_3 , to gather additional evidence of cellular properties associated with transformation. Transformed cells are capable of uncontrolled cellular proliferation. This requires the subversion of cell cycle checkpoints and sustained commitment to replicate genetic material during the synthetic phase, and separate cellular components into two daughter cells during the M phase (Malumbres & Barbacid 2001). Prior to the S phase, cells go through a sustained G1 “growth” phase during which nutrients and growth factors stimulate cells to enter the cycle from the quiescent G0 phase and continue onwards to the G1/S checkpoint.

Another crucial checkpoint is the G2/M checkpoint. This is particularly important as cells are under sustained surveillance for DNA damage in the form of double strand breaks (DSB) or single stranded DNA breaks (SSB). Under such circumstances the cell cycle is paused at the G2/M checkpoint. Recent research has suggested that the G2/M checkpoint is negligent as a

pause in the cell cycle is only introduced after a defined threshold of DNA damage (Löbrich & Jeggo 2007). This leaky G2/M checkpoint is believed to be significant in environmentally derived cancers.

To investigate the impact of arsenic trioxide exposure on the cell cycle, propidium iodide was used to stain fixed and permeabilised cells. This would allow for scoring of cellular populations within the G1, S and G2/M parts of the cell cycle. The sub G1 population was also measured via the same method which allows for the identification of internucleosomal DNA fragmentation; a hallmark of apoptosis to show the proportion of cells that are not only outside of the normal cell cycle but are undergoing cell death (Kajstura et al. 2007).



+ **Figure 4.8: Cell cycle analysis of HEK293T cells treated with 0.5 μM and 2.5 μM As₂O₃ for 72 hours.**

HEK293T cells were treated for 72 hours with 0.5 μM and 2.5 μM As₂O₃, fixed and permeabilised before staining with propidium iodide (see materials and methods for detailed information). All bars shown are ± SD. * p < 0.05, N = 3.

Treatment with 2.5 μM As₂O₃ led to a significant increase in the percentage of cells within sub G1 (Fig. 4.8). This finding was expected, as this dosage is toxic. There was no difference observed between the untreated control and 0.5 μM As₂O₃ in the sub G1 population, with less

than 10% of cells undergoing apoptosis similar to the untreated control. A significant reduction in the percentage of cells undergoing G1 growth was observed with 0.5 μM As_2O_3 and this was coupled with a significant increase in the percentage of cells in the S phase with doubled genetic material. In contrast the 2.5 μM As_2O_3 exposure had no significant impact on the percentage of cells occupying the G1 growth phase, but a significant reduction in the percentage of cells in the S phase and G2/M phase. This finding was aligned to the cell death observed at this dosage. The 0.5 μM As_2O_3 exposure resulted in a significant increase in the percentage of cells passing through the G2/M checkpoint.

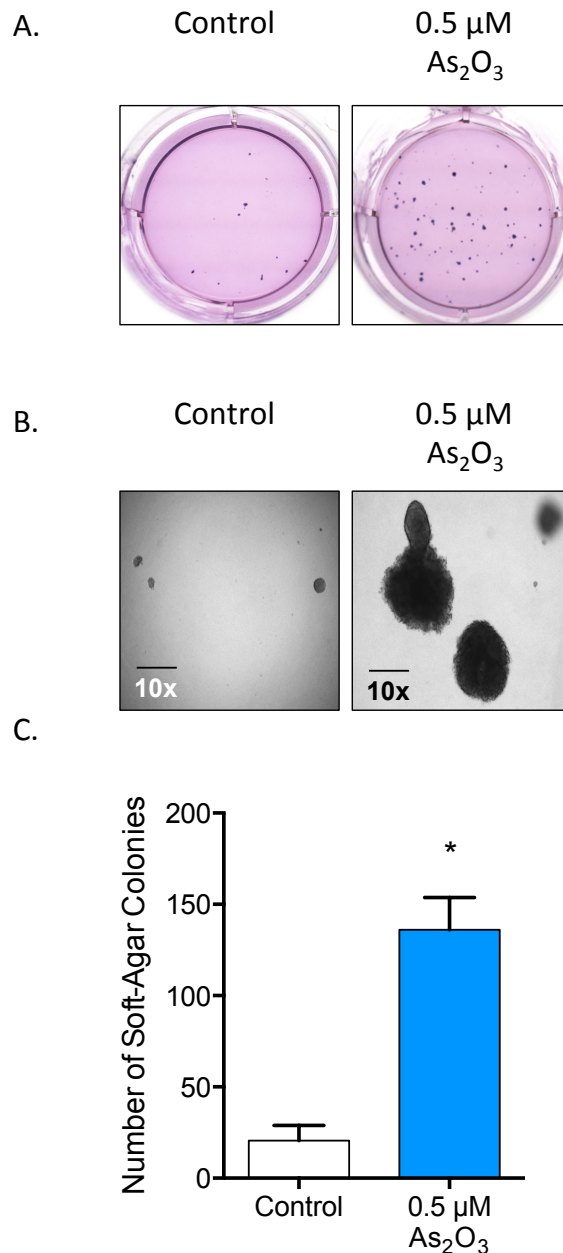
This data suggested that the 0.5 μM As_2O_3 concentration allowed for sustained and increased cellular cycling over the untreated control, whereas the 2.5 μM As_2O_3 concentration led to increased drop out of the cell cycle with a significant increase in the percentage of cells occupying the sub G1 fragmented DNA population.

4.1.8 – Exposure to 0.5 μM As_2O_3 induced anchorage-independent growth of HEK293T cells as determined by colony formation in soft agar.

Multiple cellular transformation indicators were evaluated with the 0.5 μM As_2O_3 concentration including cellular survival, cellular proliferation and cell-cycle distribution. Another significant indicator of cellular transformation is anchorage-independent growth. Normal cells undergo a form of apoptosis called anoikis when a growth surface is removed (Kopnin 2000; Frisch & Screaton 2001). In contrast, transformed cells continually grow independent of a surface anchor. This is routinely examined amongst a repertoire of cellular transformation assays to predict cellular transformation and carcinogenic potential (Creton et al. 2012).

To investigate if 0.5 μM As_2O_3 was able to induce anchorage-independent growth *in vitro*, a soft-agar colony formation assay was used. In this assay soft-agar was used to cover the bottom hydrophilic plastic coating of a tissue culture dish and then cells were seeded in semi-solid media after a 3-month exposure. This assay is considered an ideal *in vitro* indicator of malignant growth potential (Raptis & Vultur 2001).

HEK293T cells were pre-exposed to As_2O_3 for a period of 3 months prior to seeding into soft agar (Fig. 4.9). Treatment with 0.5 μM As_2O_3 resulted in a visibly clear increase in the number of colonies compared to the untreated control (Fig 4.9, A). Furthermore, inspection of the colonies under a 10 x optical zoon demonstrated that the colonies were not only larger, but some colonies had protrusions and projected growth away from the main growth colony (Fig. 4.9, B). Finally, the number of colonies in each well was counted. The 0.5 μM As_2O_3 exposure induced a significant increase in the number of colonies formed over the untreated control (Fig. 4.9, C). This suggested that 0.5 μM As_2O_3 was able induce anchorage-independent and potentially malignant growth of HEK293T cells.



+ **Figure 4.9: Anchorage-independent growth assay as determined by soft-agar colony formation in HEK293T cells treated with 0.5 μM As_2O_3 for 3 months.**
 (A) Photograph of HEK293T cells growing in soft agar after exposure to 0.5 μM As_2O_3 for 3 months. Colonies were stained with 0.005% Crystal Violet. See materials and methods for additional staining details. (B) Colonies formed in soft agar visualised under a 10 x optical magnification. (C) Number of soft agar colonies counted under a 10 x optical magnification with a hand counter. All bars shown are $\pm$ SD. * $p < 0.05$, $N = 3$.

4.1.9 – Summary: identification of a tolerated and toxic exposure of arsenic trioxide (As_2O_3) in human embryonic kidney (HEK293T) cells.

The concentration selection framework (Fig. 4.1) was used to identify a tolerated and toxic exposure of As_2O_3 in HEK293T cells. This two-step framework used a collection of assays to identify a tolerated exposure as $0.5 \mu\text{M As}_2\text{O}_3$ for exposures up to 3 months and a toxic exposure as $2.5 \mu\text{M As}_2\text{O}_3$ up to 72 hours. A summary of the results to determine this concentration selection is shown below:

- + A 1-week exposure to $2.5 \mu\text{M As}_2\text{O}_3$ led to PARP cleavage, whereas a $0.5 \mu\text{M As}_2\text{O}_3$ dosage did not (Fig. 4.2).
- + A 1-week exposure to $2.5 \mu\text{M As}_2\text{O}_3$ and higher doses including $5 \mu\text{M}$ and $10 \mu\text{M As}_2\text{O}_3$ led to gradual caspase-3 cleavage, truncation of the pro-apoptotic factor BID, phosphorylation of p53 at serine 15, decrease of MDM2 – the negative regulator of p53 and subsequently p53 stabilisation, suggesting that $2.5 \mu\text{M As}_2\text{O}_3$ was a minimal toxic dosage (Fig. 4.3).
- + Increased Caspase 3,7 and 8 activation is observed after a 72-hour exposure to $2.5 \mu\text{M As}_2\text{O}_3$, whilst reduced Caspase 3,7 activation is observed after exposure to $0.5 \mu\text{M As}_2\text{O}_3$ (Fig. 4.4).
- + A 72-hour exposure to $2.5 \mu\text{M As}_2\text{O}_3$ led to mitochondrial hypo-polarisation, a significant increase in mitochondrial superoxide generation and elevated cell death as observed by propidium iodide staining. In contrast the $0.5 \mu\text{M As}_2\text{O}_3$ exposure had no effect on the aforementioned parameters for the same exposure duration (Fig. 4.5)
- + A 15-day exposure to $0.5 \mu\text{M As}_2\text{O}_3$ led to increased cellular survival and the formation of larger colonies, whereas the $2.5 \mu\text{M As}_2\text{O}_3$ exposure gave no colony growth (Fig. 4.6).

- + A 1-week exposure to 0.5 μM As_2O_3 led to a significant increase in the proliferation of HEK293T cells. In contrast a 2.5 μM As_2O_3 exposure led to a significant decrease in proliferation (Fig. 4.7).
- + A 72-hour exposure to 0.5 μM As_2O_3 altered progression through cell-cycle checkpoints, with an increase in cells passing the G1/S and G2/M checkpoints. The 2.5 μM dosage led to a significant increase the number of cells undergoing apoptotic cell death and cell-cycle exist as observed by the increase in the sub G1 population for this exposure (Fig. 4.8).
- + A 3-month exposure to 0.5 μM As_2O_3 led to anchorage-independent growth as observed by a soft agar colony formation assay, with more and larger colonies compared to the untreated control (Fig. 4.9).

4.2 – Characterisation of Global Histone Acetylation Patterns as a Result of Tolerated and Toxic As₂O₃ Exposure in HEK293T Cells.

Once the tolerated (0.5 μ M) and toxic (2.5 μ M) As₂O₃ exposures were identified with the associated exposure durations, the next stage of this study was to characterise global histone acetylation patterns in HEK293T cells. The aim of this experiment was to assess whether or not differential histone acetylation patterns occur as a result of As₂O₃ exposure, reflecting the magnitude and duration of exposure, and the phenotypic outcome in the cells. In particular, the possibility that there are specific histone acetylation marks indicative of the carcinogenic process was investigated.

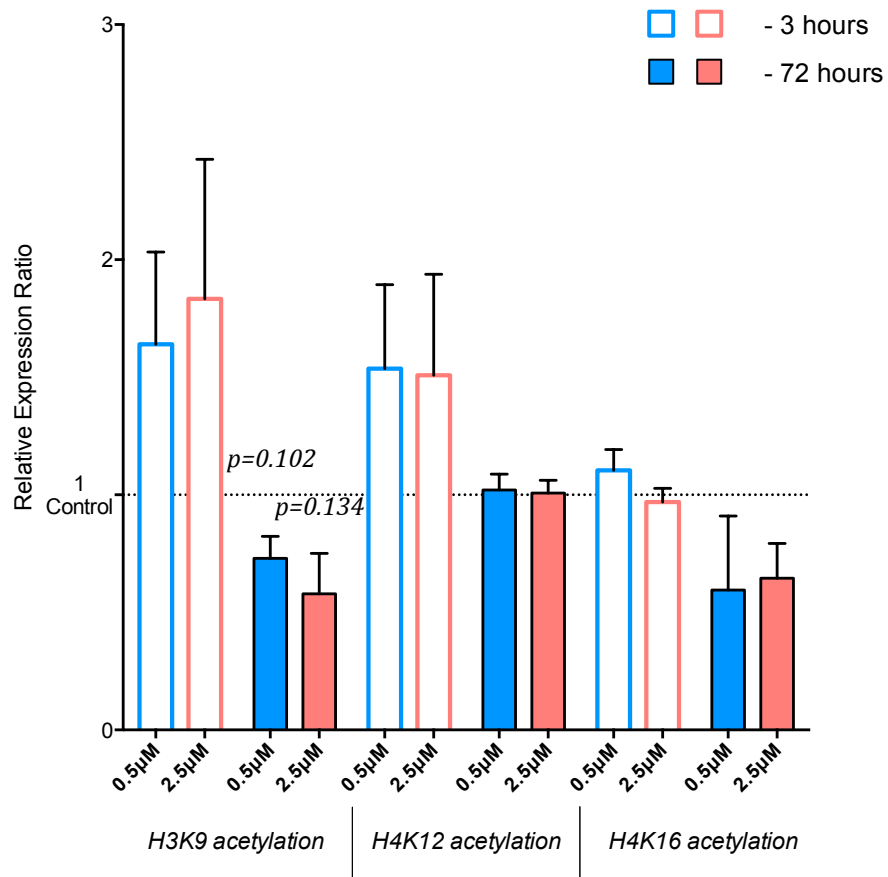
Although a wide variety of covalent modifications occur on histone tails including acetylation, methylation and phosphorylation, to name but a few, this study focused specifically on histone acetylation as it has functional consequences that impinge on transcriptional control, DNA repair, replication and condensation, making it a functionally dense chromatin mark (Kouzarides 2007). Aberrations in histone acetylation have also shown great promise as a suitable biomarker to investigate cancer progression, stratification and prognosis (Baylin & Jones 2011; Seligson et al. 2005).

4.2.1 – As₂O₃ exposure induces a temporally sensitive increase in H3K9 and H4K12 acetylation between 3 and 72 hours and a reduction in H4K16 acetylation after 72 hours.

For this present study histone H3K9, H4K12 and H4K16 acetylation was measured globally at a short-term exposure of 3 hours and compared to a longer-term exposure of 72 hours after treatment with a tolerated (0.5 µM) and toxic (2.5 µM) dosage of As₂O₃. The histone acetylation status was evaluated by immunoblotting (Fig. 4.10).

Two time points were used to identify specific arsenic-induced histone acetylation changes and to evaluate the plasticity of such events in HEK293T cells. At 3 hours, an elevation of H3K9 and H4K12 acetylation was observed upon exposure to a 0.5 µM and 2.5 µM As₂O₃. There was no change in H4K16 acetylation at 3 hours. Hypoacetylation of H4K16 was also identified at both tolerated and toxic dosages after a 72-hour exposure, with little change at the 3-hour exposure on this residue.

This experiment suggests that As₂O₃ exposure leads to temporally sensitive changes in histone acetylation as the initial increase in H3K9 and H4K12 acetylation reduces below nominal levels at 72 hours. Whilst no change is observed within 3 hours on the H4K16 acetylation status, this residue is then hypo-acetylated at 72 hours. Furthermore it suggests that the magnitude of the dosage, i.e. tolerated and toxic, has no separated direction of acetylation and rather both tolerated and toxic exposure lead in the same direction of acetylation change – hyper or hypo.



+ **Figure 4.10: Global histone H3K9, H4K12 and H4K16 acetylation in HEK293T cells exposed to As₂O₃ for 3 hours and 72 hours.** HEK293T cells were exposed to As₂O₃ for 3 hours and 72 hours to both tolerated and toxic dosages followed by histone extraction and immunoblotting. Relative expression ratios were determined by quantification of bands by densitometry analysis (see materials and methods for additional details). All concentrations along the x-axis are of As₂O₃. All bars shown are $\pm$ SD. N = 3.

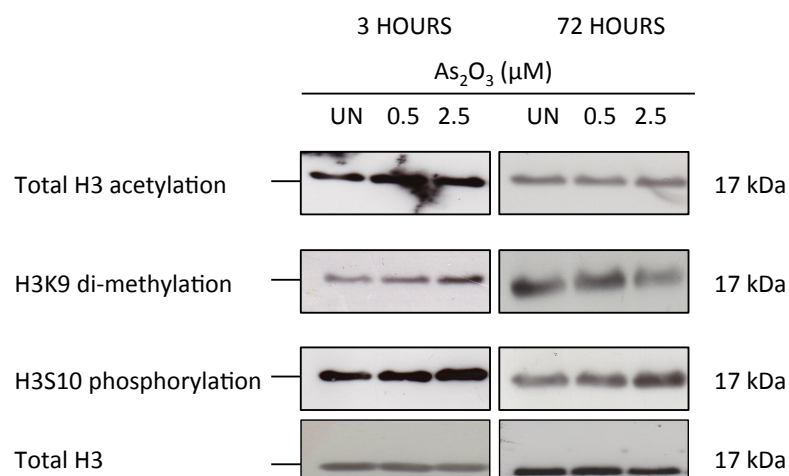
4.2.2 – As₂O₃ exposure also leads to global changes in total histone H3 acetylation, H3K9 di-methylation and H3S10 phosphorylation.

Other histone post-translational modifications were also examined including total histone H3 acetylation, H3K9 di-methylation and H3S10 phosphorylation after tolerated and toxic As₂O₃ exposure. This is due to a growing body of evidence that suggests that some histone post-translational modifications may be more or less likely to occur due to a previous event. This post-translational modification interdependency is called “crosstalk” and such events have been implicated in transcriptional control (Winter & Fischle 2010).

One such example of crosstalk is the phosphorylation of H3S10, which is believed to also assist in the acetylation of H4K16. This generates a binding site for Brd4 and P-TEFb which subsequently allows for the phosphorylation of the C-terminal domain of RNA Pol II to facilitate transcriptional elongation (J.-S. Lee et al. 2010). Furthermore, H3S10 phosphorylation is believed to be an important event in neoplastic cellular transformation (Choi et al. 2005).

H3K9 dimethylation is also in direct competition with H3K9 acetylation and forms one of the simplest examples of exclusion crosstalk as both methylation and acetylation in this case occur on the same residue (Winter & Fischle 2010). H3K9 dimethylation was also investigated, as this post-translational modification requires the availability of S-adenosylmethionine (SAM) a methyl-donating factor utilised for the metabolism of arsenic trioxide.

HEK293T cells were exposed for 3 hours and 72 hours with 0.5 µM and 2.5 µM As₂O₃ followed by histone extraction and immunoblotting for total H3K9 acetylation, H3K9 dimethylation and H3S10 phosphorylation (Fig. 4.11).



+ **Figure 4.11: Global histone H3 acetylation, H3K9 di-methylation and H3S10 phosphorylation in HEK293T cells exposed to As₂O₃ for 3 hours and 72 hours.** HEK293T cells were exposed to 0.5 μM and 2.5 μM As₂O₃ for 3 hours and 72 hours followed by histone extraction and immunoblotting. A total of 10 μg of proteins were added in each well. Total histone 3 was used as a loading control. Representative blot shown of two repeats.

A 3-hour exposure to 0.5 μM and 2.5 μM As₂O₃ resulted in a significant increase in total histone H3 acetylation. After 72 hours this increase was no longer observed. A dose-dependent increase in H3K9 di-methylation was observed at 3 hours. However at 72 hours, a reduction of H3K9 di-methylation occurred after treatment with 2.5 μM As₂O₃. At 3 hours a 0.5 μM and 2.5 μM dosage both increased the level of H3S10 phosphorylation and this increase was maintained at 72 hours for both dosages.

Both H3K9 acetylation and di-methylation increase after a 3-hour As₂O₃ exposure (Fig. 4.10/4.11). It is therefore unlikely that As₂O₃ exposure leads to post-translational variations on these residues via a crosstalk mechanism. However, it is important to note that due to the small sample size of this experiment these observations are only open to very limited interpretation.

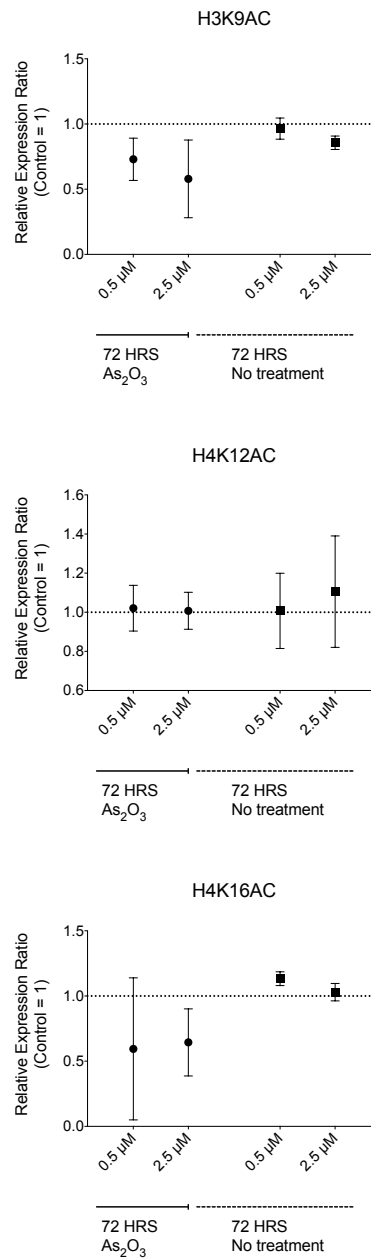
4.2.3 – As₂O₃-induced changes in H3K9, H4K12 and H4K16 acetylation are potentially reversible.

The reversibility of histone acetylation changes induced by As₂O₃ was assessed. This is because reversibility is a good marker for the causality of a specific effect, along with additional measurements such as specificity, biological gradients and consistency (Swaen & van Amelsvoort 2009).

To examine the reversibility of the global histone acetylation changes previously observed, HEK293T cells were exposed for 72 hours to 0.5 μ M and 2.5 μ M As₂O₃, after which the growth medium was changed for fresh medium with no toxicant present for a further 72 hours (Fig. 4.12). This was then followed by histone extraction and immunoblotting to evaluate histone acetylation status.

Both H3K9 and H4K16 were hypo-acetylated at 72 hours. After a 72-hour rest period without As₂O₃, the levels of acetylation appeared to normalise. H4K12 acetylation also appeared to be nominal levels after a 72-hour exposure with no real change in the acetylation status after a 72-hour rest period.

This suggests that As₂O₃-induced H3K9 and H4K16 hypoacetylation are potentially reversible events. Furthermore this data suggests that H4K12 acetylation changes may not play a significant role in As₂O₃-induced cellular transformation, rather it may have a possible involvement in an early response to the toxicant, particularly as a 72-hour exposure and a rest period does not generate substantial changes in histone acetylation.



+ **Figure 4.12: Histone acetylation profile examining the reversibility of H3K9, H4K12 and H4K16 acetylation after a 72 hour exposure to As_2O_3 followed by a 72 hour rest in HEK293T cells.**

HEK293T cells were exposed to 0.5 μM and 2.5 μM As_2O_3 for 72 hours followed by 72 hours in fresh medium without the toxicant, followed by histone extraction and immunoblotting. Relative expression ratios were determined by quantification of bands by densitometry analysis (see materials and methods for additional details). All concentrations along the x-axis are of As_2O_3 . All bars shown are $\pm$ SD. N = 3.

4.2.4 – Summary: characterisation of global histone acetylation patterns as a result of tolerated and toxic As₂O₃ exposure in HEK293T cells.

Both tolerated (0.5 µM) and toxic (2.5 µM) dosages of As₂O₃ were used to investigate the effect of toxicant exposure on the histone acetylation status on H3K9, H4K12 and H4K16 in HEK293T cells. Additional post-translational modifications on the histone tails were also investigated, including total H3 acetylation, H3S10 phosphorylation and H3K9 di-methylation. Finally a reversibility assay was conducted to investigate the plasticity of the histone acetylation changes. The results are summarised below:

- + Temporally sensitive changes in H3K9, H4K12 and H4K16 acetylation were observed in HEK293T cells after As₂O₃ exposure (Fig. 4.10).
- + At 3-hours, hyperacetylation of H3K9 and H4K12 acetylation was observed with both 0.5 µM and 2.5 µM As₂O₃, with no change in H4K16 acetylation. In contrast, a 72-hour exposure resulted in hypoacetylation in H3K9 and H4K16 acetylation with a return to nominal levels for H4K12 acetylation at both tolerated and toxic dosages (Fig. 4.10).
- + No dosage-specific effects in histone acetylation were observed, i.e. the direction of acetylation change was the same for both tolerated and toxic dosages at both time points (Fig. 4.10).
- + A 3 hour exposure to tolerated and toxic dosages of As₂O₃ led to an increase in total H3 acetylation, H3K9 di-methylation and H3S10 phosphorylation. Some of these changes were temporally sensitive, as at 72 hours, total H3 acetylation returned to nominal levels. H3K9 di-methylation was reduced specifically at the toxic exposure of 2.5 µM As₂O₃ and the profile of dose-dependent H3S10 phosphorylation remained the same at this exposure period. There was minimal evidence of epigenetic crosstalk, however due to the small sample size, these observations are limited in their significance (Fig. 4.11).

- + H3K9 and H4K16 hypoacetylation at 72-hours is potentially reversible after a 72-hour rest period without the toxicant, as the acetylation tended to return back to nominal levels (Fig. 4.12).

4.3 – Identification of the Enzymes That Control Histone Acetylation in HEK293T Cells.

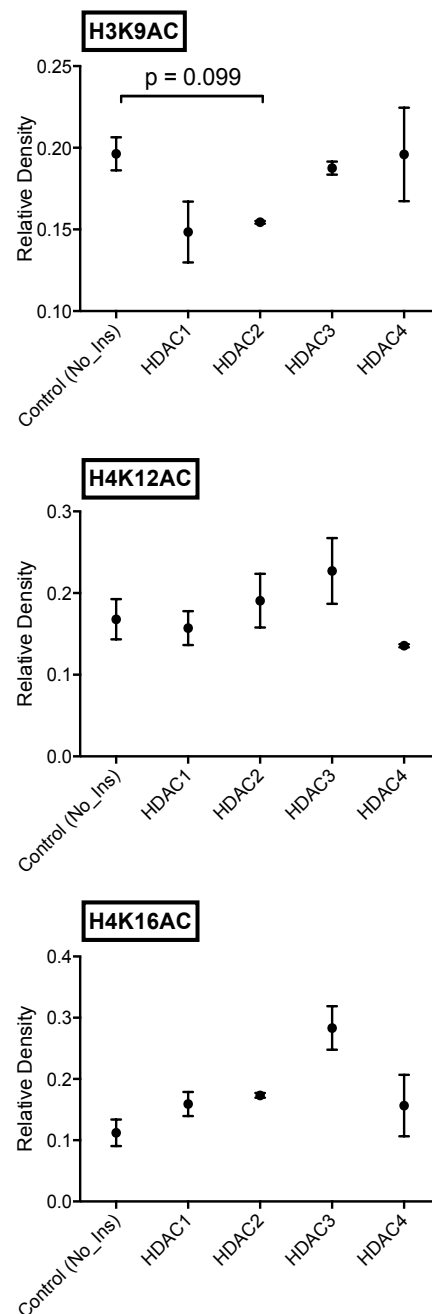
A number of lysines including H3K9, H4K12 and H4K16 were identified as being sensitive to As₂O₃ exposure. Although the separated outcomes of tolerance and toxicity were not reflected as directional differences in histone acetylation on the residues examined, there was still an impetus to identify the enzymes involved in driving the recorded acetylation changes. The reason for this was two fold. The first was that the enzymes that drive arsenic-induced global histone acetylation events have not been clearly identified, and secondly histone-modifying enzymes are involved in the transcriptional control of genes and the acetylation of other proteins; so further investigation had the potential to uncover additional mechanistic insights. Hence the next step in this study was to identify the enzymes that may be involved in facilitating the acetylation changes on these residues.

Prior research from this group identified target substrates of histone modifying enzymes by utilising transient transfections to overexpress histone deacetylases (HDACs) and histone acetyltransferases (HATs) (Hajji et al. 2010). Cells were lysed after the transfection followed by immunoblotting to identify the histone acetylation status. A similar approach was utilised in the present study.

4.3.1 – HDAC 1 and 2 deacetylate H3K9 in HEK293T cells.

HEK293T cells were transiently transfected with plasmids constitutively expressing HDAC1, 2, 3 and 4 (Fig. 4.13). The impact of overexpressing each histone deacetylase was measured on specific lysines of interest, namely H3K9, H4K12 and H4K16. The acetylation status was compared to a no-insert control.

HDAC1 and HDAC2 were effective at deacetylating H3K9 in HEK293T cells. H4K12 acetylation was not deacetylated by most of the enzymes, except for HDAC4. None of the enzymes used were effective at deacetylating H4K16 suggesting that the deacetylation of this residue is controlled by other HDACs not used here.



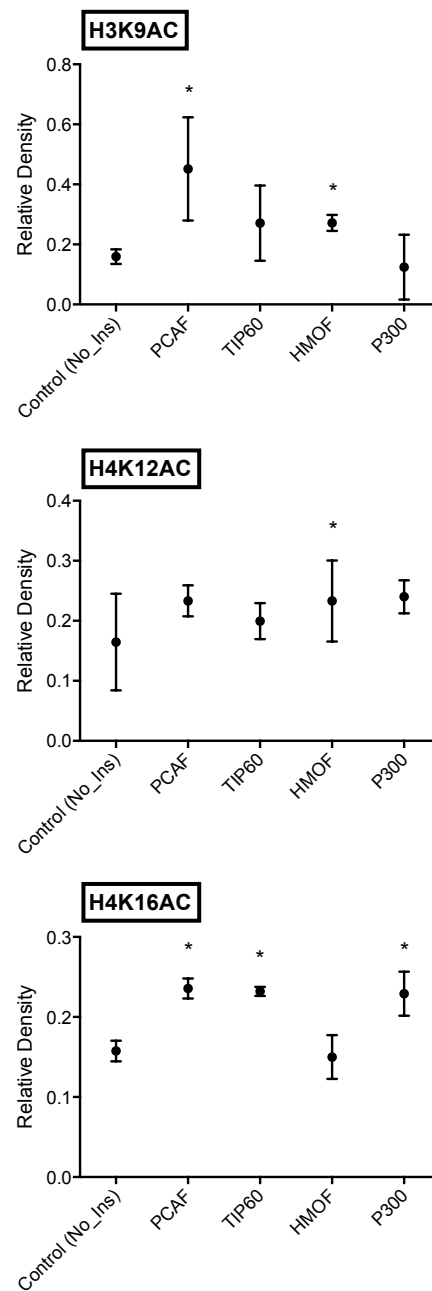
+ **Figure 4.13: Identification of histone acetylation control by histone deacetylases in HEK293T cells.**

HEK293T cells were transfected with plasmids that overexpress HDAC1, 2, 3 and 4. 20 µg of each plasmid was added to each well (see materials and methods for additional details). After transfection cells were harvested after 48-hours to ensure optimum expression of the plasmid and then subjected to histone extraction and immunoblotting to assess the histone acetylation status of H3K9, H4K12 and H4K16. All bars shown are $\pm$ SD. N = 3.

4.3.2 – PCAF and HMOF acetylate H3K9 whilst PCAF, TIP60 and P300 acetylate H4K16 in HEK293T cells.

HEK293T cells were transiently transfected with plasmids constitutively expressing PCAF, TIP60, HMOF and P300 (Fig. 4.14). This allowed for the identification of enzymes that acetylate, rather than deacetylate specific lysines. The impact of overexpressing each histone acetyltransferase was measured on the same lysines as before (H3K9, H4K12 and H4K16). The acetylation status was compared to a no-insert control.

Both PCAF and HMOF readily acetylated H3K9 in HEK293T cells. HMOF significantly increased the acetylation status on the H4K12 residue. H4K16 acetylation was the most sensitive to transfections with the HATs as PCAF, TIP60 and P300 all acetylated this residue effectively. Some redundancy was present in HEK293T cells as multiple enzymes were able to fulfil the same functional outcome in the case of both H3K9 and H4K16.



+ **Figure 4.14: Identification of histone acetylation control by histone acetyltransferases in HEK293T cells.**
HEK293T cells were transfected with plasmids that overexpress PCAF, TIP60, HMOF and P300. 20 µg of each plasmid was added to each well (see materials and methods for additional details). After transfection cells were harvested after 48-hours to ensure optimum expression of the plasmid and then subjected to histone extraction and immunoblotting to assess the histone acetylation status of H3K9, H4K12 and H4K16. All bars shown are $\pm$ SD. N = 3.

4.3.3 – Summary: identification of the enzymes that control histone acetylation in HEK293T cells.

HEK293T cells were transfected with plasmids constitutively overexpressing enzymes that deacetylate and acetylate histone residues with the intention to identify the enzymes that control the acetylation status on H3K9, H4K12 and H4K16. Cells were transfected with histone deacetylases HDAC1, 2, 3 and 4 and the histone acetyltransferases PCAF, TIP60, HMOF and P300. Cells were also transfected and treated with tolerated and toxic exposure of As₂O₃ to elucidate the impact of toxicant exposure on the functional ability of the histone modifying enzymes. The results of these assays are summarised below:

- + HDAC1 and 2 deacetylate H3K9 in HEK293T cells (Fig. 4.13).
- + PCAF and HMOF acetylate H3K9 whilst PCAF, TIP60 and P300 acetylate H4K16 in HEK293T cells (Fig. 4.14).

4.4 – Gene Expression Analysis of HDACs and HATs Under Tolerated and Toxic As₂O₃ Exposure in HEK293T Cells.

Prior experiments were able to identify the enzymes involved in the acetylation and deacetylation of specific lysines in HEK293T cells. HDAC1 and HDAC2 were shown to deacetylate H3K9 whilst PCAF and HMOF were shown to fulfil the opposing function by acetylating this residue. Both, TIP60 and P300 also had an effect on the acetylation of H4K16 in HEK293T cells.

Whilst the function of these enzymes appeared to be disrupted in the presence of As₂O₃ further assays were conducted to investigate the mRNA expression levels of these enzymes under tolerated and toxic exposure conditions and to see whether these levels could explain the variance in global histone acetylation levels observed previously. This was carried out in an attempt to understand the driving factors behind the changes in As₂O₃-induced global histone acetylation observed on the residues of interest, most notably the H3K9 and H4K16 hypoacetylation events as these residues remain hypo-acetylated after 72 hours.

4.4.1 – Increased expression of HDAC2 contributes to As₂O₃-induced H3K9 hypoacetylation at 72 hours.

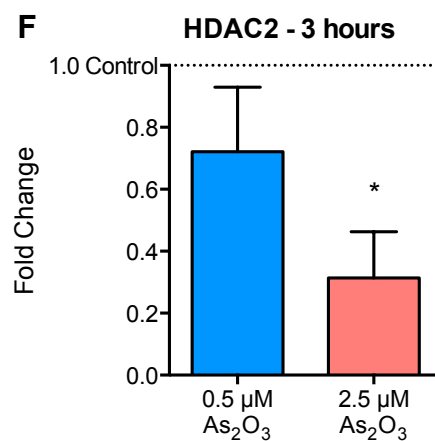
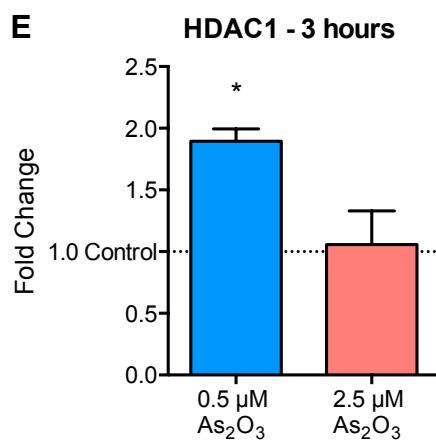
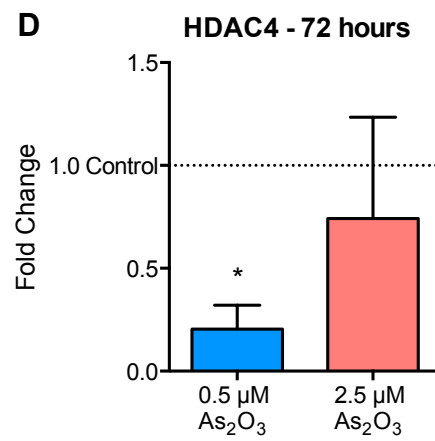
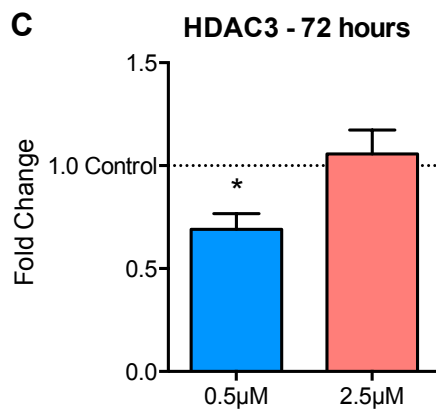
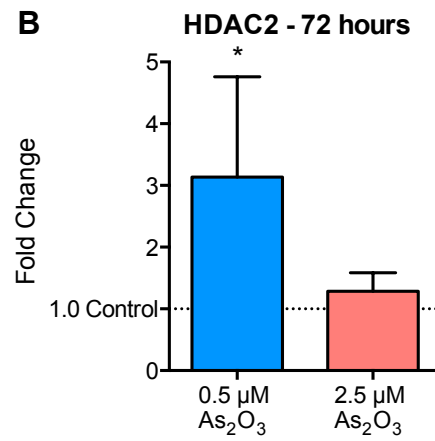
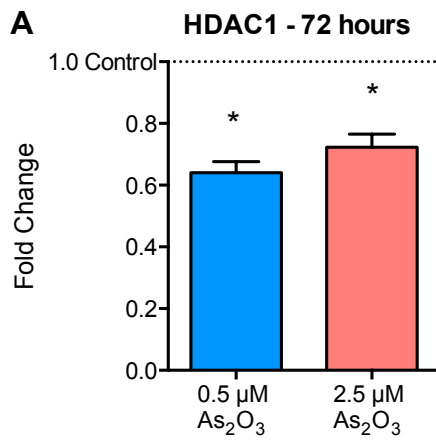
HEK293T cells were exposed to tolerated (0.5 µM) and toxic (2.5 µM) dosages of As₂O₃ for 72 hours. A shorter 3-hour exposure was also used. Cells were then harvested and subjected to a two-step qRT-PCR process, firstly total RNA was extracted followed by a reverse transcriptase reaction to convert the RNA into cDNA. To ensure effective reverse transcription of all RNA, a reverse transcriptase with a higher thermal stability which allowed for a higher reaction temperature and effective denaturation of RNA secondary structures. The cDNA formed was then used as a template for the qRT-PCR reactions. To quantify the expression of the enzymes

of interest, primers were designed to target only relevant cDNA molecules that have been synthesised from the target mRNA molecules.

Initially the expression levels of HDAC1, 2, 3 and 4 was evaluated at 72 hours. The expression levels of HDAC1 and 2 were also examined at 3 hours as these enzymes were identified previously as being instrumental in controlling H3K9 acetylation status (Fig. 4.15).

HDAC1 expression was significantly reduced at 72 hours after tolerated and toxic exposure to As_2O_3 (Fig. 4.15, A). Given that HDAC1 was previously shown to deacetylate H3K9, this finding challenged the possibility of As_2O_3 -induced H3K9 hypoacetylation to be a HDAC1 mediated event. However, at the tolerated exposure there is a significant increase in the expression of HDAC2 and a slight increase in expression at the toxic exposure (Fig. 4.15, B). This suggests that As_2O_3 -induced H3K9 hypoacetylation is driven by HDAC2. Both HDAC3 and HDAC4 expression was significantly reduced at the tolerated exposure (Fig. 4.15, C, D).

HDAC1 and 2 expression levels were also measured after a shorter 3-hour exposure (Fig. 4.15, E, F). At this time point global H3K9 acetylation was previously shown to be elevated. HDAC1 expression was significantly elevated at 3-hours after a 0.5 μM As_2O_3 dosage. HDAC2 expression was slightly reduced at both dosages and significantly reduced at the 2.5 μM As_2O_3 dosage. The reduction in HDAC2 expression levels at 3 hours is likely to assist in the global H3K9 hyperacetylation event due to reduced deacetylation.



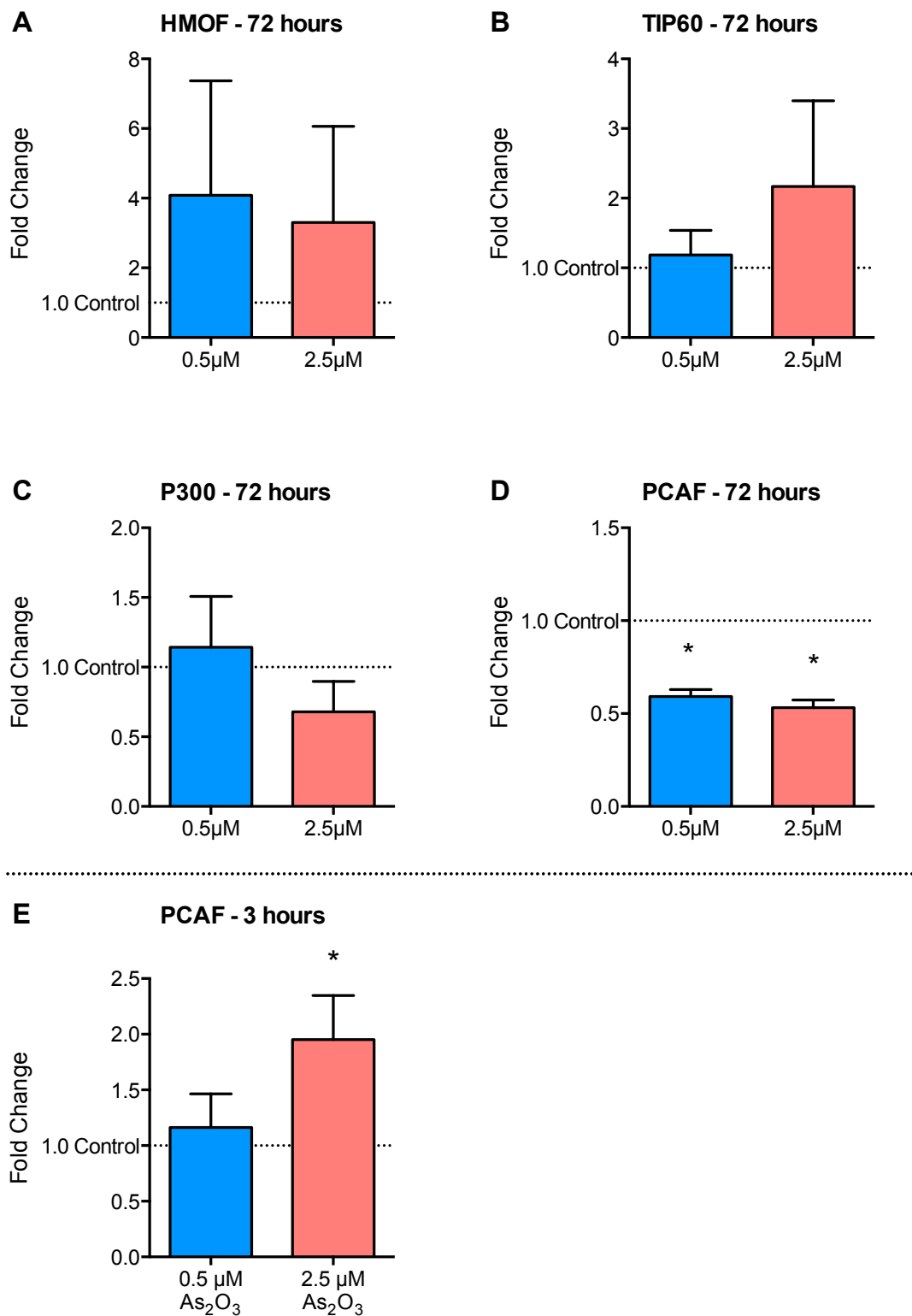
- + **Figure 4.15: Gene expression profiles of HDAC1, 2, 3 and 4 at 72 hours and HDAC1 and 2 at 3 hours after a tolerated and toxic exposure to As₂O₃ in HEK293T cells.**
(A) HDAC1 mRNA expression level at 72 hours, (B) HDAC2 mRNA expression level at 72 hours, (C) HDAC3 mRNA expression level at 72 hours, (D) HDAC4 mRNA expression level at 72 hours, (E) HDAC1 mRNA expression level at 3 hours and (F) HDAC2 mRNA expression level at 3 hours. A total of 5 µg of extracted RNA was converted into cDNA. Data was quantified via the standard curve method. Primers were designed by utilising the Primer-BLAST bioinformatic tool. See materials and methods for additional details. Data presented is fold change normalised to the untreated control. All bars shown are ± SD. N = 3.

4.4.2 – Increased expression of PCAF drives As₂O₃-induced H3K9 hyperacetylation at 3 hours and reduced expression at 72 hours assists H3K9 hypoacetylation.

HEK293T cells were exposed to tolerated and toxic exposures of As₂O₃, followed by RNA extraction, reverse transcription and qRT-PCR as mentioned previously. To begin, the expression levels of HMOF, TIP60, P300 and PCAF were evaluated at 72 hours (Fig. 4.16). The expression level of PCAF was also examined at 3 hours, as this enzyme was able to acetylate H3K9.

Whilst some gene expression changes were observed for HMOF, TIP60 and P300 these were not significant (Fig. 4.16, A, B, C). PCAF expression however was significantly reduced at 72 hours after tolerated and toxic As₂O₃ exposure (Fig. 4.16, D). Given that PCAF was shown to acetylate, H3K9 the reduced expression of this enzyme was potentially responsible for the hypoacetylation of this residue at 72 hours. To investigate this further a 3-hour exposure was able to show a small increase PCAF expression after treatment with 0.5 μ M As₂O₃ and a significant increase in expression after a 2.5 μ M As₂O₃ treatment (Fig. 4.16, E). This occurs in concert with global H3K9 hyperacetylation at the 3-hour time point.

By examining the data from the gene expression assays together, it becomes clear that HDAC2 and PCAF fulfil functionally opposing enzymatic actions on the H3K9 substrate. At 3 hours the reduced HDAC2 expression combined with the elevated PCAF expression allows for H3K9 hyperacetylation. This is inverted at 72 hours with an increase in HDAC2 expression and a reduction in PCAF expression leading co-operatively to H3K9 hypoacetylation.



- + **Figure 4.16: Gene expression profiles of HMOF, TIP60, P300 and PCAF at 72 hours and PCAF at 3 hours after a tolerated and toxic exposure to As₂O₃ in HEK293T cells.**
 (A) HMOF mRNA expression level at 72 hours, (B) TIP60 mRNA expression level at 72 hours, (C) P300 mRNA expression level at 72 hours, (D) PCAF mRNA expression level at 72 hours, (E) PCAF mRNA expression level at 3 hours. A total of 5 µg of extracted RNA was converted into cDNA. Data was quantified via the standard curve method. Primers were designed by utilising the Primer-BLAST bioinformatic tool. See materials and methods for additional details. Data presented is fold change normalised to the untreated control. All bars shown are ± SD. N = 3.

4.4.3 – Summary: gene expression analysis of HDACs and HATs under tolerated and toxic exposure to As₂O₃ in HEK293T cells.

In an effort to understand the enzymes driving histone acetylation changes on specific lysines the relative levels of HDACs and HATs were quantified by qRT-PCR following As₂O₃ exposure. Of particular interest were the enzymes involved in deacetylating and acetylating H3K9, which were previously shown to be HDAC2 and PCAF. The results of these experiments are summarised below:

- + Elevation of the HDAC2 mRNA levels assists H3K9 hypoacetylation at 72 hours (Fig. 4.15).
- + Reduction of the PCAF mRNA at 72 hours occurs in concert with an elevation of HDAC2 mRNA leading to H3K9-hypoacetylation (Fig 4.15, 4.16).
- + Elevation of the PCAF mRNA levels assist H3K9 hyperacetylation at 3 hours (Fig 4.16, E).

4.5 – Unifying Histone Modifications and Histone Modifying Enzymes to Gene Promoters via Chromatin Immunoprecipitation and Global Chromatin Conformation Analysis.

Prior experiments were able to identify a number of specific events, including As₂O₃-induced changes in H3K9 acetylation and variations in HDAC2 and PCAF mRNA expression levels. To explicitly assess the consequence of these changes on gene expression a chromatin immunoprecipitation assay was conducted. The assay determines the presence and relative enrichment of proteins binding to specific gene loci. Through the utility of this method it is then possible to investigate the transcriptional control and activation of specific promoters with a particular focus on genes that have consequences between apoptosis and cellular proliferation.

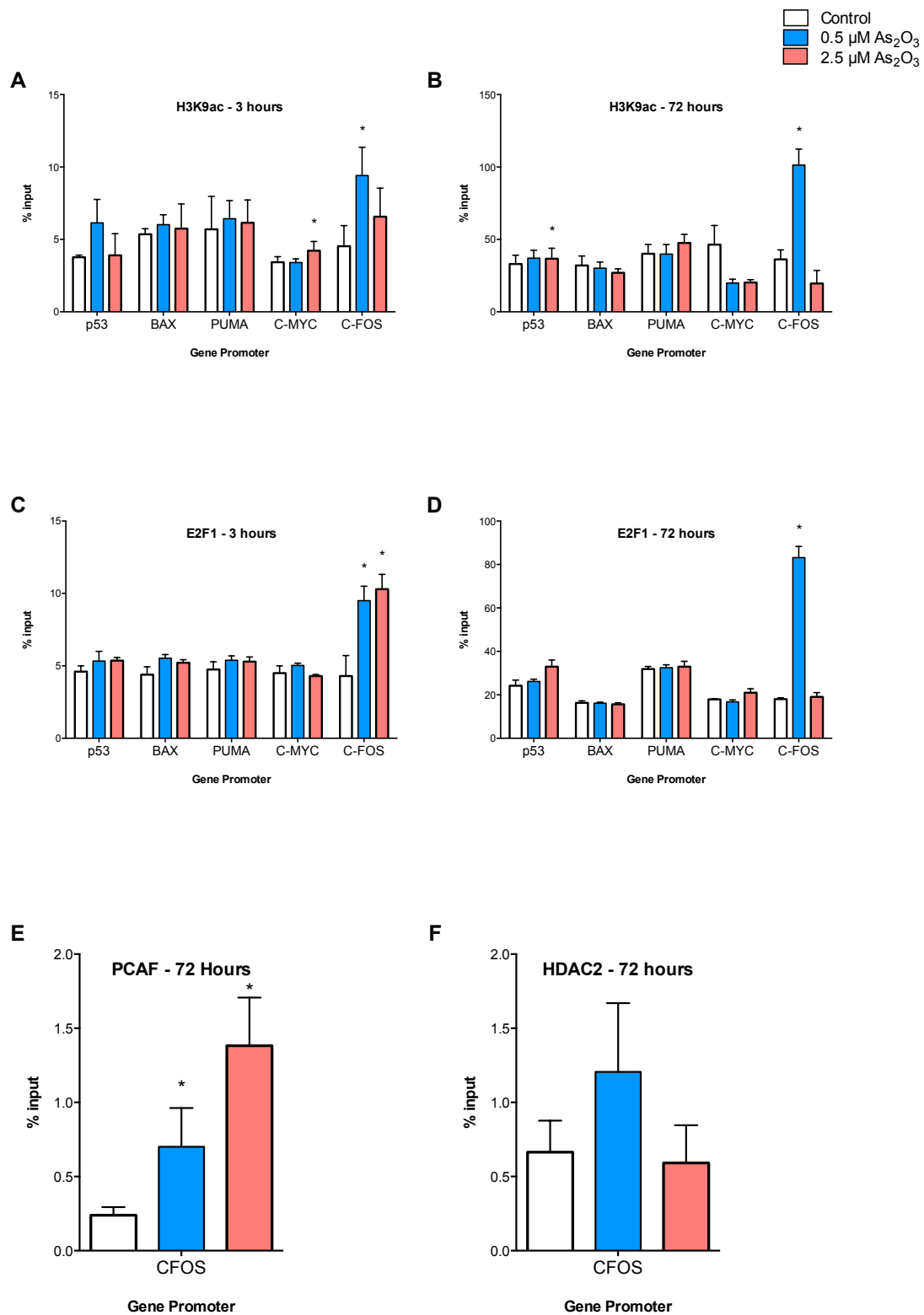
To do this a candidate transcriptional factor was also selected, as histone acetylation and histone-modifying enzymes alone would be unlikely to initiate transcription. E2F1 was chosen as it has been shown to influence decisions between cellular proliferation and cell death (Hallstrom et al. 2008). E2F1 and H3K9 acetylation interactions were examined at a number of gene promoters including the tumour suppressor (*TP53*), apoptotic genes (*BAX* & *PUMA*) and proto-oncogenes (*MYC* & *FOS*).

4.5.1 – As₂O₃-induced H3K9 acetylation and E2F1 transcription factor binding co-occurs on the *FOS* gene promoter.

HEK293T cells were exposed to As₂O₃ for 3 and 72 hours. Treatment with 0.5 µM As₂O₃, followed by anti-acetylated H3K9 antibody precipitation significantly enriched the *FOS* promoter fragment (Fig. 4.17, A). This suggested the presence of a localised increase of H3K9 acetylation at the *FOS* promoter. Using the same duration of exposure and an anti-E2F1 antibody,

enrichment at the *FOS* promoter was observed at both 0.5 μM and 2.5 μM As_2O_3 exposures, which was indicative of increased E2F1 binding at the *FOS* promoter (Fig. 4.17, C). After 72-hours, dosage specific responses were identified as significantly elevated binding to the *FOS* promoter occurred specifically at the tolerated 0.5 μM As_2O_3 exposure, with both E2F1 and H3K9 acetylated antibody pull-downs (Fig. 4.17, B, D).

The relative enrichment of PCAF and HDAC2 at the *FOS* promoter was also investigated after a 72-hour exposure to determine if these enzymes are involved in the maintenance of H3K9 acetylation (Fig. 4.17, E, F). Tolerated 0.5 μM As_2O_3 exposure increased binding of PCAF at the *FOS* promoter and also at the toxic 2.5 μM As_2O_3 treatments. No significant difference in HDAC2 binding was observed at either exposure.



- + **Figure 4.17: Tolerated As₂O₃ exposure leads to the recruitment of E2F1 and PCAF with H3K9 co-occurrence at the FOS promoter.**
 (A, B, C, D) Chromatin immunoprecipitation of H3K9ac and E2F1 using anti-acetylated H3K9 and anti-E2F1 antibodies after exposure to 0.5µM and 2.5µM As₂O₃ at two time-points, 3 hours and 72 hours. (E, F) Chromatin immunoprecipitation with anti-HDAC2 and PCAF antibodies at 72 hours. Precipitated chromatin was then subjected to qRT-PCR promoter analysis. Statistical significance is depicted by * for p<0.05 calculated by a two-tailed Student's T test compared to the untreated control group with N=3. All bars plotted are mean ± SD.

4.5.2 – Tolerated exposure to As₂O₃ leads to E2F1 recruitment, PCAF binding and H3K9 acetylation at the *FOS* promoter leading to an increase the FOS mRNA transcript.

At 72 hours and only at the tolerated dosage of 0.5 µM As₂O₃ a number of proteins (E2F1, PCAF and acetylated H3K9) are co-localised at the *FOS* promoter. QRT-PCR was carried out to investigate if these interactions are sufficient for gene expression by measurement of FOS mRNA. In addition to this a number of other proto-oncogenes were also investigated including *JUN* and *MDM2* as these genes are synonymous with cancer stem cell expansion, p53 regulation and proliferation (Ito et al. 2002; Jiao et al. 2010).

HEK293T cells were exposed to 0.5 µM and 2.5 µM As₂O₃ for 3 hours and 72 hours followed by RNA extraction, cDNA synthesis and qRT-PCR as described previously.

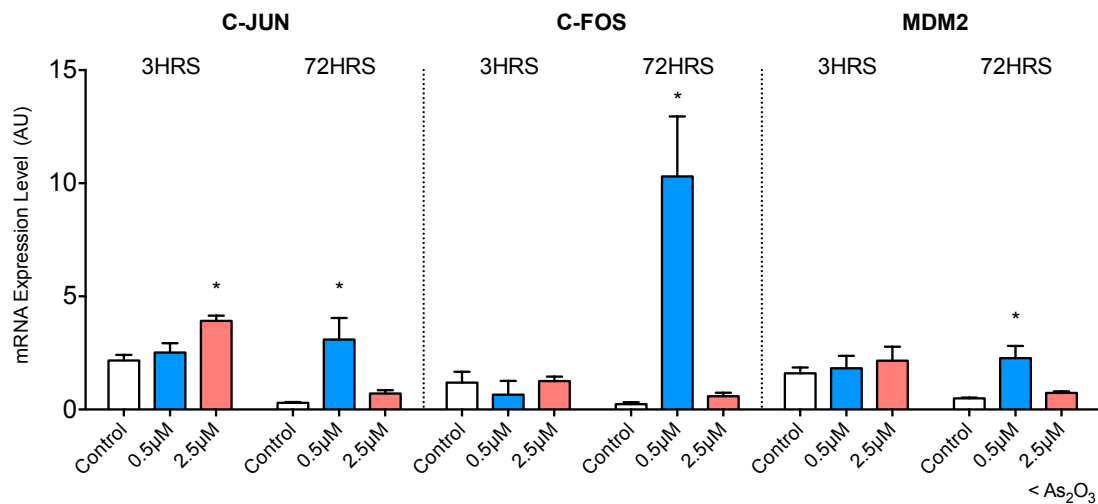


Figure 4.18: Tolerated exposure induces the expression of JUN, FOS and MDM2. Normalised expression ratios are shown for JUN, FOS and MDM2 mRNA. Total RNA was isolated from 0.5 µM and 2.5 µM As₂O₃ treated HEK293T cells to be used for the qRT-PCR analysis. Statistical significance is depicted by * for p<0.05 calculated by a two-tailed Student's T test compared to the untreated control group with N=3. All bars plotted are mean ± SD.

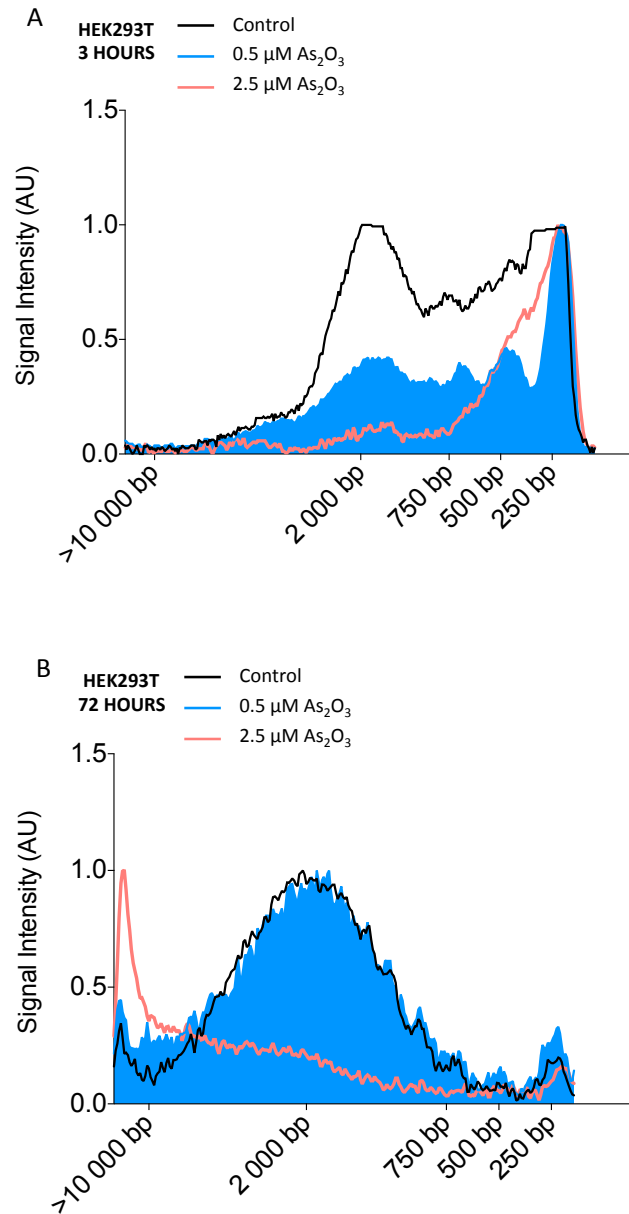
After 3-hours of exposure, the expressional changes observed are subtle with a significant increase only in *JUN* expression with the 2.5 µM As₂O₃ treatment (Fig. 4.18). After 72-hours of exposure the differences are more pronounced between the tolerated and toxic exposures. This is highlighted by the expression of *FOS*, which is highly elevated at the 0.5 µM As₂O₃ exposure. This increase was dosage-specific, as the toxic 2.5 µM As₂O₃ exposure resulted in no significant elevation. A similar expression pattern was observed for *JUN*. The expression of MDM2 mRNA was significantly increased at the tolerated 0.5 µM As₂O₃ exposure in the HEK293T.

4.5.3 – Global chromatin conformation analysis via MNase sensitivity assay demonstrates time dependent chromatin and dosage-specific chromatin conformation changes as a result of As₂O₃ exposure.

Whilst perturbations in histone acetylation have significant consequences on transcriptional control it also has an impact on global chromatin architecture. To investigate this further cells

treated with As_2O_3 were subjected to an MNase sensitivity assay which is an effective method to chart changes in higher-order chromatin structure (V. W. Zhou et al. 2010). The method relies on the relative compactness and density of nucleosomes, where areas of lower nucleosome densities are more susceptible to MNase digestion, which introduces a double strand break within nucleosomal linker regions (Anon 2005).

HEK293T cells were exposed to 0.5 μM and 2.5 μM As_2O_3 for 3 hours and 72 hours followed by MNase digestion after each time-point (Fig. 4.19). Rapid relaxation occurred after 3-hours with 0.5 μM and 2.5 μM As_2O_3 exposures as the digestion pattern was positively skewed towards the 250 to 500 base pair size range, relative to the 2,000 base pair size (Fig. 4.19, A). The 2.5 μM As_2O_3 treatment led to greater chromatin relaxation than the 0.5 μM As_2O_3 treatment after 3-hours. This relaxation was reversed after 72-hours, where the toxic 2.5 μM As_2O_3 treatment led to chromatin condensation (Fig. 4.19, B). At 72-hours the tolerated 0.5 μM As_2O_3 exposure has the same digestion pattern as the control, with the majority of the DNA in the 2,000 base pair size range, with a small elevation in the 250 base pair range compared to the control.



+ **Figure 4.19: MNase sensitivity assay on HEK293T cells treated with 0.5 μM and 2.5 μM As₂O₃ at 3 and 72 hours.**
 MNase sensitivity assay was used to assess the relative levels of chromatin relaxation between two treatments 0.5μM and 2.5μM As₂O₃. A total of 0.025 units of MNase were used to carry out digestions. (A) 3-hour time point. (B) 72-hour time point. Digested chromatin was separated on an agarose gel, photographed under a UV lamp and digitally analysed using ImageJ to plot the signal intensity against the DNA ladder.

4.5.4 – Summary: unifying histone modifications and histone modifying enzymes to gene promoters via chromatin immunoprecipitation.

Chromatin immunoprecipitation was used to unify the previous findings that As₂O₃ exposure leads to global H3K9 hypoacetylation and disruption in the expression levels of the enzymes regulating the acetylation status of this residue, namely PCAF and HDAC2. This assay was also used to investigate the recruitment of E2F1 at apoptotic and proto-oncogenic promoters. The data from these experiments are summarised below:

- + A 3-hour 0.5 μ M As₂O₃ exposure increased H3K9 acetylation at the *FOS* promoter (Fig. 4.17, A).
- + A 3-hour 0.5 and 2.5 μ M As₂O₃ dosage leads to the recruitment of E2F1 at the *FOS* promoter (Fig. 4.17, C)
- + At 72 hours, only a tolerated (0.5 μ M As₂O₃) dosage led to H3K9 hyperacetylation and E2F1 binding at the *FOS* promoter, which remains contrary to the global H3K9 hypoacetylation (Fig. 4.17, B, D).
- + Increased binding of PCAF at the *FOS* promoter was observed at the tolerated and toxic exposures at 72 hours, with no difference in HDAC2 binding (Fig. 4.17, E, F).
- + At 72 hours, only a tolerated (0.5 μ M As₂O₃) dosage leads to the co-occurrences of E2F1, PCAF and H3K9 acetylation at the *FOS* promoter and *FOS* induction with a measurable increase in *FOS* mRNA (Fig. 4.18).
- + At 72 hours, only a tolerated (0.5 μ M As₂O₃) dosage leads to the increased expression of *JUN* and *MDM2* (Fig 4.18).
- + As₂O₃ exposure at 3 hours leads to global chromatin relaxation in contrast to 72 hours where a tolerated exposure leads to nominal chromatin conformation and the toxic exposure leads to chromatin condensation (Fig. 4.19).

Results: As₂O₃-induced MicroRNA Aberrations and the Consequences on Histone Acetylation.

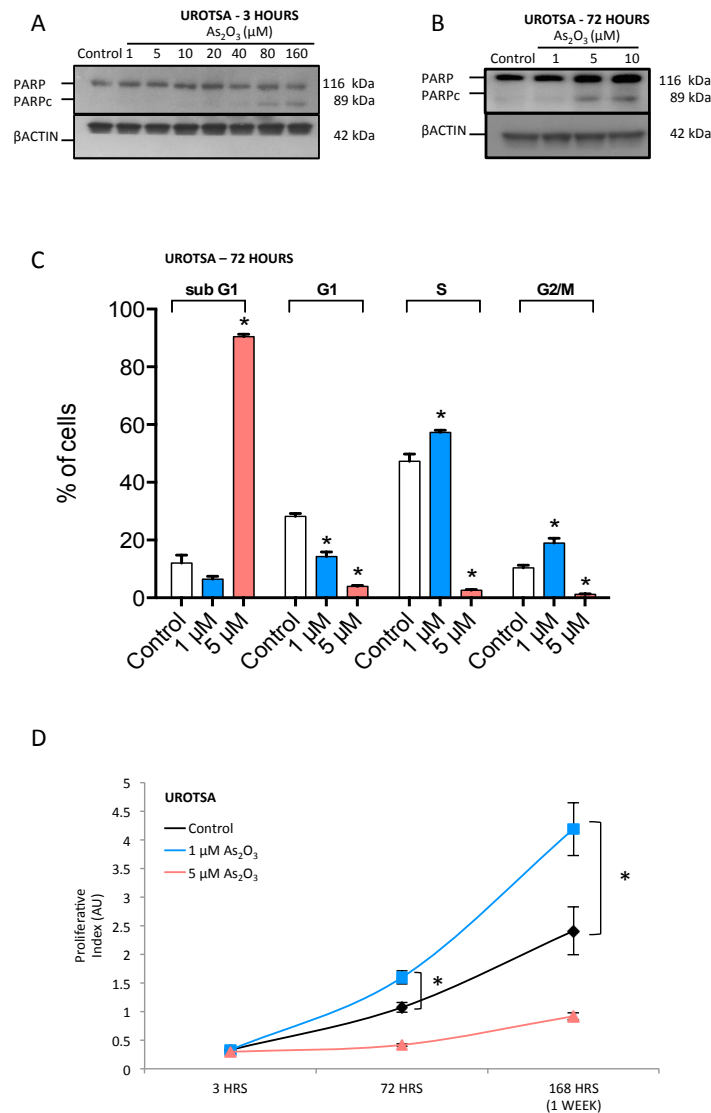
4.6 – Identification of a Tolerated and Toxic Exposure of Arsenic Trioxide (As₂O₃) in Human Urothelial (UROtsa) Cells.

The identification of tolerated and toxic dosages of As₂O₃ in UROtsa cells utilised PARP cleavage analysis, cell cycle analysis and a WST-1 proliferation assay. Similar to before, two time-points were investigated, 3 hours and 72 hours. As previously mentioned (see materials and methods), UROtsa cells are a preferential choice to investigate arsenic-induced toxicity and cellular transformation, but were only acquired towards the latter stages of this present research due to a lack of local depositories.

4.6.1 – At 3 hours, 5 µM and 80 µM As₂O₃ dosages are tolerated and toxic respectively, whereas 1 µM and 5 µM As₂O₃ dosages are tolerated and toxic respectively at 72 hours in UROtsa cells.

Time-dependent tolerated As₂O₃ exposures were identified through PARP cleavage analysis. At the 3-hour time point, 5 µM As₂O₃ was tolerated and 80 µM As₂O₃ was toxic (Fig. 4.20, A). At

the 72-hour time point, 1 μM As_2O_3 was tolerated and 5 μM As_2O_3 was toxic (Fig. 4.20, B). After 72-hours, a 1 μM As_2O_3 exposure led to a significantly redistributed cell cycle, particularly an elevated commitment to S-phase and progression through the G2/M checkpoint (Fig. 4.20, C). This was in contrast to the 5 μM As_2O_3 toxic exposure, that led to a significant increase in the sub-G1 cell population and reduced S-phase commitment and migration through the G2/M checkpoint. Positive proliferation was observed after a 72 hours and 1-week exposure to a 1 μM As_2O_3 dose, whilst 5 μM led to a reduced proliferative index compared to the untreated control (Fig. 4.20, D).



+ Figure 4.20: Identification of tolerated and toxic dosages of As₂O₃ in UROtsa cells at 3 hours and 72 hours.

(A) UROtsa cells were exposed to increasing dosages of As₂O₃ for 3 hours starting from 1 μM to 160 μM followed by protein extraction and immunoblotting for PARP cleavage. (B) UROtsa cells were exposed to increased dosages of As₂O₃ for 72 hours starting from 1 μM to 10 μM As₂O₃ followed by protein extraction and immunoblotting for PARP cleavage. Total amount of protein loaded was 80 μg. (C) UROtsa cells were treated for 72 hours with 1 and 5 μM As₂O₃, fixed and permeabilised before staining with propidium iodide (see materials and methods for detailed information). Bars shown are ± SD. * p < 0.05, N = 3. (D) UROtsa cells were treated with 1 μM and 5 μM As₂O₃. Full calculation methods for the proliferative index are to be found in the material and methods section. Curves shown are ± SD. * p < 0.05, N = 6.

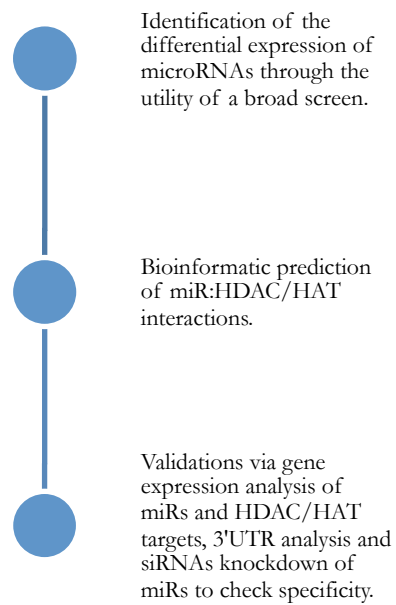
4.6.2 – Summary: – identification of a tolerated and toxic exposure of arsenic trioxide (As_2O_3) in human urothelial (UROtsa) cells.

Tolerated and toxic dosages of As_2O_3 were identified in the UROtsa cell line via PARP cleavage analysis, cell cycle analysis and a WST-1 proliferation assay. Dosages were identified for both 3 and 72 hours. The results are summarised below:

- + At 3 hours, 5 μM and 80 μM As_2O_3 dosages are tolerated and toxic respectively.
- + At 72 hours, 1 μM and 5 μM As_2O_3 dosages are tolerated and toxic respectively.

4.7 – Differential Expression of MicroRNAs and Bioinformatic Assisted prediction of miR:HDAC/HAT interactions.

A central component of this second study was to identify novel As_2O_3 sensitive microRNA mediated attenuation mechanisms of the HDAC or HAT machinery. Such mechanisms would have an explicit impact on histone acetylation. A framework was devised to identify such interactions with the assistance of bioinformatic prediction (Fig. 4.21).

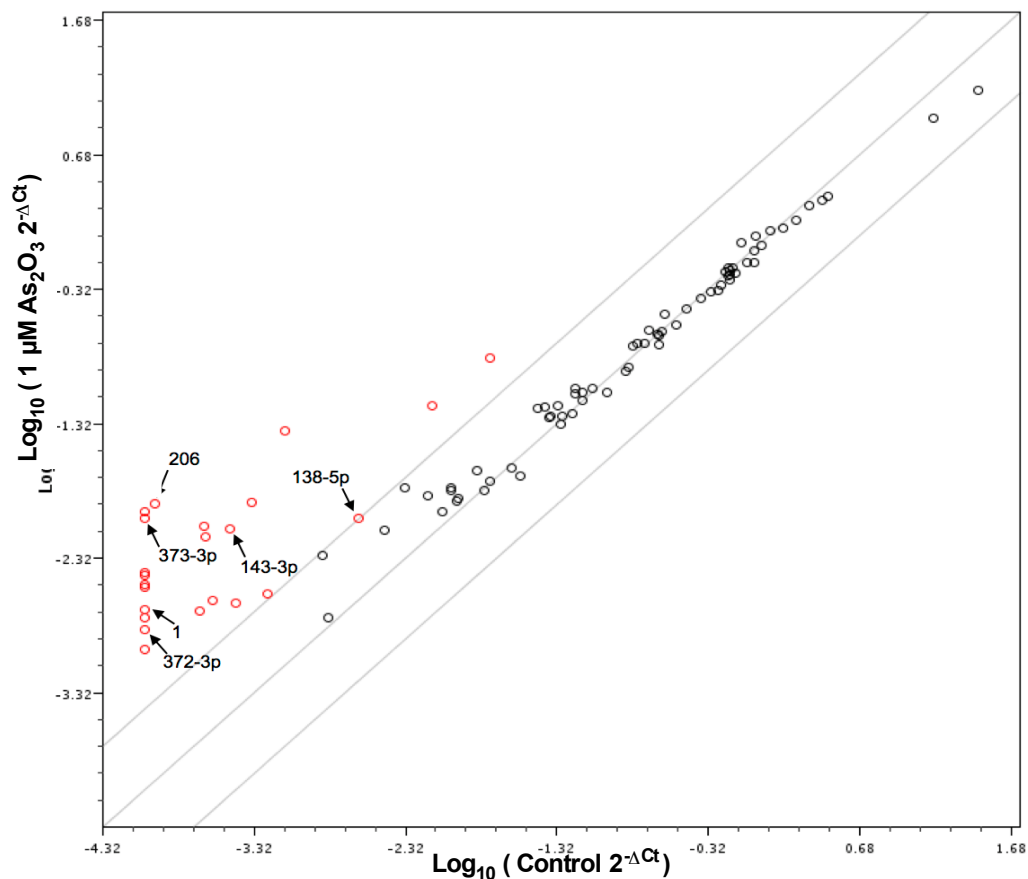


+ Figure 4.21: Framework to identify differentially expressed microRNA with potential interactions with HDAC/HATs through a bioinformatic prediction.

This research framework was used to identify differentially expressed microRNAs, predict potential interactions with HDACs and/or HATs and validate such interactions through qRT-PCR, 3'UTR reporting and siRNA knockdown of candidate microRNAs.

4.7.1 – 26 microRNAs exhibit elevated expression after tolerated exposure to 1 μ M As_2O_3 for 72 hours of which 6 were predicted to interact with histone acetylation machinery.

Using the tolerated As_2O_3 exposure identified for the 72-hour exposure a miR PCR array was used to measure the differential expression of 89 miRs against an untreated control (Fig. 4.22). A total of 26 miRs exhibited elevated expression after tolerated As_2O_3 exposure. There is a 4-fold boundary marked on the scatter plot in both directions. MiRs that are up-regulated and fall outside of the boundary are marked as red. The 26 miRs identified were then passed through the miRWalk bioinformatic program to predict HDAC and HAT targets. This predicted gene-miRNA interaction search was limited to 3'UTR miRNA binding sites for each gene, a minimum seed length of 7 and a p-value stringency set at 0.05. The miRWalk prediction was enhanced by an additional 5 prediction programs capable of similar comparative analysis including DIANA-mt, miRanda, miRDB, RNA22 and TargetScan. MiRs that were cumulatively predicted to interact by 5 or more programs are listed (Table. 9). Of the 26 miRs that exhibited increased expression, 4 miRs were predicted to interact with the HDAC4 mRNA, 1 miR was predicted to interact with the HDAC5 mRNA. All 6 programs predicted the PCAF mRNA as a potential target of miR-372. To identify other microRNAs that may not be covered by the PCR array, a bi-directional prediction approach was employed, where the PCAF mRNA was put through the miRWalk program as a target to identify additional predicted microRNA interactions. This process led to the identification of miR-106b in addition to miR-372 as potential attenuators of the PCAF mRNA (Table. 10).



+ **Figure 4.22: Scatter plot showing the differential expression of 89 microRNAs between an untreated control and a 1 μM As_2O_3 treatment in UROtsa cells.** UROtsa cells were exposed to 1 μM As_2O_3 for 72 hours followed by RNA extraction, reverse transcription of mature microRNAs followed by qRT-PCR. The broad screen PCR array used was the Human Cancer PathwayFinder (Qiagen/ SA Biosciences – CAT# MIHS-102Z). Data was processed and analysed using the Qiagen Data Analysis Centre (<http://www.qiagen.com/us/products/genes%20and%20pathways/data-analysis-center-overview-page/>, accessed: 10.10.14). Symbols in red are for those miRNAs that were up-regulated following treatment with As_2O_3 . A 4-fold boundary is marked on the scatter plot in both directions. N=1.

- + **Table 9: MicroRNAs up regulated as a result of tolerated exposure to 1 μ M As₂O₃ for 72 hours and the associated *in silico* predicted targets of the HDAC and HAT machinery.**
Key: 1 = predicted target, 0 = no predicted interactions.

Mature ID	Fold Regulation	Gene	DIANAmt	miRanda	miRDB	miRWalk	RNA22	Targetscan	SUM
hsa-miR-372	15.6	PCAF	1	1	1	1	1	1	6
hsa-miR-206	116	HDAC4	1	1	1	1	0	1	5
hsa-miR-373-3p	104	HDAC4	1	1	0	1	1	1	5
hsa-miR-143-3p	23.9	HDAC4	1	1	0	1	1	1	5
hsa-miR-1	22.1	HDAC4	1	1	1	1	0	1	5
hsa-miR-138-5p	4.07	HDAC5	1	1	0	1	0	1	4

- + **Table 10: Highest scoring predicted microRNA interactions with the target PCAF mRNA as generated by the miRWalk bioinformatic tool.**
Key: 1 = predicted target, 0 = no predicted interactions.

Gene	Mature ID	DIANAmt	miRanda	miRDB	miRWalk	RNA22	Targetscan	SUM
PCAF	hsa-miR-372	1	1	1	1	1	1	6
	hsa-miR-106b	1	1	1	1	1	1	6

4.7.2 – Summary: differential expression of microRNAs and bioinformatic assisted prediction of miR:HDAC/HAT interactions.

Through the utility of a PCR array combined with bioinformatics it was possible to identify differentially expressed microRNAs and then predict miR:HDAC/HAT interactions. The results are summarised below:

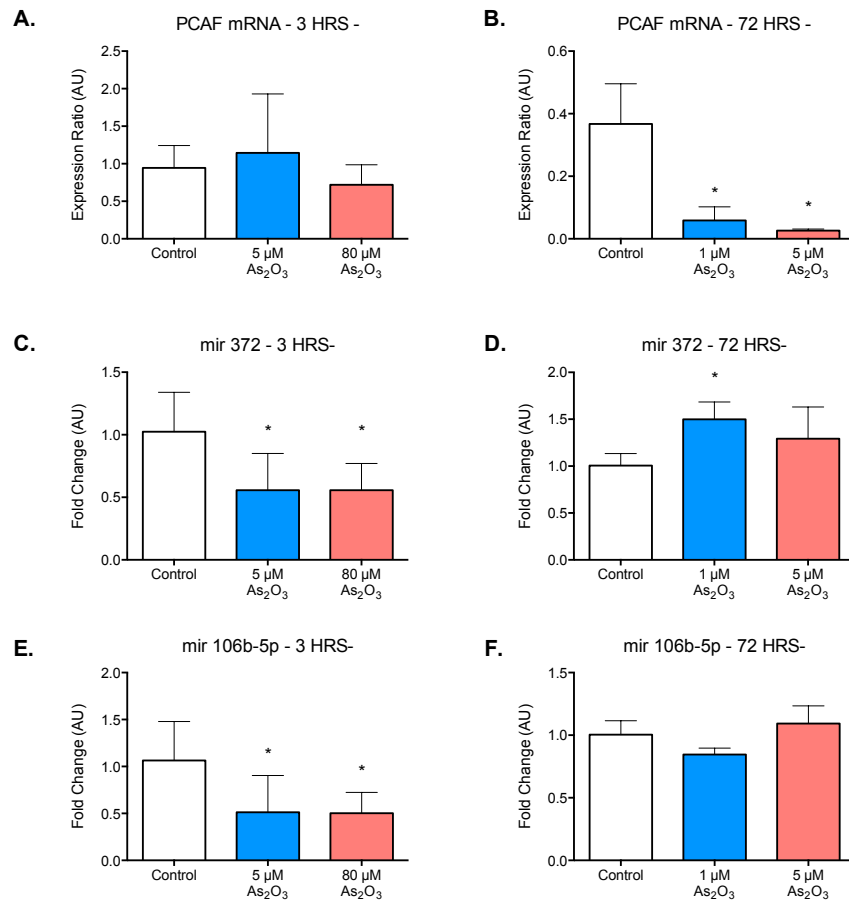
- + 26 microRNAs exhibit elevated expression after tolerated exposure to 1 μM As_2O_3 for 72 hours (Fig. 22).
- + 6 were predicted to interact with histone acetylation machinery (Table 8).
- + All 6 programs predicted the PCAF mRNA as a potential target of miR-372 (Table 8).
- + miR-106b was also identified as a potential attenuator of the PCAF mRNA (Table 9).

4.8 – Seeking an Inverse Correlation between the Expression of the MicroRNA and the Expression of its Target mRNA.

MicroRNAs are small nucleotide sequences that often act as negative regulators of gene expression by binding to the 3'UTR of target mRNAs (Krol et al. 2010). Hence a simplified hypothesis would be that the expression of a particular microRNA would be inversely correlated to that of its target. This particular relationship was investigated in the context of miR-372 and miR106b with the potential PCAF mRNA target.

4.8.1 – As₂O₃ exposure leads to a time-dependent increase in miR-372 expression and decrease in PCAF mRNA levels.

To examine the possibility of miR-mediated attenuation mechanisms occurring after As₂O₃ exposure, the relative abundance of PCAF mRNA, miR372 and miR106b was validated by qRT-PCR. Specifically there was a focus to determine the relationship between expression of the effector microRNA and its target. There was no significant change in PCAF mRNA levels after a 3-hour exposure to both tolerated and toxic doses of As₂O₃ (Fig. 4.23, A). After a 72-hour exposure PCAF mRNA levels were significantly decreased at both tolerated and toxic As₂O₃ exposures (Fig. 4.23, B). After 3-hours a significant reduction in miR-372 levels with tolerated 5 μ M As₂O₃ exposure and toxic 80 μ M As₂O₃ (Fig. 4.23, C). In contrast, after a 72-hour exposure miR-372 expression is significantly elevated at the tolerated 1 μ M As₂O₃ exposure and although not statistically significant, an elevation at the toxic 5 μ M As₂O₃ exposure is observed too (Fig. 4.23, D). MiR-106b expression was significantly reduced at 3 hours, with no significant changes at 72 hours (Fig. 4.23, E, F).



+ **Figure 4.23: MiR372, miR106b and PCAF mRNA expression levels as quantified by qRT-PCR in UROtsa cells exposed to tolerated and toxic exposure of As_2O_3 at 3 hours and 72 hours.**

(A, B) PCAF mRNA levels after exposure to tolerated and toxic dosages of As_2O_3 at 3 hours and 72 hours. Data presented as expression ratios from the standard curve relative quantification method. (C, D, E, F) miR372 and miR106b-5p levels after exposure to tolerated and toxic dosages of As_2O_3 at 3 hours and 72 hours. Data was presented as fold change and quantified via the Pfaffl method. Bars shown are $\pm$ SD. * $p < 0.05$, $N = 3$.

4.8.2 – Summary: seeking an inverse correlation between the expression of the microRNA and the expression of its target.

The expression levels of miR372, 106b and the potential target PCAF were evaluated to seek inverse correlations as a potential marker for targeted interactions. The results are summarised below:

- + The expression level of miR372 and PCAF are inversely correlated at 72 hours (Fig. 4.23, A, B, C, D).
- + The expression profile of miR106b was reduced at 3 hours, with no change at 72 hours (Fig 4.23, E, F).

4.9 – Checking for MicroRNA-mediated Attenuation of Histone Modifying Enzymes and the Outcomes on Histone Acetylation.

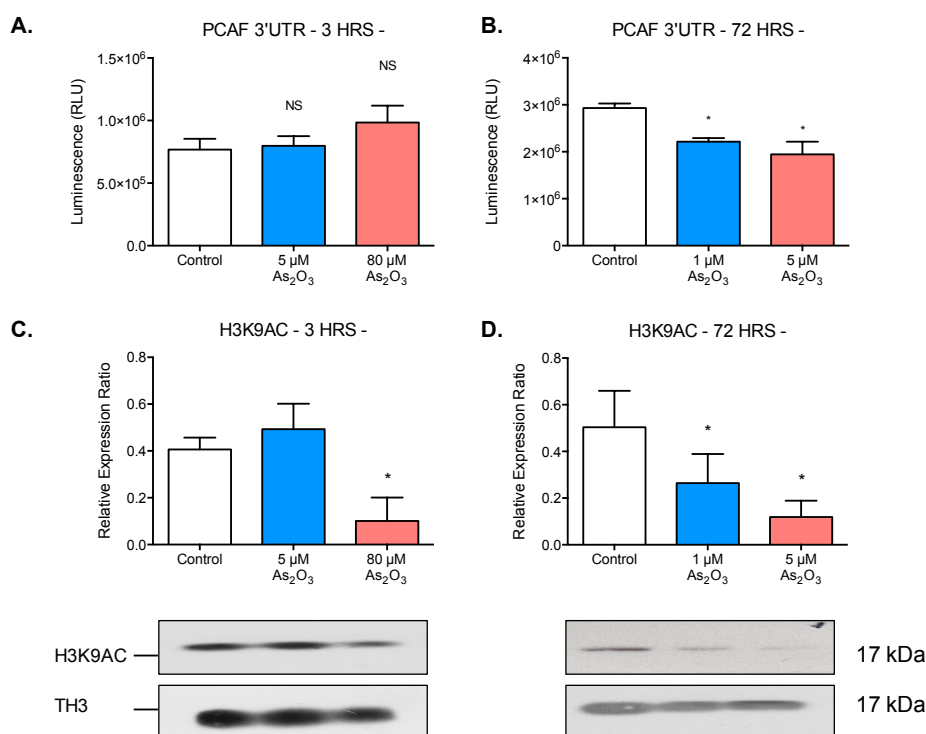
As an inverse relationship between the expression of miR372 and PCAF mRNA was identified at 72 hours, further experiments were conducted to check for PCAF mRNA attenuation via the 3'UTR. The impact of this attenuation on histone acetylation was also investigated.

4.9.1 – A temporally dependent PCAF attenuation mechanism leads to global H3K9-hypoacetylation after tolerated and toxic As₂O₃ exposure.

UROtsa cells were transfected with a PCAF 3'UTR luciferase reporter and treated with tolerated and toxic dosages of As₂O₃. At the 3-hour time point after As₂O₃ exposure no significant binding occurred at the PCAF 3'UTR leading to no significant change in luminescence from the

luciferase reporter (Fig. 4.24, A). At the 72-hour time-point a significant reduction in luminescence was observed after tolerated and toxic exposure (Fig. 4.24, B). This data suggests the PCAF mRNA is attenuated by a 3'UTR dependent mechanism and it is time-dependent due to the lack of attenuation at 3-hours.

Global H3K9 acetylation levels were also investigated. A significant decrease was observed after a 3-hour exposure to 80 μ M As_2O_3 (Fig. 4.24, C). No significant changes were noted for the tolerated exposure. At 72 hours, both the tolerated and toxic dosages led to hypoacetylation of H3K9 and this occurred together with PCAF 3'UTR attenuation (Fig. 4.24, D).

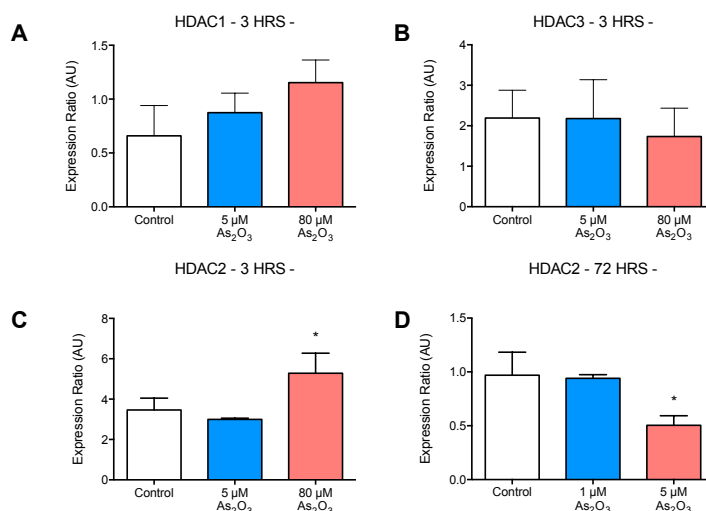


- + **Figure 4.24: PCAF 3'UTR attenuation assay and global H3K9 acetylation levels after tolerated and toxic As₂O₃ exposure in UROtsa cells.**
 (A, B) UROtsa cells were transfected with a PCAF 3'UTR luciferase reporter and a control PPIA 3'UTR luciferase reporter in a separate plate. Both were treated with tolerated and toxic exposures of As₂O₃. Luminescence measurements were taken in a GloMax 96 Microplate Luminometer. Bars shown are $\pm$ SD. * $p < 0.05$, N = 6. Luciferase activity was normalised to the PPIA 3'UTR luciferase reporter, on the second plate. A reference to the normalisation details can be found on page 88. (C, D) UROtsa cells were exposed to tolerated and toxic dosages of As₂O₃ followed by histone extraction and immunoblotting. Graphs shown were calculated by a densitometry analysis. Bars shown are $\pm$ SD. * $p < 0.05$, N = 3

4.9.2 – Elevated HDAC2 expression potentially leads to global H3K9-hypoacetylation after a 3-hour toxic 80 μ M As₂O₃ exposure.

At 3 hours there was PCAF 3'UTR attenuation yet there was a reduction in H3K9 acetylation after a toxic 80 μ M As₂O₃ exposure. To investigate the possibility that this is mediated through HDAC-dependent mechanism, the gene expression profiles of a number HDACs were investigated.

UROtsa cells were exposed for 3 hours to 5 μ M and 80 μ M As₂O₃ dosages. The expression of HDAC1, 2 and 3 was investigated via qRT-PCR.



- + **Figure 4.25: HDAC1, 2 and 3 mRNA expression levels as quantified by qRT-PCR in UROtsa cells.**
(A, B, C) HDAC1, 3 and 2 expression levels at 3 hours after exposure to 5 μ M and 80 μ M As₂O₃. (D) HDAC2 mRNA expression level at 72 hours after exposure to 5 μ M and 80 μ M As₂O₃. Bars shown are $\pm$ SD. * $p < 0.05$, N = 3

No significant change in HDAC1 and 3 expression levels are observed at 3 hours (Fig. 4.25, A, B). HDAC2 expression levels are significantly elevated at 3 hours after a toxic exposure to 80 μ M As₂O₃ (Fig. 4.25, C). This is inverted at 72 hours where the HDAC2 expression levels are significantly reduced at the toxic exposure (Fig. 4.25, D). This suggests that the H3K9 hypoacetylation observed after a toxic exposure to As₂O₃ at 3 hours is potentially driven by HDAC2 and is independent of the PCAF 3'UTR attenuation mechanism.

4.9.3 – Summary: checking for microRNA mediated attenuation of histone modifying enzymes and the outcomes on histone acetylation.

MicroRNA mediated attenuation of PCAF was evaluated by transfecting UROtsa cells with a PCAF 3'UTR luciferase reporter followed by As₂O₃ treatment. Global H3K9 acetylation was also evaluated to establish the impact of potential attenuation mechanisms. The results are summarised below:

- + PCAF 3'UTR mediated attenuation occurs after tolerated and toxic exposure to As₂O₃ at 72 hours only and this is likely to be microRNA-mediated (Fig. 4.24).
- + H3K9 is hypo-acetylated at 72 hours after tolerated and toxic exposure to As₂O₃ (Fig. 4.24, D).
- + Toxic exposure to As₂O₃ leads to H3K9 hypoacetylation at 3 hours (Fig. 4.24, C).

- + Toxic exposure to As_2O_3 potentially leads to HDAC2 driven H3K9 hypoacetylation at 3 hours (Fig. 4.25, C).
- + H3K9 hypoacetylation after toxic exposure to As_2O_3 at 3 hours is likely to be independent of microRNA mediated attenuation mechanisms.

Discussions

5.1 – Summary

This thesis describes two studies designed to explore the core hypothesis that tolerated As₂O₃ exposure leads to disruptions in HDACs, HATs, and miRs, which contribute to aberrations in histone acetylation and gene expression favouring cellular transformation. Whilst the first study examined histone acetylation explicitly, the second study focused on identifying novel “crosstalk” mechanisms between microRNAs and histone modifying enzymes, which ultimately impinge on histone acetylation.

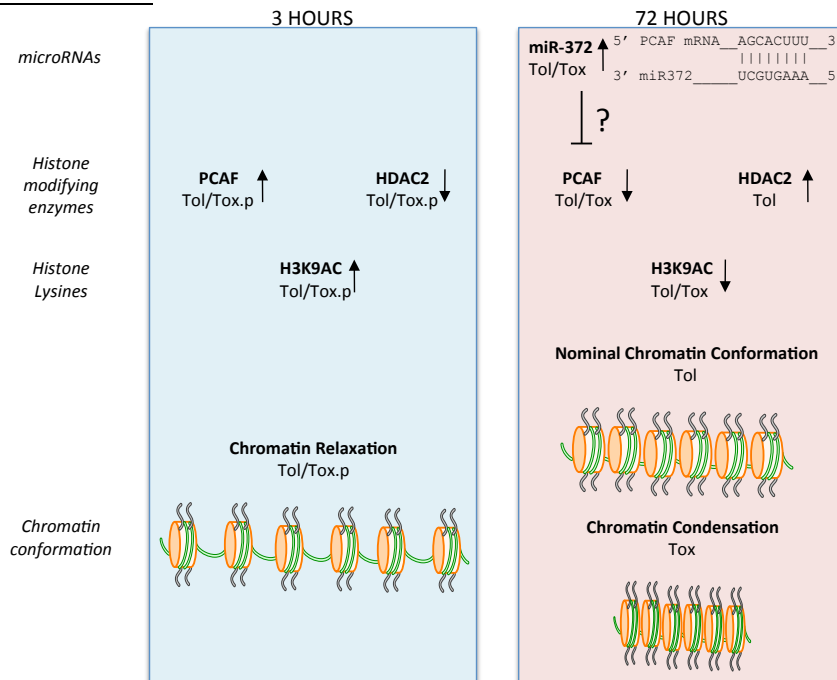
A number of assays identified concentrations of As₂O₃ that were either tolerated (0.5 µM) or toxic (2.5 µM) to HEK293T cells following exposure for 72 hours. Exposure of cells to a tolerated concentration of As₂O₃ led to increased cellular survival, anchorage-independent colony formation, cell-cycle progression and proliferation. At the same time, these cells maintained nominal mitochondrial function and intracellular function as determined using a number of markers, including but not limited to caspase activation, P53 stabilisation and PARP cleavage. This tolerated and toxic exposure led to changes in the HDAC2/PCAF mRNA expression ratio and as both of these enzymes target the H3K9 residue, As₂O₃ exposure gave rise to temporally dependent changes in global H3K9 acetylation. Global H3K9 hypoacetylation occurred at 72 hours for both tolerated and toxic exposures, giving poor mechanistic differentiation between these separated cellular outcomes. Further study led to the identification of PCAF recruitment,

E2F1 binding and H3K9 acetylation at the *FOS* proto-oncogenic promoter leading to an elevation in *FOS* mRNA levels at the tolerated concentration compared to the untreated control. This assembly of protein factors at the *FOS* promoter did not occur at the toxic concentration, and there was no significant induction of the gene and no cellular survival. Hence at the promoter-specific level it is possible to identify an epigenetic mechanism with a trajectory of cellular transformation over toxicity. These assays collectively address the aims of the first study.

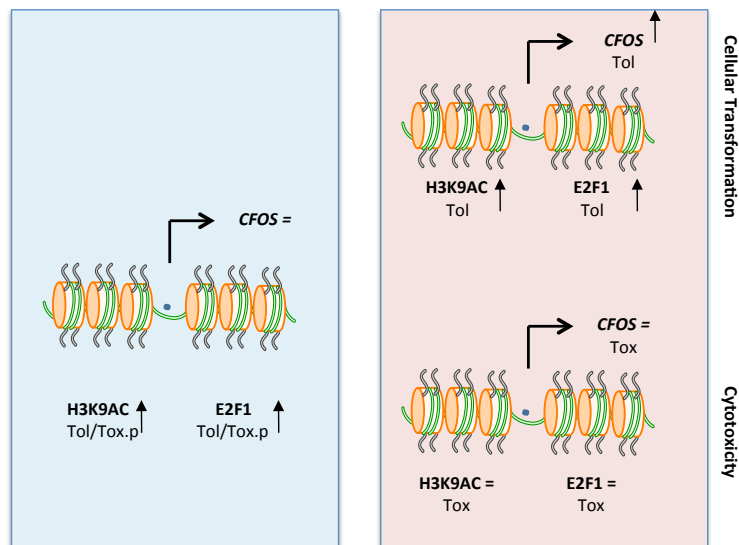
Using the human urothelial UROtsa cell line, tolerated and toxic concentrations of As_2O_3 were identified for two exposure durations – 3 hours and 72 hours. A limited repertoire of assays was used to identify these exposures focusing on PARP cleavage analysis, cell-cycle progression and cellular proliferation. Nevertheless, 5 μM and 80 μM As_2O_3 concentrations were considered tolerated and toxic respectively at a 3-hour exposure. At 72 hours 1 μM and 5 μM As_2O_3 were considered tolerated and toxic respectively. Using the longer exposure duration and a tolerated concentration of As_2O_3 , a broad PCR screen was utilised to quantify the differential expression of microRNAs that have significant roles in human cancers. Whilst microRNAs may have lots of different targets within the cell, this second study focused on identifying microRNA-mediated attenuation of histone modifying enzymes. Bioinformatic analysis of the differentially expressed miRs, of which there were 26, allowed for the identification of 6 predicted interactions with the histone acetylation machinery. Two of the six predicted interactions impinged on the histone acetyltransferase PCAF. The miRs predicted to interact were miR372 and miR106-b. Both were chosen for further analysis, as these were not previously reported to interact with PCAF. This not only helped to narrow the research profile of this study, but at the time it was considered a valuable avenue to explore given role of PCAF in the prior study. By utilising qRT-PCR it was shown that miR372 and PCAF were inversely correlated at 72 hours for both tolerated and toxic exposures, giving rise to the possibility of an attenuation mechanism. Further analysis of cells transfected with a PCAF 3'UTR luciferase reporter, demonstrated a decrease in luminescence, suggesting that a microRNA was binding to the PCAF 3'UTR leading to its degradation.

Combined with the global H3K9 hypoacetylation observed in UROtsa cells at 72 hours at both tolerated and toxic doses, it became clear that this microRNA-mediated attenuation of PCAF had consequences on global H3K9 acetylation. This study did fall short of connecting this attenuation mechanism specifically to miR372, due the absence of a combined anti-miR372 siRNA and PCAF 3'UTR luciferase reporter assay. Nevertheless, it is worth postulating that miR372 attenuates PCAF after As₂O₃ exposure for the purpose of future investigations. These experiments address the aims of the second study. A summary of the mechanistic data generated in this thesis is shown below (Fig. 5.1).

Global Effects



Promoter Specific Effects



+ Figure 5.1: Global and promoter-specific mechanisms in As₂O₃-induced cellular transformation.

Schematic mechanism outlining HDAC2 and PCAF mediated global acetylation changes in H3K9 followed by subsequent E2F1 recruitment to the *FOS* promoter. Tolerated and toxic dosages lead to separated mechanistic outcomes after 72 hours of exposure where a toxic dosage leads to cytotoxicity and chromatin condensation, whilst tolerated exposure leads to continued *FOS* expression. Tolerated and toxic dosages are abbreviated to the following: “Tol” for 0.5 μ M As₂O₃, “Tox.p” (toxic pathway) for 2.5 μ M As₂O₃ at 3 hours and “Tox” for 2.5 μ M As₂O₃ at $\geq$ 72 hours in this schematic. “Tol” and “Tox” for the miR372 are 1 μ M and 5 μ M As₂O₃ respectively.

5.2 – The Concentration Selection Framework.

Central to this course of research was to identify As₂O₃-induced epigenetic perturbations, with an awareness of the associated physiological outcomes of these changes. An *in vitro* framework was used to choose a tolerated concentration of As₂O₃ prior to epigenetic characterisation; this would ensure epigenetic information is appropriately contextualised with a given outcome.

This framework was designed to identify suitable concentrations and durations of exposure to use when investigating the epigenetic events contributing to arsenic-induced carcinogenesis. Whilst many studies have investigated mechanisms of arsenic-induced carcinogenesis in cellular models, a wide range of concentrations have been used, such as 0.001 – 10 µM for As(III) and 0.05 – 3 µM for MMA(III) (Arrigo 1983; Jensen et al. 2008; J. Li et al. 2003; Chu et al. 2011; Jo et al. 2009). The key rationale behind some of these concentrations is that 1 – 10 µM As(III) equates to a real-world plasma level of 250 – 1000 µg/L, associated with arsenic-induced illnesses (X. Zhou et al. 2008).

Some studies have focused on using low concentrations and longer exposures. For example, human keratinocytes (HaCaT) treated with 0.5 µM and 1 µM arsenite for 20 passages allowed for the detection of chromosomal abnormalities (Chien et al. 2004). Treatment of human uroepithelial cells (SV-HUC-1) with a variety of concentrations and arsenic species for 25 passages led to the identification of 11 hypermethylated genes, whilst treatment of UROtsa cells with 50 nM MMA(III) for 12 weeks revealed global hypoacetylation of histones (Chien et al. 2004; Su et al. 2006; Ge et al. 2013). These studies were anchored in cellular transformation as an endpoint, as shown by cellular aggregation assays, cellular size measurements, anchorage independent growth, colony formation and tumour formation on injection of arsenic treated cells into nude mice.

There is no fixed paradigm of cellular transformation assays to use when constructing a concentration selection framework for such studies. Rather than focusing purely on cellular transformation, this thesis utilised a two-component framework in which toxic exposures (pro-anticancer) were distinguished from those associated with tolerance and transformation (pro-cancer). This approach allowed for a scientific contribution to the debate regarding the paradoxical effects of arsenic trioxide as both a potent anticancer agent and a carcinogen. Whilst it may be effective for some malignancies, As₂O₃ treatment may not be appropriate for kidney and urothelial cancers as studies reported here have shown tolerated exposures may lead to cellular transformation rather than cell death. The dichotomy of arsenicals as potent cancer treatments and as cancer-causing compounds is still under intense investigation; hence any insights into epigenetic aberrations leading to toxicity over tolerance and vice versa would be advantageous.

5.3 – Cellular Physiological Outcomes After Tolerated and Toxic As₂O₃ Exposure.

One of the clearest outcomes of toxic As₂O₃ exposure is caspase activation and cell death. Arsenic trioxide-induced cell death is well studied as its a very effective chemotherapeutic particularly in the treatment of leukaemia (Soignet et al. 1998). Exposure of cells to 6.25 µM As₂O₃ leads to the activation of a number of caspases including Caspase-3, -7 and -9, PARP cleavage and cell death in pancreatic cancer cells (X. Li et al. 2003). A similar response was observed in this study with a toxic 2.5 µM As₂O₃ exposure for 72 hours, resulting in an increase in Caspase-3, -7, -8 activity, PARP cleavage and cell death. This suggested that the HEK293T cell line had a nominal apoptotic response to a toxic exposure of As₂O₃. At a tolerated exposure, there was no significant increase in Caspase-3 and -7 activation, rather a significant reduction was observed at this concentration. This physiological effect is a favourable outcome for cellular survival and transformation.

Although a significant reduction in Caspase-3 activity following arsenic exposure has not been reported before, one study investigated apoptotic mechanisms in normal human keratinocytes (NHK) using both low and higher concentrations of As₂O₃. This study demonstrated that at 0.5 – 32 nM As₂O₃ exposure there was no change in Caspase-3 activation compared to the untreated control, whereas higher exposure of 0.5 µM - 10 µM As₂O₃ leads to Caspase-3 activation and cell-cycle arrest at the G2/M checkpoint (Bi et al. 2010). However, the role of Caspase-3 in As₂O₃-induced cell death has been shown to be less important than previously expected. In the study by Scholz *et al.* they demonstrated the presence of a caspase-independent necrotic cell death pathway that propagates via the mitochondria as a result of As₂O₃ exposure (Scholz et al. 2005). Similarly in the present study, Caspase-3 processing and activation are weak after As₂O₃ exposure and z-VAD-fmk, a broad-spectrum inhibitor of caspases did not inhibit cell death. This suggests that an alternative caspase-independent cell death pathway is available, but in the case of a tolerated exposure, neither the core-apoptotic or necrotic pathways are utilised by the exposed cells to initiate cell death and avoid cellular transformation.

An increase in Caspase-8 activation is also observed at the tolerated concentration, yet the data suggests that this is not sufficient to continue the cascade further to Caspase-3 activation, PARP cleavage and cell death. Prior research has demonstrated that As₂O₃-induced apoptosis does indeed occur via the FAS/FAS ligand pathway (G.-F. Yang et al. 2010; Liao et al. 2004). Although the initiator Caspase-8 is upstream to the effector Caspase-3 in this pathway, it has been shown that moderate activation of Caspase-8 can occur without further activation of Caspase-3 – specifically after a 48-hour exposure to 1 µM sodium arsenite in normal human keratinocytes (Liao et al. 2004). Together these findings suggest that tolerated As₂O₃ exposure leads to a partial apoptotic response that is not sufficient to initiate cell death via the executioner activity of Caspase-3, hence cells remain alive in the presence of As₂O₃.

Tied intimately with caspase activation is mitochondrial dysfunction. As expected, a tolerated 0.5 μM As_2O_3 exposure does not lead to mitochondrial depolarisation or the generation of mitochondrial reactive oxygen species. Therefore at the tolerated exposure mitochondrial function is nominal. In contrast, the toxic 2.5 μM As_2O_3 exposure significantly increases mitochondrial ROS in addition to mitochondrial membrane depolarisation. Under this circumstance mitochondrial ROS and depolarisation led to cell death. In the review by Shi *et al.*, the two prevailing ideas regarding the source of arsenic-induced ROS are that it originates from the mitochondria, as arsenic-induced intracellular ROS can be diminished by treatment with rotenone and inhibition of NADH oxidase or from arsenic-derived intermediates that originate from the metabolic processing of the toxicant (H. Shi et al. 2004). Arsenic-induced ROS is believed to contribute to both carcinogenicity and toxicity. In the context of carcinogenicity, normal murine keratinocytes (HEL30) exposed to 10 μM and 50 μM sodium arsenite demonstrate elevated proliferation via interleukin-1 α , a growth factor which is transcribed with the assistance of redox sensitive transcription factors AP-1 and NF κ B (Corsini et al. 1999). With regards to toxicity, exposure of human hepatocellular carcinoma cells to 2.5 – 50 μM As_2O_3 leads to the increased induction of ROS within 24 hours, DNA damage as measured by comet assay, and cytotoxicity (Alarifi et al. 2013). As the data presented in this thesis focused purely on measuring the mitochondrial ROS, it does not contribute to the debate regarding the origin of the ROS, however it does demonstrate that at the tolerated exposure, an elevation of mitochondrial ROS is not required for cellular transformation.

The remaining cellular physiological outcomes after As_2O_3 exposure were measures of cellular transformation including cellular survival, proliferation, colony formation in soft agar and cell cycle analysis. Similar to this thesis, some studies have utilised an XTT assay, which is similar to the WST-1 assay to measure cellular proliferation, clonogenic assays to investigate cellular survival and the soft-agar assay to examine anchorage-independent growth. Human keratinocytes exposure to 1 μM arsenic for 72 hours led to an increase in cellular proliferation as demonstrated

by XTT assay and BrdU assimilation (C.-H. Lee et al. 2011). A significant proliferative increase was observed after tolerated 0.5 μM As_2O_3 exposure in the HEK293T cell line after 1 week. Although this is a longer exposure to the mentioned study, similar results were observed when using the UROtsa cell line, where a 1 μM As_2O_3 exposure for 72 hours also resulted in a significant increase in proliferation. HaCaT cells exposed to 100 nM arsenite for 20 weeks showed an increase in soft agar colony formation, and the increased expression of metalloproteinase-9 an enzyme crucial in assisting the ability of cells to invade the extra-cellular matrix (Sun et al. 2009). Human airway epithelial (NuLi-1) cells exposed to 1 μM As_2O_3 for 34 weeks also exhibited anchorage-independent growth (Y. Liu et al. 2014). These findings agree well with what is presented in this thesis, as HEK293T cells exposed for 12 weeks to 0.5 μM As_2O_3 exhibited anchorage-independent growth with significantly more colonies than passage matched untreated controls.

The data presented in this thesis regarding cellular proliferation and the magnitude of arsenic exposure can be classified as a hormetic response due to the non-linear relationship between the variables (Cook & Calabrese 2006). It is hypothesised that a hormesis signalling circuit is structured so that the a variable – such as cellular proliferation, can be altered via a sensor protein that can detect the environmental stress and recruit transcription factors to specific genes to modulate a physiological response (Q. Zhang et al. 2008). In the case of a toxicant such as arsenic, this response may counteract the toxic response seen at higher doses. Some recent research using *C. elegans* suggests that a hormetic response following arsenic exposure is related to a transient increase in ROS, as the use of a ROS scavenger was able to attenuate a *C. elegans* survival response (Schmeisser et al. 2013). Schmeisser et al., also reported an increased expression of mitochondrial and stress response genes following a 48 hour 100 nM arsenite exposure. Furthermore, 67.1% of the genes also had transcription factor consensus motifs for SKN-1, an orthologue of NRF2. Zhang et al., state that NRF2 can bind to antioxidant response elements which are situated in the promoter regions of many antioxidant genes allowing for

elevated antioxidant capacity and the restoration of a redox equilibrium (Q. Zhang et al. 2008). It is possible that the mitochondrial ROS profile seen at 72 hours following a 0.5 μM As_2O_3 presented in this thesis is actually the restored redox equilibrium. Interrogation at an earlier time point may have been able to capture the ROS levels in flux prior to restoration. The increased expression of PGC1- α , NRF1 and mtTFA all of which have been implicated in mitochondrial biogenesis and function has been observed in biopsies collected from patients with arsenic-induced skin cancers (C.-H. Lee et al. 2011). Chih-Hung Lee et al., also demonstrate a similar effect in primary keratinocytes treated with arsenic at concentrations less than 1 μM , along with an elevation in mitochondrial biogenesis, intracellular ATP levels and mitochondrial oxygen consumption rate all of which contribute to aberrant proliferation.

Tolerated exposure to As_2O_3 led to a redistributed cell cycle, with significant increases in the proportion of cells progressing through the G1/S and G2/M checkpoints for both HEK293T and UROtsa cell lines. This tessellates with existing evidence that demonstrates how low dose (0.01 – 1 μM) As_2O_3 exposure leads to cell cycle progression in normal breast epithelial cells (MCF10A) via the expression of CDC6 and cyclin D1 (Y. Liu et al. 2010). There is also emerging evidence to suggest that cells that are in late G1 or S-phase of the cell cycle are more sensitive to MMA(III)-induced chromosomal aberrations compared to cells that are in the G0 or early G1-phases (Kligerman et al. 2010). This is due to the presence of efficient base excision repair mechanisms in the initial growth phase of the cell cycle. Prior research with the UROtsa cell line was also able to identify elevated S-phase cellular populations with a 2 μM and 4 μM iAs^{3+} exposure for 72 hours (Simeonova et al. 2000). This occurred in concert with an associated decrease in the G0/G1 phase populations, which was also found in the present study. Whilst these aberrations in the cell cycle assist in cellular transformation, a redistributed cell cycle has also been reported in cancer cell lines exposed to 6.25 μM As_2O_3 in the context of toxicity (X. Li et al. 2004). In this study human pancreatic, colonic and breast cancer cell lines all demonstrated an increase in the sub-G1 apoptotic population after a 72-hour exposure to 6.25 μM As_2O_3 and a

reduction in cellular survival and viability. This elevation in the sub-G1 population is also observed after a toxic exposure to As₂O₃ in HEK293T and UROtsa cell lines.

Another interpretation of the cell cycle data is the possibility of a transient arsenic-induced cell cycle pause leading to acute synchronisation followed by progression through the cell cycle. When cells are synchronised by serum starvation and then released, an increase in cells occupying S and G2/M phases can be observed towards the latter end of the doubling time (M. Chen et al. 2012). The doubling times of HEK293T and UROtsa cells are 24 hours and 30 hours respectively (http://www.lgcstandards-atcc.org/products/all/ACS-4500.aspx?geo_country=gb#specifications, accessed 07.2015) (Rossi et al. 2001). Given that the cell cycle data is at 72 hours, this should have allowed for 3 complete cycles for the HEK293T cells and 2.4 cycles for the UROtsa cells. So with the UROtsa cells at least, the accumulation of cells in S and G2/M phases may represent the latter 0.6 of the cycle, if arsenic-induced cell cycle synchrony occurred. Whilst this remains a possibility this is difficult to corroborate without data at multiple time points, and indeed this may be a consideration for future investigations. There is some data to suggest that arsenic exposure leads to cell cycle arrest in immortalised human fibroblasts and that exit from cell cycle arrest at the G2/M checkpoint is contingent on a p53-mediated response (McNeely et al. 2006). Cells negative for p53, exhibited reduced kinetics through the G2/M checkpoint. In addition to pausing, arsenic-induced stretching of the cell cycle period has been observed in human myeloid leukaemia cells treated with 5 µM sodium arsenite, where progression through each checkpoint takes longer, during the observation period of 24 to 48 hours post-treatment (McCollum et al. 2005).

If acute synchrony were to occur, such evidence should be observable in immediately after treatment. Human TK6 lymphoblastoid cells exposure to low level arsenic trioxide, 0.1 µM – 5 µM for 24 hours, did not lead to any significant changes in the cell-cycle distribution compared with untreated controls, with only the 5 µM exposure generating an increase in G2/M, but not at

a statistically significant level. The authors suggest that partial synchrony is not a physiological response to As₂O₃ exposure (Danaee et al. 2004).

Interestingly accumulation cells within the S and G2/M phases of the cell cycle has also been observed in cells undergoing apoptosis, in contrast to the data presented in this thesis. Acute promyelocytic leukemia (NB4-R1) cells exposed to 25 µM tetra-arsenic tetra-sulphide (As₄S₄), demonstrated a gradual increase in the percentage of cells occupying S and G2/M phases of the cell cycle, through measurements taken at T=0 hours, T=24 hours and T=48 hours (Y. Wang et al. 2015). These cells exhibited PARP cleavage, Caspase-3 cleavage, apoptotic cell death and internucleosomal DNA fragmentation. Whilst HEK293T and UROtsa cells treated with tolerated As₂O₃ did lead to an accumulation of cells in the S and G2/M phases of the cell cycle, the WST-1 proliferation assay demonstrated that these cells proliferate faster than untreated controls and do not undergo apoptosis.

An additional factor that may affect the cellular response to As₂O₃ exposure is the transport of the toxicant into the cellular environment and its metabolic processing. A number of active and passive transporters are involved in the influx and efflux of arsenicals depending on its speciation and methylation state (Zangi & Filella 2012). Arsenic trioxide transport is primarily facilitated by aquaglycoporins, AQP 3, 7, 9 and 10, glucose permeases, GLUT1, GLUT2 and GLUT5 and organic anion transporting polypeptides, OATPB and OATPC (Maciaszczyk-Dziubinska et al. 2012). One of the better-characterised influx mechanisms is the uptake of As₂O₃ via AQP9. Site-directed mutagenesis of AQP9 affected the uptake of glycerol and As₂O₃ equally, suggesting that the translocation of both compounds is potentially via the same pathway (Porquet & Filella 2007; Z. Liu et al. 2004). Although many aquaporins are bidirectional transporters As₂O₃ can be methylated by AS3MT and SAM, leading to the formation of additional metabolites that are exported via glucose permeases and ABC transporters with the latter involved in the efflux of glutathione-S-conjugated forms of arsenic (Maciaszczyk-Dziubinska et al. 2012). The levels of

these proteins were not explicitly analysed in the context of this thesis, however a search through publically available gene expression data sets pertaining to the HEK293T and UROtsa cell lines (HEK293T – <https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-21092/>, UROtsa – <https://www.ebi.ac.uk/arrayexpress/experiments/E-GEOD-26828/>) allows one to comment on As₂O₃ assimilation. Whilst the expression of most arsenic compatible transporters in the HEK293T cell line was absent bar GLUT1, UROtsa cells were found to express AQP3, GLUT1 and GLUT5, suggesting that more influx and efflux mechanisms are available to UROtsa cells. Furthermore, both cells lines readily express AS3MT and glutathione synthetase – GSS, suggesting that biotransformation of As₂O₃ is possible for both cell lines. A comparison between the two gene expression arrays also shows that GSS expression in the UROtsa cell line is 1.24 fold greater than in HEK293T cells, which is particularly significant as the depletion of glutathione by buthionine sulfoximine (BSO) leads to an IC₅₀ drop from 100 µM As(III) to 15 µM As(III) in UROtsa cells (Bredfeldt et al. 2004). The enhanced transport options and metabolic capacity of the UROtsa cell line may help to explain why these cells can tolerate As₂O₃ more so than the HEK293T cell line in the present study.

5.4 – Tolerated and Toxic As₂O₃ Exposure Affects Global Histone Acetylation.

Using tolerated and toxic As₂O₃ exposures this thesis reports temporally sensitive changes in global H3K9, H4K12 and H4K16 acetylation in both HEK293T and UROtsa cell lines. Significant findings include the global hypoacetylation of H3K9 in both HEK293T and UROtsa cells exposed to tolerated and toxic As₂O₃ concentrations after a 72 hour exposure. Global H4K16 hypoacetylation is also observed in the HEK293T cell line after exposure to both 0.5 µM and 2.5 µM As₂O₃ exposures at 72 hours. There is also a temporal component to the findings as a shorter 3-hour exposure to 0.5 µM and 2.5 µM As₂O₃ led to global H3K9 and H4K12 hyperacetylation and whilst the former residue became hypo-acetylated, the latter became

nominal at 72-hours. Prior research has identified histone acetylation changes *in vitro* and *in vivo* in response to arsenic exposure. Some molecular epidemiology studies have also been successful in identifying histone post-translational modifications as a result of arsenic exposure. The observed reduction of H4K16 acetylation presented in this thesis is in concert with previous research that showed H4K16 hypoacetylation after exposure to 3 mM As₂O₃ and 1 mM MMA(III) (Jo et al. 2009). A distinction between the aforementioned study and the present thesis is that the exposure period of 7 days was cytotoxic for both concentrations, whereas the 0.5 µM As₂O₃ exposure used for 72 hours in HEK293T cells was not toxic. Notably, this exposure still gave rise to H4K16 hypoacetylation. A follow up study utilised a tolerated dosage of 1 µM As(III) to expose UROtsa cells for 7 days, which revealed a reduction in global H4K16 acetylation via quantitative mass spectrometry (Chu et al. 2011). In this example, global H4K16 hypoacetylation occurred in the context of tolerance rather than toxicity, demonstrating in light of the previous study the challenges involved in characterising the significance of this event. Changes on this residue still require further investigation, as prior research from this group has demonstrated that H4K16 acetylation can protect cancer cells from cytotoxic drug treatment and may potentially be used as a marker of malignancy (Hajji et al. 2010). Under this circumstance loss of H4K16 acetylation should lead to cytotoxicity, yet this does not occur at the tolerated dosage. A combinatorial approach through the examination of multiple global residues may provide a clearer indication of cellular outcome.

Hepatocarcinoma cells (HepG2) exposed to 7.5 µM sodium arsenite for 24 hours showed a significant increase in global H3K9 acetylation (Ramirez et al. 2007). During this short exposure there was no cell death, similar to the data presented at 3 hours for HEK293T cells and for the 5 µM As₂O₃ exposure of the UROtsa cell line. This suggests that initial arsenic exposure may hyper-acetylate H3K9. However, there is a temporally dependent switch in the profile of H3K9 acetylation as a 72-hour exposure results in H3K9 hypoacetylation. This has also been demonstrated effectively in molecular epidemiology studies that have capitalised on the benefit of

taking samples from long exposures at environmentally relevant dosages. Samples from 40 participants of a clinical trial in Bangladesh were used for urinary arsenic concentration measurements and regressed against global H3K9 acetylation levels of circulating peripheral blood mononuclear cells (Chervona et al. 2012). In this study it was found that urinary arsenic concentration and the acetylation of H3K9 were inversely correlated, similar to the relationship found in this thesis. Another study exposed pregnant mice to arsenic and examined the effect of exposure on embryonic development and genome-wide H3K9 acetylation (Cronican et al. 2013). Brain tissue examined from these exposed pups also demonstrated global H3K9 hypoacetylation and cognitive impairment in a first of its kind study to demonstrate the effects of prenatal arsenic exposure. It is unclear in these examples whether or not hypoacetylation of this residue is explicitly associated with cellular transformation, as long-term exposures have also led to H3K9 hyperacetylation. Steel workers exposed to a variety of inhalable metallic compounds including arsenic, demonstrate H3K9 hyperacetylation in blood leukocytes in line with the number of years of employment (Cantone et al. 2011). These conflicting reports regarding the directionality of H3K9 acetylation and H4K16 acetylation demonstrate that global measurements may not be useful in delineating the separated outcomes of toxicity and tolerance. This is reflected in the data within this thesis, as tolerated and toxic dosages do not provide separated directions of acetylation on the residues examined.

The global levels of modification of other residues were also examined for this thesis, although the small sample size does impose limitations on the interpretations of the findings. A dose-dependent increase in H3S10 phosphorylation was observed at both 3 and 72-hour time points. Previous studies examining the impact of arsenic exposure on this residue have been conducted on short exposure durations. For example human diploid fibroblast WI-38 cells treated with 400 μM sodium arsenite led to an increase in H3S10 phosphorylation over a number of time intervals between 10 minutes and 60 minutes. Similarly the JB6 mouse epidermal cell line Cl41 was exposed to 10 μM sodium arsenite for 15 to 240 minutes, which resulted in a time-dependent

increase of H3S10 phosphorylation for up to 60 minutes. This was then followed by a reduction for the remaining exposure duration (Z. He et al. 2003). These studies are insightful as they utilise normal cell lines, however the short exposures limit the possibility of associating elevated H3S10 phosphorylation to cellular transformation. In the acute promyelocytic leukaemia NB4 cancer cell line, which is particularly sensitive to arsenic exposure, a dose-dependent increase in H3S10 phosphorylation was also observed by interval exposures between 0.2 μM to 1.6 μM As_2O_3 (J. Li et al. 2002). Concentrations of 0.2 μM to 0.4 μM As_2O_3 did not induce cell death, but did lead to an increase in H3S10 phosphorylation, whilst higher dosages did lead to cell death with the same outcome. Whilst there is considerable supporting evidence to suggest that As_2O_3 -induced H3S10 phosphorylation is a genuine toxicant-induced occurrence, the significance of the event is unclear.

H3K9 di-methylation was also examined after tolerated and toxic exposure to As_2O_3 . At 3-hours there is an apparent dose-dependent increase and at 72 hours there is no significant difference between the untreated control and the tolerated exposure. A slight reduction after a toxic exposure is also reported. Again, given the small sample size of this experiment it is unclear whether or not histone methylation is effectively adjusted from arsenic exposure. There is also evidence to suggest arsenic exposure does not affect histone methylation. For example, HepG2 carcinoma cells treated with 7.5 μM sodium arsenite for 24 hours showed no discernible impact on histone methylation across a number of lysines including, H3K4me(1,2,3), H3K9me(1,2,3), H4K29me3 and H3K27me3 and remains as one of the few studies that examined such a broad range of histone methylations (Ramirez et al. 2007). In contrast, exposure of human lung carcinoma A549 cells to 1 μM to 10 μM As(III) led to an increase of H3K9 di-methylation and a decrease in H3K27 tri-methylation (X. Zhou et al. 2009). This exposure was not cytotoxic, which is notable as a 2.5 μM As_2O_3 exposure for 72 hours led to a reduction in H3K9 di-methylation and cytotoxicity. Although this information alone is not sufficient to conclude that it as a protective mark, a similar study also introduced a normal human bronchial epithelial BEAS-2B

cell line and used exactly the exact same profile of arsenite trioxide exposure, which led to an increase in H3K9 di-methylation without cytotoxicity (X. Zhou et al. 2008). These findings in addition to those presented in the thesis provide indications that both phosphorylation and methylation events on histone tails may also play a role in arsenic-induced tolerance or toxicity.

To date, there is insufficient data interrogating the reversibility of arsenic-induced histone post-translational modifications. One study by Zhou *et al.*, did attempt to address this issue, demonstrating that 1 μ M arsenite exposure leads to H3K4 tri-methylation, which remains for 7 days after toxicant removal before returning to nominal levels (X. Zhou et al. 2009). In the present study, HEK293T cells were exposed for 72 hours and then allowed to rest in fresh growth medium in the absence of the toxicant for a further 72 hours. The acetylation status of H3K9, H4K12 and H4K16 were all examined. Hypoacetylation of H3K9 and H4K16 both appeared to normalise after the rest period, this suggested that the arsenic-induced histone acetylation changes are reversible. H4K12 acetylation was nominal at 72 hours and this also remained static after the rest period. Understanding the potential for reversibility is considered a fundamental cornerstone in the assesement of environmentally derived deleterious epigenetic events (Reamon-Buettner et al. 2008; McGowan & Szyf 2010).

Given the disruptions seen the cell cycle in both HEK293T and UROtsa cells, it is worth discussing the intrinsic relationship between the cell cycle and histone organisation, as effective DNA replication involves the cyclical disruption of chromatin (Bannister & Kouzarides 2011). Faithful DNA replication leads to challenges on histone post-translational modification, particularly those that are involved in maintaining an established pattern of gene expression. This concept of epigenetic maintenance during the vulnerable time of DNA replication has led to the proposal of replicative stress, where environmentally derived perturbations during replication may contribute to aberrant epigenetic changes and genomic instability leading to carcinogenesis (Alabert & Groth 2012). Whilst there is evidence to suggest that global histone H4

monoacetylation and trimethylation levels alter during the cell cycle, a direct comparison between non-tumourigenic and leukaemia cells with similar doubling times, to account for cell cycle fluxes in histone PTMs demonstrated a global reduction in H4K16 acetylation in the tumourigenic cell line (Fraga et al. 2005). Moreover, this global hypoacetylation was confirmed in a comparison between normal tissues and primary tumours. Whilst a global reduction in H3K9 and H4K16 acetylation was observed in the present study, further time points in addition to the 3 and 72 hours used, may help to further corroborate that these arsenic-induced global histone acetylation changes are indeed due to the toxicant rather than cyclical effects tethered to the cell cycle.

5.5 – Tolerated and Toxic As₂O₃ Exposure Alters the Expression of Enzymes Regulating Histone Acetylation.

In addition to the aberrant arsenic-induced histone acetylation events reported, there is growing evidence that the enzymes involved in regulating these acetylation events are also disrupted by arsenic exposure. A prevailing hypothesis about arsenic is that it behaves as an apparent HDAC inhibitor (HDACi) and indeed there is evidence to support this. For example in hepatocarcinoma HepG2 cells, exposure to 7.5 μ M, 10 μ M and 15 μ M sodium arsenite inhibited overall HDAC activity at a comparable intensity to the well documented HDACi trichostatin A (TSA) (Ramirez et al. 2007). Additionally in multiple myeloma NCI-H929 cells, exposure to 4 μ M As₂O₃ also inhibited overall HDAC activity from cellular lysates (Qu et al. 2012). Whilst these studies helped motivate investigations into HDAC expression for this thesis, there is an important distinction to be made. The properties of HDAC inhibition demonstrated previously were in the context of arsenic as a therapeutic agent to induce cytotoxicity in cancer cell lines. Having said that, the latter study also investigated the treatment of NCI-H929 cells with As₂O₃ at a non-cytotoxic dose of 4 μ M, after which no HDAC inhibition was observed (Qu et al. 2012).

In this thesis, the role of HDACs and HATs were investigated by first identifying the enzymes that are effective at adjusting the acetylation status of a particular lysine and secondly through the examination of HDAC/HAT expression. HDAC2 and PCAF were identified as regulators of H3K9 acetylation in HEK293T cells. Subsequent investigation into HDAC2 and PCAF mRNA levels by qRT-PCR, demonstrated that the initial increase in global H3K9 acetylation is potentially caused by an adjustment in the HDAC2 to PCAF expression ratio. The direction of this imbalance is inverted at the 72-hour time point, where at the tolerated exposure of 0.5 μ M As₂O₃, HDAC2 expression is significantly elevated combined with a significant reduction in PCAF expression leading to global hypoacetylation of the H3K9 residue at the 72-hour time point in HEK293T cells.

Although aberrant PCAF expression in the context of arsenic-induced carcinogenicity has not yet been documented, both PCAF and HDAC2 have been implicated in cancer. The role of PCAF is not definitive in carcinogenesis, but there is a possibility that PCAF has tumour suppressive properties (Zheng et al. 2013). Furthermore, loss of function mutations in the PCAF gene have been reported in epithelial cancers (Berdasco & Esteller 2010). Indeed if PCAF were exerting a tumour suppressive effect, the reduction in its expression observed after a 72-hour tolerated exposure could contribute to cellular transformation. However, due to the lack of studies utilising arsenicals in the investigation of PCAF expression and function, it may be suitable to examine wider functional data of this HAT in the context of cancer. There are complexities regarding the role of PCAF in cancers due to the presence of a functional duality within the enzyme. In its traditional role as a HAT, it is often found in transcriptional activation complexes. It has also been shown to degrade MDM2 via an intrinsic ubiquitin E3 ligase activity and thus help nuance p53 levels (Linares et al. 2007; Infante et al. 2014). PCAF can also directly target P53 at lysine 320 after DNA damage to assist in repair (L. Liu et al. 1999). This may be significant in the context of arsenic-induced carcinogenesis, due to the previously discussed ROS-dependent DNA damage mechanisms at tolerated arsenic exposure. Thus the reduced expression of this enzyme reported

at 72 hours after a tolerated exposure, speculatively may forgive DNA damage without stimulating repair – an outcome assistive to cellular transformation.

The expression levels of other HATs were also investigated in this thesis. Whilst HMOF/MYST1 expression was not significantly elevated, there was an indicative increase at both tolerated and toxic exposures at 72 hours. Researchers were able to show using UROtsa cells exposed to 1 μM – 10 μM As(III) and 0.3 μM – 3 μM MMA(III), that HMOF expression was required for cellular viability in the presence of the As(III) and MMA(III) (Jo et al. 2009). The increase observed may contribute to cellular survival, but it is unclear as to why the indicative increase occurs at both tolerated and toxic exposures and also why at both exposures PCAF expression is reduced. This lack of dosage-specific directionality makes PCAF expression alone a relatively poor biomarker in arsenic-induced cellular transformation.

Bi-directional mRNA expression of HDAC2 has been reported previously in human prostate epithelial RWPE-1 cells exposed for 6 months to 100 pg/mL As (Treas et al. 2013). This exposure led to the induction of HDAC2, 1, 3, and 7. But when the dosage was increased to 100 ng/mL As this led to a significant decrease in HDAC2 and 7 expression. Both exposures were tolerated; suggesting that direction of HDAC2 expression does not dictate an outcome between tolerance and toxicity. Although a bi-directional expression profile is also reported in this thesis due to an initial reduction at 3-hours, this is subsequently increased after a tolerated 72-hour exposure only. HDAC2 expression is also believed to be instrumental in the maintenance of urothelial cancers as a gene expression profiling study of 18 urothelial cancer cell lines was able to identify the upregulation of HDAC2 in 11 of these cell lines compared to normal uroepithelial controls (Niegisch et al. 2013). The effectiveness of HDAC2 as a marker for either maintenance or as a treatment response was still debated in the concluding segments, as reported mRNA and proteins levels did not always match and treatment with the pan-HDACi suberoylanilide hydroxamic acid (SAHA) had mixed outcomes. Yet, the apparent specificity of HDAC2

overexpression at the tolerated exposure in this thesis cannot be ignored. HDAC2 is believed to interact with cell cycle components, where siRNA-mediated silencing of HDAC2, inhibits progression through the G1/S checkpoint in hepatocarcinoma cells (Noh et al. 2011). This is particularly significant as tolerated As₂O₃ exposure led to a redistributed cell cycle to favour cellular proliferation by passing both G1/S and G2/M checkpoints in both cell lines.

The potential role of other HDACs in arsenic-induced carcinogenesis has been investigated further in UROtsa cells exposed for 12 weeks to 50 nM MMA(III) (Ge et al. 2013). The mRNA expression levels of HDAC1, 3, 4, 5, 7 and 9 were increased along with a time-dependent increase in HDAC activity at 4, 8 and 12 weeks of exposure. In contrast, a 72-hour exposure in HEK293T cells results in a significant decrease in HDAC1, 3 and 4 expression at the tolerated concentration. Treatment with the HDACi SAHA in the aforementioned study resulted in a significant reduction in HDAC2, 4, 5, 6, 7 and 8 mRNA levels, reduction in overall HDAC activity and the prevention of colony formation in soft-agar.

By reviewing existing literature along with the evidence presented in this thesis, it is clear that HDAC and HAT expression profiles alone are not entirely reliable biomarkers of either cellular transformation or toxicity. This is particularly due to the presence of conflicting reports regarding the direction of expression and the associated outcomes.

5.6 – Elucidating Epigenetic Aberrations at the Promoter-specific Level Helps to Clarify Cellular Outcomes in As₂O₃ Exposure.

Measurement of global histone acetylation profiles and the expression levels of histone modifying enzymes alone did not appear to be effective in separating between arsenic-induced tolerance and toxicity. As a result there was a requirement to unify the data onto gene promoters.

This was made possible by chromatin immunoprecipitation as it brought the global observations of histone hypoacetylation and the aberrant expression profiles of PCAF and HDAC2 into the transcriptional control space.

Some studies have characterised histone aberrations at the promoter specific level after low-dose arsenic exposure. For example, UROtsa cells transformed by a 52-week exposure to 1 μM As(III) and 50 nM MMA(III) led to the differential histone H3 hypo- and hyperacetylation of >50 gene promoters (Jensen et al. 2008). A number of these genes including *DBC1*, *FAM83A*, *ZSCAN12* and *C1QTNF6* found to have hypo-acetylated histone H3 at the promoter regions, exhibited a reduction in expression. A follow up study using the same cell lines, identified hyperacetylation of histone H3 at the *WNT5A* gene, which is found overexpressed in three bladder cancer cell lines (Jensen et al. 2009). UROtsa cells transformed by sodium arsenite exposure overexpress the *MT3* gene, which is expressed highly in urothelial cancers (Somji et al. 2011). A similar induction of the *MT3* gene can be achieved with the HDACi MT-275, which results in an increase in H4 acetylation at the gene promoter. Similar interactions have also been reported in the context of toxicity. Acute promyelocytic leukemia NB4 cells exposed to 1.6 μM As₂O₃ show increased phosphoacetylation of histone H3 at the *CASPASE10* gene locus, leading to increased expression and apoptosis (J. Li et al. 2002). These studies demonstrate the role of dysfunctional arsenic-induced histone acetylation events in the regulation of genes critical in cellular transformation. However, histone acetylation events alone are not able to adjust gene expression; rather it is the utilisation of additional components such as transcription factors that help communicate adjustments in the histone tail to the transactivation of genes.

To assess the significance of global H3K9 hypoacetylation in the transcriptional control of proto-oncogenes and apoptotic genes, chromatin immunoprecipitation was used to focus on the transcription factor E2F1, due to its dual role in proliferation and apoptosis (Korah et al. 2012). This present study identified the co-occurrence of promoter-specific H3K9 acetylation and E2F1

at the *FOS* promoter, a well-established proto-oncogene. At the 72-hour time point this interaction was significantly strengthened at the tolerated concentration only. E2F1 has been previously shown to interact in the promoter region of *FOS* and the retinoblastoma control element (RCE) and regulate the transcription of the pRb protein, an important regulatory protein in balancing cellular proliferation and apoptosis (Mimaki et al. 2005).

After 3 hours H3K9 is hyper-acetylated at the *FOS* promoter similar to the global acetylation profile. This localised acetylation is able to stimulate significant E2F1 binding at both tolerated and toxic exposures. After 72 hours of exposure, H3K9 acetylation is maintained at the *FOS* promoter specifically at the tolerated treatment in both cell lines, and although global H3K9 acetylation is reduced at this exposure, E2F1 transcription factor binding is still secure at the *FOS* promoter leading to transcription. It has been previously documented that E2F1 is able to recruit other histone acetyltransferases such as GCN5 to acetylate localised H3K9 residues (Guo et al. 2011). This thesis suggests that a similar mechanism is involved as the elevated binding of PCAF at the *FOS* promoter occurs in concert with E2F1 after a tolerated treatment of 72 hours. This allowed for the maintained H3K9 acetylation at the promoter. Although a similar increase in PCAF binding is observed at the toxic exposure, this does not occur with E2F1 binding at the promoter, leaving *FOS* expression and H3K9 acetylation nominal. Furthermore, combined transfection with PCAF and treatment at the toxic dosage, leads to inhibited acetylation activity on the H3K9 substrate compared to transfected untreated controls.

A study by Bi *et al.*, was able to show a significant increase in E2F1 expression after a low concentration exposure to 0.5 nM – 32 nM As₂O₃ for 7 days in human keratinocytes (Bi et al. 2010). This exposure elevated cellular proliferation and although an explicit mechanism was not stated at the time, the data in this thesis helps reconcile the significance of this finding. Another point of discussion is the relationship between E2F1 and PCAF. One study has attempted to investigate this, going so far as suggesting that E2F1 binding efficiency and transactivation ability

can be enhanced by PCAF-dependent acetylation of the transcription factor (Nagy & Tora 2007). It is however difficult to assess if this is the case in the present study as no explicit measurements of E2F1 acetylation were carried out.

The induction of *FOS* has also been reported previously in human fibroblast WI-38 cells exposed to 50 μM – 400 μM sodium arsenite (J. Li et al. 2003). In this study, the exposure period was short at only 120 minutes, but successfully identified an 8.6 fold increase in *FOS* expression along with a change in histone H3K14 acetylation at the *FOS* promoter. This increased expression of *FOS* is corroborated in this thesis also. The same study also reported an increase in *JUN* expression, which was also reported in this thesis. MDM2 mRNA expression levels were also elevated specifically at the tolerated exposure acting as an additional contributor of cellular transformation.

The data reported in this thesis demonstrates how arsenic-induced changes in global histone acetylation are not always reflected at the promoter specific level. Rather early perturbations in global histone acetylation, lead to transcription factor binding and promoter specific effects. The proposed mechanism in this thesis is that arsenic exposure leads to an adjustment in HDAC2 and PCAF expression and hyperacetylation of the H3K9 residue within 3 hours. This allows for the early recruitment of E2F1, which after 72-hours leads to PCAF mediated H3K9 hyperacetylation at the promoter and sustained transcription. This promoter specific effect occurs against a background of global H3K9 hypoacetylation.

This understanding of arsenic-induced global acetylation information followed by focused profiling at a specific gene promoter locus is a significant step in understanding arsenic-induced carcinogenesis from perturbations in histone post-translational status.

5.7 – Disruption of Global Chromatin Conformation by Tolerated and Toxic As_2O_3 in a Time-dependent Manner.

Transcriptional regulation and higher order chromatin conformation are intrinsically tethered by histone acetylation events which adjust the electrostatic properties of the histones (Shogren-Knaak 2006). Chromatin relaxation is observed in a dose-dependent manner in addition to increases in H3K9 and H4K12 acetylation at the 3-hour time point. Extension of the exposure to 72 hours led to chromatin condensation in the toxic exposure but normalisation of the chromatin architecture at the tolerated exposure in HEK293T cells. This demonstrates the reversibility of initial relaxation and this co-occurs with the global hypoacetylation of H3K9 and normalisation of H4K12 acetylation in HEK293T cells. Similar condensation is observed as a result of arsenic trioxide initiated apoptosis in the treatment of hepatocarcinoma (Alarifi et al. 2013). This may explain why initial E2F1 binding at the toxic exposure is not maintained after a 72-hour exposure, as the chromatin is severely condensed and E2F1 binding becomes unfavourable at the *FOS* promoter.

5.8 – The Potential for As_2O_3 -induced Crosstalk Between MicroRNAs and Histone Modifying Enzymes.

In a review by Bailey & Fry, existing literature was examined with the remit to understand arsenic-associated epigenetic changes and highlight studies with clear functional consequences (Bailey & Fry 2014). To quote the concluding remarks of the paper, the authors suggest that more research is needed “...to examine the interplay between multiple epigenetic components that likely work in unison to mediate the toxic effects of arsenicals”.

Either through the presence of multiple disrupted epigenetic mechanisms running concurrently, or through explicit “crosstalk” between the tripartite arms of epigenetics, there is indeed a lack of information on the deregulation of multiple epigenetic processes in the context of arsenic-induced carcinogenesis. This thesis has attempted to address this by seeking novel microRNA interactions with histone modifying enzymes under tolerated and toxic As₂O₃ exposure. Amongst the limited existing literature of arsenic-induced transformation, one study stands out because it presents evidence of arsenic-induced aberrations in DNA methylation, histone acetylation and microRNAs simultaneously. Herbert *et al.*, were able to demonstrate that an increase in H4K16 acetylation in normal human epidermal keratinocytes (NHEK) exposed to 0.5 μM As(III) was due to the miR34a-directed attenuation of SIRT1 (Herbert et al. 2014). Furthermore, long-term exposure led to DNA hypermethylation of both SIRT1 and miR-34a promoters and the subsequent reduction in H4K16 acetylation. This temporally dependent reduction in H4K16 acetylation was also reported in this thesis. Furthermore, this study asserts the notion that multiple epigenetic mechanisms can lead to arsenic-induced malignant transformation.

For this present study, a PCR array was used to identify differential expression profiles of miRs followed by a bioinformatic analysis upon these miRs to predict interactions with the histone acetylation machinery. Of the 26 miRs that were up-regulated 6 were predicted to interact with the histone modifying enzymes HDAC4, HDAC5 and PCAF. Out of these enzymes, PCAF was the highest scoring predicted target of the miRs, including miR-372 and miR-106b. Independent of the PCR array, miR-372 expression was validated by qRT-PCR to report a significant decrease at 3 hours exposure, which is then inverted at 72 hours.

PCAF mRNA levels at 3 hours were unchanged, but at 72 hours both a tolerated and toxic exposure to As₂O₃ resulted in a reduced PCAF mRNA. This was inversely correlated to miR-372 expression at this time-point suggesting that PCAF mRNA could potentially be attenuated by miR-372. Further analysis through the utility of a PCAF 3'UTR-luciferase reporter was also able

to identify PCAF mRNA degradation mediated via its 3'UTR. This also resulted in a significant decrease in H3K9 acetylation at 72 hours. However a planned experiment to diminish miR-372 through the use of a siRNA and examine the possibility of PCAF mRNA rescue did not come to fruition due to time constraints. Hence, miR-372 mediated attenuation of PCAF remains a possibility through correlative evidence, but due to the lack of an explicit experiment to investigate the interaction; a solid conclusion cannot be made. This is certainly a point of future investigations, as it may add further detail to the previously mentioned arsenic-induced global H3K9 hypoacetylation effect.

The body of exiting literature regarding arsenic-induced carcinogenesis and the differential expression profiles of miRs is relatively small but of growing interest. One study exposed the human leukemic (Jurkat) T cell line to 2 μ M sodium arsenite at tolerated concentrations (Sturchio et al. 2014). In this study, cells formed larger colonies and clumped together more readily along with the down-regulated expression of 8 miRs. An *in silico* analysis of the miRs identified predicted targets that were validated by qRT-PCR to be inversely correlated to the miR expression profiles, including *RNF4*, *TGF β 1*, *SP1* and *CYP1A1*. Sturchio *et al.*, go on to state that these genes are implicated in cell-cycle progression, proliferation and apoptosis. In addition to this study, human lung epithelial cells (BEAS-2B) transformed by arsenic exhibited a 100-fold reduction in miR-199-5p expression compared to untreated controls (J. He et al. 2014). When miR-199-5p was overexpressed in these arsenic-transformed cells, it impaired malignant growth via direct targeting of HIF-1 α and COX-2. Another study, used human prostate epithelial (RWPE-1) cells malignantly transformed by arsenic exposure to also demonstrate the aberrant expression of miRs (Ngalame et al. 2014). Ngalame *et al.*, report that the reduced expression of a number of miRs, including miR-134, miR-155, and miR-372, was inversely correlated to the expression of target mRNAs including a number of *RAS* oncogenes. These studies describe mechanisms where the down-regulation of miRs contributed to the unregulated expression of other genes responsible for malignant transformation. In contrast, all of the miRs differentially

expressed in this thesis after a tolerated 1 μM As_2O_3 exposure for 72 hours were in fact overexpressed, suggesting that the respective targets of these miRs may in fact be interacting with target mRNAs for attenuation.

Due to the lack of a longer time-point it is unclear if the elevated expression of these miRs sustain over prolonged As_2O_3 exposure. This is particularly important, as there is some evidence to suggest that arsenic exposure does induce temporally dependent changes in the expression of miRs. In one such study, PBMCs were exposed to 2 μM sodium arsenite for 72 hours, which led to a significant increase in miR-2909 for an exposure period up to 48 hours but this then dropped dramatically to almost nominal levels at 72 hours (Sharma et al. 2013). The increased expression of miR-2909 attenuated KL4, a tumour suppressor protein involved in the suppression of cyclin D1 by competing with the SP1 transcription factor at the *CCND1* promoter ultimately having consequences on cell cycle control.

This thesis is the first to describe elevated miR-372 expression after tolerated arsenic exposure, therefore there are no existing publications exploring the significance of this induction in the context of arsenic-induced carcinogenesis. However there is mounting evidence to suggest miR-372 has a significant role in carcinogenesis in general. For example, miR-372 has been shown to potentiate an increase in cellular proliferation through the inhibited regulation of cyclin-dependent kinases (Voorhoeve et al. 2005). Hepatocarcinoma tissues compared to normal adjacent liver tissues exhibit a significant elevation in miR-372 expression and ectopic expression assists in cellular proliferation and invasion of human hepatocarcinoma cell lines (H. Gu et al. 2013). MiR-372 is also believed to have proto-oncogenic properties, particularly in B-cell lymphoma and testicular cancer (L. He et al. 2007; Silahatoglu & Stenvang 2010). There is an indicative increase in miR-372 at the toxic 5 μM As_2O_3 exposure that this thesis postulates is also responsible for PCAF attenuation.

To date there is no research that describes the exact mechanism of miR-372 induction following environmental stimulation, either through a toxicant or by other means. However, it is well established that microRNAs are pre-transcriptionally regulated via control imposed by transcription factors, epigenetically controlled through DNA methylation and histone post-translational modifications, in addition to post-transcriptional restrictions that target the processing and maturation of miRs (Ha & V. N. Kim 2014). One can postulate that miR-372 induction by arsenic occurs through a combinatorial mechanism involving transcription factors and epigenetic regulation. Such mechanisms have been reported previously. For example, E2F1 has been implicated in a miRNA regulatory circuit which includes the proto-oncogene c-MYC (O'Donnell et al. 2005). O'Donnell et al., demonstrate that c-MYC can simultaneously activate E2F1 transcription and facilitate the miRNA-mediated attenuation of its translation via a cluster of miRs situated on chromosome 13. This finding is pertinent as a 500 nM arsenite exposure can lead to the overexpression of c-MYC and cellular transformation of the rat liver epithelial cell line – TRL 1215 (H. Chen et al. 2001). E2F1 has also been shown to explicitly regulate the polycistronic miR-17-92 cluster through the presence of functional E2F1 binding sites within its promoter region (Woods et al. 2007). Furthermore, the presence of a c-MYC-miR17-19-E2F1 regulatory network has been postulated to help cells decide between a proliferative and apoptotic cell fate, similar to the core mechanism presented in this thesis (Coller et al. 2007). This thesis has also provided evidence of arsenic-induced perturbations of histone PTMs and higher order chromatin conformation. Such perturbations may also assist in the induction of miR-372, as promoter regions of miRs have differential histone marks including, H3K4me3, H3K9/K14ac and H3K27ac (Kuchen et al. 2010). Additionally promoter elements belonging to miRs are depleted of nucleosomes, whilst short depleted regions of 70 nucleotides allow for the transcriptional initiation and expression of miRs (Schanen & X. Li 2011).

A finding that at first appears contradictory to the postulated miRNA-mediated attenuation mechanism of PCAF is the H3K9 hypoacetylation observed at 3 hours after a toxic 80 μ M As₂O₃

exposure. At this time point, miR-372 and 106b expression are significantly downregulated making a miR-dependent mechanism for this hypoacetylation improbable. Further investigations into the expression of histone modifying enzymes, identified a significant increase of HDAC2 expression at only the toxic 80 μM As_2O_3 , making it more likely to be the cause of H3K9 hypoacetylation at 3 hours. This is in contrast to the data presented in the HEK293T cell line for potentially two reasons. The first is that both 0.5 μM and 2.5 μM As_2O_3 exposures are tolerated at 3 hours in HEK293T cells, whereas the 80 μM As_2O_3 at 3 hours is actually toxic. Secondly the HDAC2 induction observed here could be an attempt at cellular survival, and through examination of the toxic 5 μM As_2O_3 exposure at 72 hours, it is clear that HDAC2 expression is significantly diminished. Indeed elevated HDAC2 expression has been well characterised in urothelial bladder cancer, with the examination of 348 tissue samples from 174 patients (Poyet et al. 2014). So it is notable that overexpression is observed specifically at the tolerated 0.5 μM As_2O_3 exposure in HEK293T cells, which through further assays clearly lead to cellular transformation, unlike the 80 μM As_2O_3 exposure in UROtsa cells that leads to PARP cleavage – a solid apoptotic marker.

5.9 – Broader Implications

The incorporation of epigenetic data into existing risk assessment frameworks is of considerable benefit, but not without some challenges. Risk assessment frameworks usually include a number of steps including the identification of the hazard, a dose-response assessment, a quantification of the exposure and a characterisation of the risk. The latter may include toxicokinetic / dynamic data with respect to the toxicant (http://www.epa.gov/risk_assessment/health-risk.htm, accessed: 01.15).

Epigenetic markers may be included in such risk assessments as biomarkers for a particular disease state following exposure to an environmental toxicant. The challenge is answering the questions regarding the epigenetic alterations themselves, for example are these epigenetic alterations dose-responsive, is the observed response specific to the toxicant, is it a genome-wide event or a promoter-specific event and do such events actually lead to a clear biological endpoint? (Ray et al. 2014). To some extent this thesis has contributed to answering these questions by attempting to draw out specific cellular outcomes at specific doses, and in the process, characterise dosage-specific epigenetic events. This has allowed for the identification of global H3K9 hypoacetylation and H3K9 hyperacetylation at the *FOS* promoter at the tolerated exposure of 0.5 μM As_2O_3 .

This thesis also contributes to the debate regarding the placement of the risk threshold for arsenic exposure. In a news review by Schmidt, published in EHP, a number of scientists voice their concerns regarding the extrapolation of data from high-dose Taiwanese studies to allow for the placement of risk thresholds by the EPA (Schmidt 2014). Whilst some agree that the threshold is low enough to avoid deleterious health outcomes, others suggest that its not low enough, and that actually very low-dose arsenic exposure can still lead to negative health outcomes. The report continues by citing a number of studies that have identified low-dose impacts following arsenic exposure. This thesis helps by contributing to this body of knowledge, which may hopefully be integrated into future risk assessments of arsenic exposure.

The evaluation of on-going exposures and the associated risks requires the identification of good biomarkers. This thesis demonstrates the challenge in identifying suitable biomarkers for arsenic-induced carcinogenesis particularly with regards to de-regulated epigenetic mechanisms. Two significant insights have emerged, the first being the requirement to characterise exposure concentrations by determining the relevance of the exposure prior to the measurement of epigenetic aberrations. Secondly, more promoter-specific epigenetic aberrations need to be

identified that collectively lead to cellular transformation. The latter point is particularly suitable for the development of novel therapies, as the identification and characterisation of novel epigenetic pathways that lead to cancer, particularly those that impinge on specific gene targets, allow for the development of effective therapies (Dawson & Kouzarides 2012).

5.10 – Future Prospects

This thesis presents additional mechanistic detail to arsenic-induced cellular transformation by starting with the analysis of histone post-translation modifications. More research is needed to understand As₂O₃-induced disruptions on histone acetylation machinery. Systematic analysis of functional impediments imposed by As₂O₃ on HATs and HDACs would be of a particular insight as well as an investigation into a wider range of enzymatic families. A preliminary experiment of this sort was attempted and could be developed further (Appendix. AP1). Some of these enzymes also target non-histone proteins to alter the acetylation status of these proteins and function. This may provide a cellular network of disruption originating from the aberrations on the histone acetylation machinery.

The identification of more promoter targets regulated by histone acetylation as a result of tolerated As₂O₃ exposure may help to further our understanding of arsenic-induced cellular transformation – potentially through the utility of ChIP-Seq. This study was able to identify a significant increase in H3K9 acetylation at the *FOS* promoter leading to *FOS* induction. To check if this is really crucial for cellular transformation, this gene could be silenced with siRNAs to examine the effect it has on soft-agar growth following long-term 0.5 µM As₂O₃ exposure. If soft agar formation is impeded, it would validate the necessity of *FOS* induction in arsenic-induced cellular transformation. Whilst the 0.5 µM As₂O₃ exposure is tolerated *in vitro* it is difficult to extrapolate this to a concentration of environmental relevance. Hence a molecular

epidemiology study would help in examining if this potential biomarker is exhibited in those drinking arsenic contaminated water, against those that do not. Although preliminary experiments have not yet been conducted, it may be possible to spin-down shed urothelial cells from urine samples for this examination, in addition to the more standard collection of PBMCs.

A significant number of miRs were overexpressed as a result of tolerated As₂O₃ exposure in UROtsa cells (Appendix. AP3). The roles of these microRNAs are still not fully understood, which could be elucidated with further bioinformatic processing, either through a pathway analysis or a larger scale attempt to identify novel targets. Such methods have been attempted before with some success (Nie et al. 2013). This thesis proposes that miR-372 attenuates PCAF mRNA, which could be examined by transfecting cells with an antisense RNA against miR-372 to see if the PCAF mRNA levels are rescued. Additionally, miR-372 could be overexpressed in cells, followed by an examination of the PCAF mRNA levels to see if it is diminished. It is also possible to mutate the proposed seed site within the PCAF mRNA 3'UTR by utilising a genomic engineering tool such as CRISPR-Cas9 to destabilise the miRNA:transcript interaction, leading to a rescue in PCAF mRNA levels. This will also help define a region of specificity between the miR-372 and its potential target.

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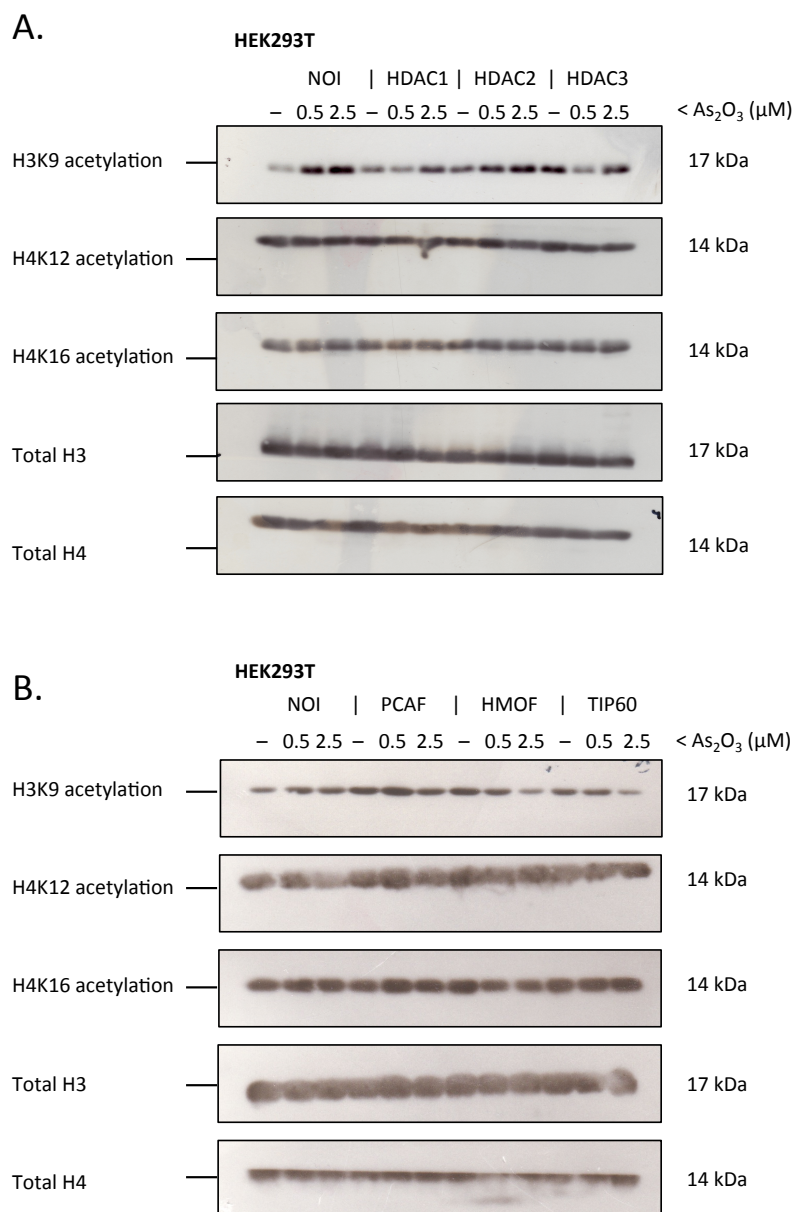
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Appendices

Appendix 1 – As₂O₃ exposure and functional outcomes of histone modifying enzymes on target lysines.

A number of histone modifying enzymes were shown to have functional consequences on residues of interest. HEK293T cells were therefore exposed to 0.5 μ M and 2.5 μ M As₂O₃ for a short duration (3-hours) to identify if these functions were disrupted by the presence of As₂O₃. Cells were transfected with the histone deacetylases HDAC1, 2 and 3 in addition to the histone acetyltransferases PCAF, HMOF and TIP60. The acetylation status of H3K9, H4K12 and H4K16 was evaluated by immunoblotting (Fig. AP1).

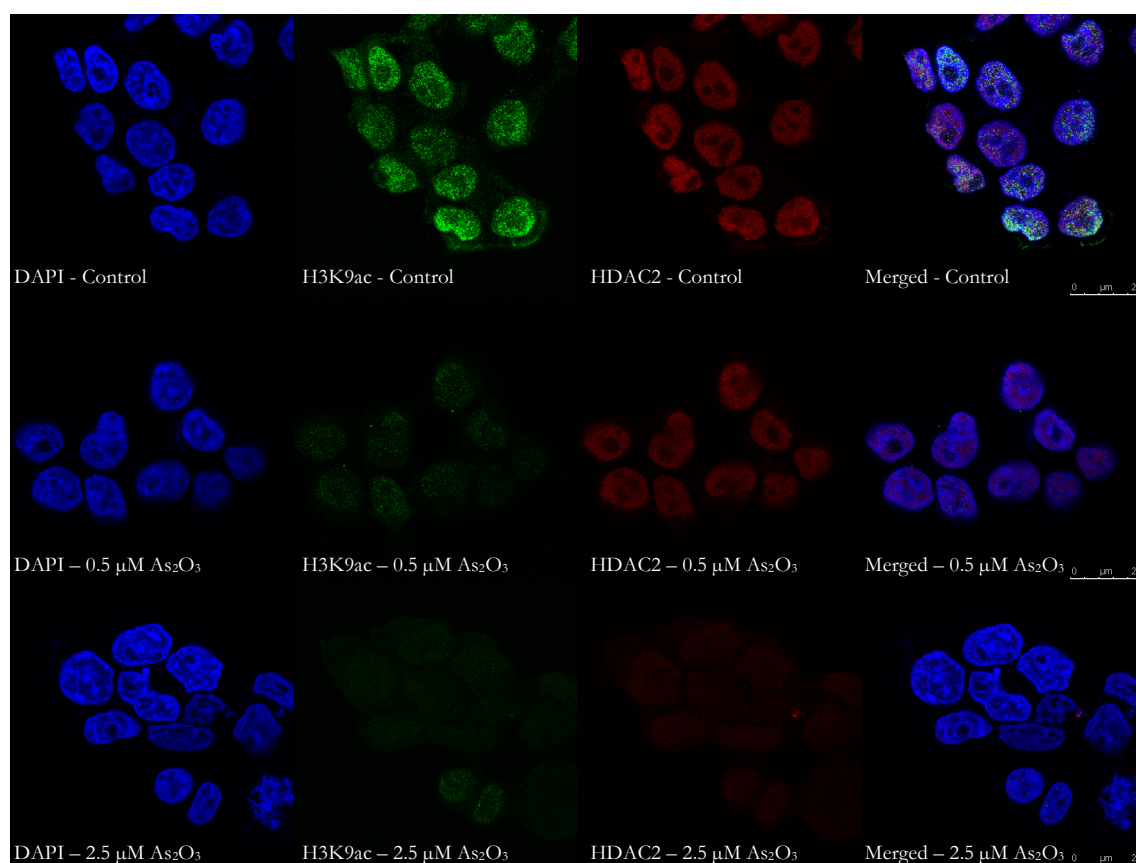


+ Figure AP1: Transfections with HDACs and HATs in HEK293T cells followed by As₂O₃ treatment for 3 hours.

HEK293T cells were transfected with plasmids constitutively overexpressing HDAC 1, 2, 3, PCAF, HMOF and TIP60. Cells were also treated with 0.5 μM and 2.5 μM As₂O₃ followed by histone extraction and immunoblotting to evaluate the histone acetylation status on H3K9, H4K12 and H4K16. A total of 10 μg of proteins were added in each well. Total histone 3 and 4 was used as the loading controls. N=1.

Appendix 2 – Confocal Image: H3K9ac and HDAC2 after 72 hours As₂O₃ exposure in HEK293T cells.

HEK293T cells were exposed to 0.5 μ M and 2.5 μ M As₂O₃ for 72 hours. Cells were then harvested and prepared for immunocytochemistry with antibodies against H3K9 and HDAC2. See materials and methods for additional details.



+ **Figure AP2: Immunofluorescent image of H3K9ac and HDAC2 following 0.5 μ M and 2.5 μ M As₂O₃ exposure in HEK293T cells.**

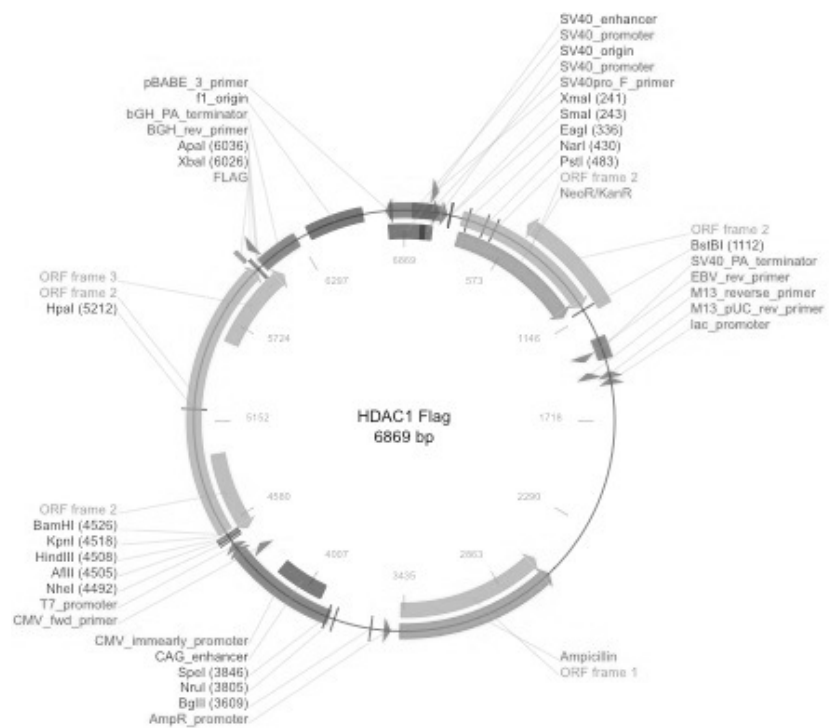
HEK293T cells were seeded on coverslip and treated. Cells were then fixed and permeabilised prior to incubation with primary antibodies against acetylated H3K9 and HDAC2. See materials and methods for additional details.

Appendix 3 – Up-regulated microRNAs from the PCR array.

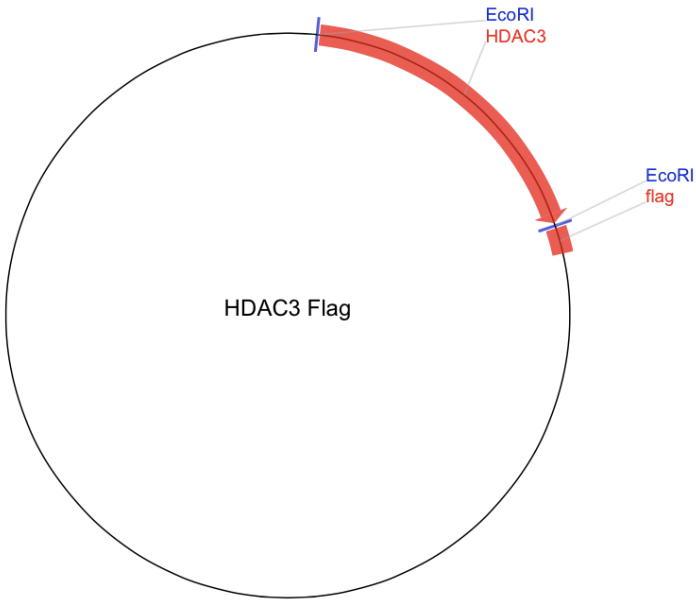
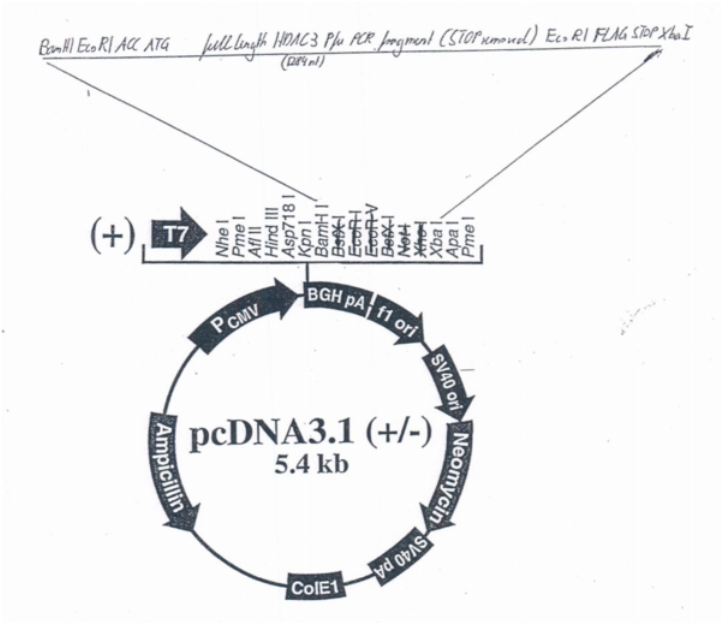
+ Table 10: Full list of up-regulated microRNAs as a result of tolerated 1 μM As_2O_3 exposure for 72 hours in human urothelial cells (UROtsa).

Mature ID	Fold Regulation
hsa-miR-218-5p	200.9464
hsa-miR-122-5p	117.6263
hsa-miR-206	115.9828
hsa-miR-373-3p	104.3341
hsa-miR-196a-5p	55.4558
hsa-miR-215	41.1
cel-miR-39-3p	39.5846
hsa-miR-10b-5p	36.8621
cel-miR-39-3p	33.6881
hsa-miR-133b	32.2509
hsa-miR-10a-5p	30.5578
hsa-miR-214-3p	26.9697
hsa-miR-143-3p	23.8889
hsa-miR-1	22.1167
hsa-miR-144-3p	19.3273
hsa-miR-372	15.6021
hsa-miR-184	11.0906
hsa-miR-142-5p	9.3727
hsa-miR-150-5p	9.0115
hsa-miR-155-5p	9.0003
hsa-miR-181d	8.3447
hsa-miR-127-5p	6.0986
hsa-miR-124-3p	4.4923
hsa-miR-138-5p	4.0656
hsa-miR-134	3.7094
hsa-miR-335-5p	3.419

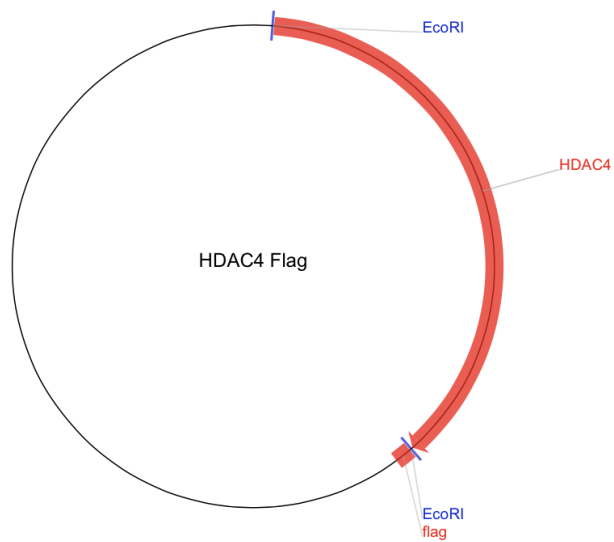
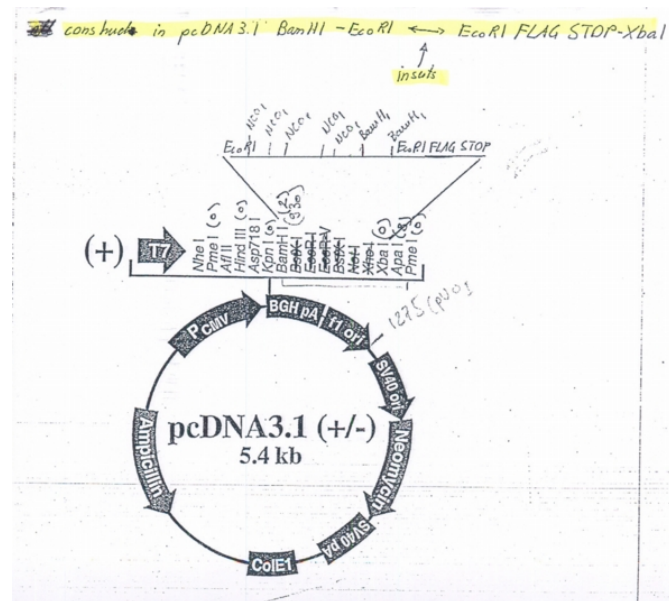
HDAC1



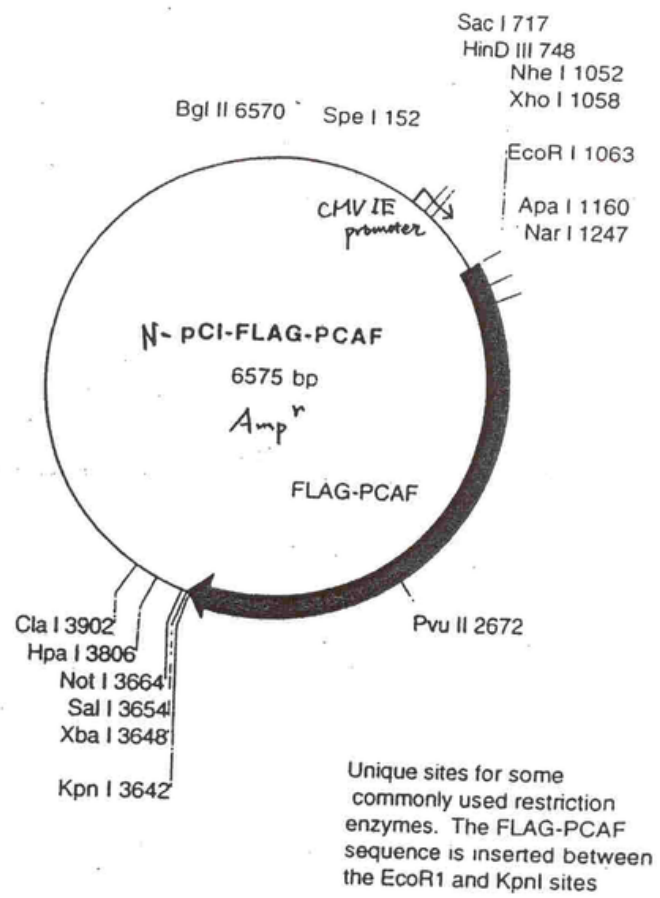
HDAC3

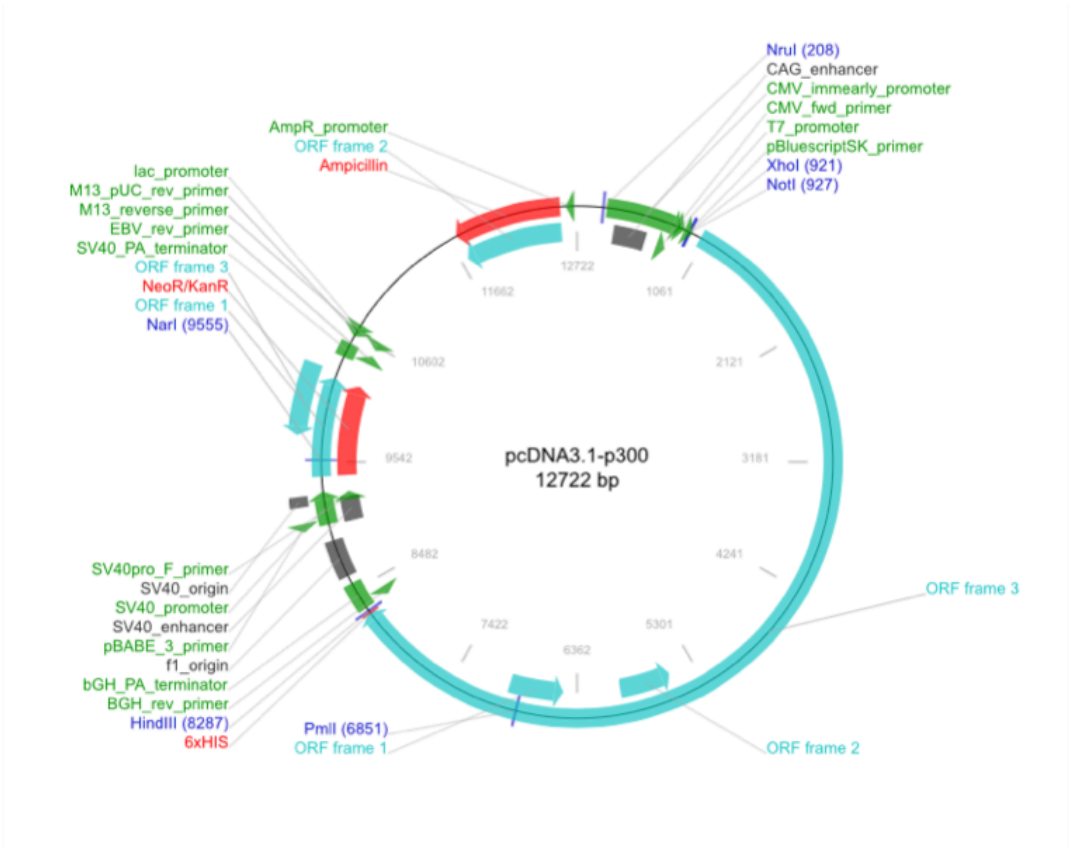


HDAC4



PCAF





3'UTR Reporter Backbone Vector

Base pairs: 3910

RPL10 Promoter: 33-307

RenSP reporter gene (synthetic renilla luciferase; includes mODC PEST): 350-1408

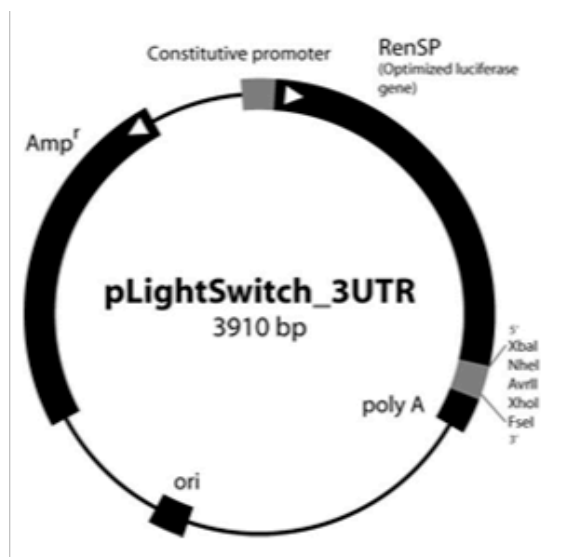
Multiple cloning region 2 (UTR region): 1409-1446

SV40 late poly(A) region: 1455-1676

ColE1-derived plasmid replication origin: 2001

Synthetic Beta-lactamase (Amp^r) coding region: 2792-3652

Synthetic poly(A) signal/transcriptional pause site: 3757-3910



3'UTR Reporter Inserts

The below represents the sequence inserted into the 3'UTR Reporter Backbone Vector to generate the 3'UTR PCAF(KAT2B) plasmid used in the present study.

These constructs were acquired commercially from Switchgear Genomics via Active Motif.

Product ID	S810567
Vector name	pLightSwitch_3UTR
Gene symbol	KAT2B
Insert length	1989
Restriction pair	NHE1XHO1

NOTE

Sequence

```
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