Characterization of the role of the Aurora B kinase in quiescent lymphocytes

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Doctor of Philosophy
Declaration of originality

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Abstract

The role of the Aurora B kinase in mediating cellular functions outside mitosis was investigated. A SILAC-based approach was used to show that Aurora B interacts with proteins implicated in gene regulation and chromatin organization in G1 activated B cells. Among these interactors, I identified the PRC1 component Ring1B as a new partner of Aurora B. Both proteins are bound to the promoters of highly expressed genes in resting B and T lymphocytes. Quiescent resting B cells require the maintenance of a transcriptional programme that keeps the cells viable and ready to proliferate upon encounter with a specific antigen. Aurora B and Ring1B physically co-occupy the same promoters in resting B cells but, upon activation, Aurora B is replaced by MSK1 on its target genes, whereas Ring1B binding is preserved.

Analysis of the active promoters in resting B cells shows that other PRC1 components, such as Cbx7 and Bmi1, and the histone deubiquitinase USP16, together with Aurora B and Ring1B, bind almost exclusively to the regulatory elements of active promoters and are not found at repressed genes. Binding of PRC2 components and deposition of the H3K27me3 and H2Aubq marks are largely confined to silent genes. By employing conditional knockout mouse models, I showed that removal of either Aurora B or Ring1B results in a global reduction in the binding of unphosphorylated and serine 5-phosphorylated RNA Polymerase II to active promoters. This phenomenon is also accompanied by a reduction in the production of transcripts from Aurora B and Ring1B target genes and reduced cell viability. Aurora B is also required to maintain high levels of phosphorylated H3S28 and low levels of the repressive H2Aubq mark at active promoters. These results identify a new role for both Aurora B and Ring1B in the regulation of active transcription in quiescent lymphocytes.
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<thead>
<tr>
<th>Acronym</th>
<th>Full name</th>
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<tr>
<td>2A-HUB</td>
<td>2A-histone ubiquitinase</td>
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<tr>
<td>4-OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>ACF</td>
<td>Assembly-complementing factor</td>
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<td>AEBP2</td>
<td>AE binding protein 2</td>
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<td>Akap8l</td>
<td>A kinase (PRKA) anchor protein 8-like</td>
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<td>Ap1m1</td>
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<td>APC/C</td>
<td>Anaphase promoting complex/cyclosome</td>
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<td>B lymphoma Mo-MLV insertion region 1 homolog</td>
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<td>Clpp</td>
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<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
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<td>Interleukin</td>
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<td>Interleukin 2 receptor, gamma</td>
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<td>Immature</td>
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<td>Metaxin 1</td>
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<td>Nuclear receptor co-repressor</td>
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<td>Natural killer</td>
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<td>Nr4a1</td>
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<td>Tumor protein 53</td>
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<td>PBAF</td>
<td>Polybromo-associated BAF</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
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<td>PC</td>
<td>Plasma cell</td>
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<td>Pc</td>
<td>Polycomb</td>
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<td>Polycomb group protein</td>
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<td>PCGF</td>
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<td>Pleiohomeotic</td>
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<td>Phosphoinositide 3-kinase</td>
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<td>PIAS3</td>
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<td>Pmf1</td>
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<td>Protein phosphatase</td>
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<td>Rat sarcoma</td>
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<td>Retinoblastoma</td>
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<td>RNA polymerase II</td>
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<td>Robo1</td>
<td>Roundabout, axon guidance receptor, homolog 1</td>
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<td>rpm</td>
<td>Revolutions per minute</td>
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<td>Reads Per Million of mapped reads</td>
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<td>Roswell park memorial institute</td>
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<td>Rtr</td>
<td>RNA Pol II subunit</td>
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<td>Runx1</td>
<td>Runt-related transcription factor 1</td>
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<td>RYBP</td>
<td>RING1 and YY1 binding protein</td>
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Synthesis

S2p Pol II Serine 2-phospho RNA Pol II
S5p Pol II Serine 5-phospho RNA Pol II
S7p Pol II Serine 7-phospho RNA Pol II
SAC Spindle assembly checkpoint
SAGA Spt-Ada-Gcn5 acetyltransferase
SANT Swi3, Ada2, N-Cor, and TFIIB
SDS Sodium dodecyl sulfate
SET Su(var), Enhancer of zeste, Trithorax
Sgo1 Shugoshin
SIGNR C-type lectin domain family 4, member M
SILAC Stable isotopic labelling with amino acid in culture
SKAP Small kinetochore-associated protein
SKP1 Kinetochore-associated protein
snRNA Small non coding RNA
SP1 Specificity protein 1
Srgn Serglycin
SUMO Small Ubiquitin-like Modifier
SUVR3H1 Suppressor of variegation 3-9 homolog 1
Suz12 Suppressor of zeste 12
SWI/SNF Archetypal remodeller complex with Snf2p (Swi2p) at its core
SWR1 SWI/SNF related protein
TAFs TBP associated factors
TBP TATA-binding protein
TBS Tris-buffered saline
TCR T-cell receptor
TD-60 Telophase disk protein of 60 kda
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<td>Transcription factor</td>
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<tr>
<td>TGF-b</td>
<td>Transformin growth factor-β</td>
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</tbody>
</table>
| T
\(_H\) | T-helper |
| TLE     | Transducin-like enhance |
| TLK-1   | Tousled-like kinase-1 |
| Top3a   | Topoisomerase (DNA) III alpha |
| Tpd52l2 | Tumor protein D52-like 2 |
| Tregs   | T regulatory |
| Tris    | Trisaminomethane |
| TSPs    | Thymus-settling progenitors |
| TSS     | Transcriptional start site |
| Tzfp    | Testis zinc finger protein |
| U       | Unit |
| UbcH5c  | Ubiquitin carrier protein D3 |
| USP     | Ubiquitin-specific proteases |
| Vps37b  | Vacuolar protein sorting 37 homolog B |
| w/v     | Weight on volume |
| WAF     | Cyclin-dependent kinase inhibitor 1A |
| Yaf2    | YY1-associated factor 2 |
| YY1     | Ying yang 1 |
| Zbtb24  | Zinc finger and BTB domain containing 24 |
| ZRF1    | Zuotin-related factor 1 |
| µg      | Microgram |
| µl      | Microliter |
| µM      | Micromolar |
Acknowledgements

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Chapter 1 - Introduction

1.1 Overview

The main aim of this study has been to identify new roles of the Aurora B kinase outside mitosis. In particular, my PhD project has focused on the interaction between Aurora B and the Polycomb group protein Ring1B. I have shown that these proteins form a functional complex bound to active promoters in resting B and T lymphocytes and regulate transcription of their target genes in the quiescent state.

To introduce the results that I have obtained, I will firstly review the published literature on quiescence, the stage of the cell cycle in which this functional interaction takes place. Secondly, I will describe the cell system that I used to study this interaction: murine lymphocytes. I will then review some key aspects of the regulation of gene expression in mammals, as these concepts are important for understanding the mechanisms behind the transcriptional programme that maintains resting lymphocytes in a quiescent state and ready for activation. This will be followed by a description of the known functions of the Aurora B kinase in regulating mitosis and cytokinesis. Finally, I will deal with the roles of the Polycomb group proteins in regulating gene expression.

1.2 Quiescence

1.2.1 Quiescence as an adaptive mechanism

Quiescence (also termed G0) is defined as a reversible exit from the cell cycle, characterised by a reduced size and metabolic activity. Most cells in an
adult organism are non-proliferating and some are in a resting state and resume proliferation upon encounter with the appropriate stimulus. A classical example is resting lymphocytes. In the immune system of higher eukaryotes, each subset of quiescent lymphocytes will proliferate only upon encounter with a specific antigen. This cell population will expand in order to respond to a specific antigen but, otherwise, the demand of energy and nutrients of these cells is modest.

Quiescence is remarkably conserved from *E. coli* to humans (O'Farrell, 2011). In microorganisms such as yeast, quiescence is mostly an adaptive response to a lack of nutrients (Laporte et al., 2011). In the absence of essential nutrients such as carbon, yeast arrests its cell cycle during the G1-phase and enters quiescence (De Virgilio, 2012). Prototrophic yeast strains are able to synthesise essential compounds for their metabolism and this ability is utilised in quiescence (François and Parrou, 2001). During quiescence, *S. cerevisiae* accumulates glycogen and reinforces its cell wall (De Virgilio, 2012). This series of events requires the reorganization of the transcriptional programme and, indeed, even in relatively simple organisms such as yeast, a quiescence-specific pattern of gene expression exists.

The quiescence programme causes one fourth of the entire genome of *S. cerevisiae* to become differentially expressed. Most of the genes are silenced and the overall transcriptional activity of *S. cerevisiae* is reduced. Despite the global reduction in transcription, RNA Polymerase II is present at most promoters and its release from the Mediator complex is likely to be the limiting factor in transcription (see section 1.4.2) (Radonjic et al., 2005) Moreover, the overall structure of yeast DNA becomes more compacted (Schäfer et al., 2008). The global reduction in transcription reduces the translation of new proteins (Boucherie, 1985). Proteosomal degradation is high at the onset of quiescence but is progressively reduced meaning that protein turnover becomes quite slow under these conditions (Bajorek et al., 2003). Mitochondrial respiration is reduced
to adjust the metabolism to new conditions and limit the oxidative stress (Cyrne et al., 2003). However, quiescent yeast strains exhibit a high resistance against oxidants (Costa and Moradas-Ferreira, 2001).

Similar to yeast, serum starvation causes mammalian cells to adopt a quiescent programme characterised by reduced metabolism and protein turnover, global reduction of transcription and chromatin compaction. The notion that quiescence is also actively regulated in mammalian cells comes from pioneering studies where it was observed that 3% of the mRNA in resting fibroblasts is not present in proliferating cells and vice versa (Williams and Penman, 1975). Interestingly, many of the genes upregulated upon serum starvation are repressed in tumours and an antagonizing role for quiescence in cancer development has been proposed (Liu et al., 2007). Indeed, tumours often arise in adult tissues, where most of the cells are non dividing (Malumbres and Barbacid, 2001). The mechanisms that govern the decision between proliferation (observed in cancer cells) and quiescence are often disrupted in tumours (Sherr, 2000).

In addition to serum starvation, contact inhibition and loss of adhesion are also commonly employed to induce quiescence in cultured cells. These mechanisms might more closely reflect the type of situations encountered by cells in an organism, as serum deprivation is not a physiological phenomenon. The size of an organism is limited and the different tissues are compartmentalised and can proliferate and expand within certain physical limits. Therefore, contact inhibition might reflect a physiological condition that imposes a block to cell proliferation and induces the formation of a reservoir of quiescent cells ready to replace the dead ones. For example, endothelial cells spend most of their life in quiescence at a high density, where competition for nutrients might also play a role in the maintenance of this state. However, upon tissue lesion and loss of cells they resume proliferation to heal the wound (Lee and Gotlieb, 1999).
Many cell types, such as mammary epithelial cells, require the presence of a layer of feeder cells and exhibit anchorage-dependence to these cells. Upon loss of adhesion, different pathways lead to the arrest of proliferation and the engagement of a quiescence programme (Sequeira et al., 2007). Interestingly, in cultured fibroblasts, serum starvation, contact inhibition and loss of adhesion lead to different patterns of differential gene expression at the onset of quiescence but, over time, these three separate programmes converge towards a unique one (Coller et al., 2006).

1.2.2 Quiescence as a developmental stage

Quiescence is not exclusively an adaptive response to unfavourable growing conditions that allows a normally proliferating cell to survive in a transiently unfavourable environment. Certain cell types are by default resting and can remain alive for several months to years (Tough and Sprent, 1994). Examples include the endothelial cells present in veins and arteries, satellite cells in skeletal muscles, hepatic and pancreatic stellate cells in mammals.

Mature resting B and T lymphocytes (also termed B and T cells) are also an important example of quiescent cells. Resting B cells arise in the spleen where transitional B lymphocytes cease proliferation, complete their development and becomes mature and quiescent (see section 1.3.4). Resting T lymphocytes are released from the thymus to the peripheral lymph nodes as mature cells (see section 1.3.8). Both cell types reside in the lymphoid organs in a resting state where they are poised to vigorously proliferate and differentiate (as described in detail below) only upon encounter with the appropriate antigen.

Interestingly, the comparison between in vitro induced quiescence in fibroblasts (Coller et al., 2006) and naïve T lymphocytes (Diehn et al., 2002) shows a 71% overlap in the downregulated genes but only 15% of upregulated genes are shared between the datasets. What might explain this difference is the
observation that quiescence programmes tend to downregulate genes implicated in proliferation, which are often the same among cell types. On the other hand, cell-type specific genes that control tissue-specific functions required for survival need to be upregulated. Cell type-specific transcriptional factors are often implicated in the regulation of the quiescence programmes and cooperate with cell cycle inhibitor proteins in the onset of quiescence.

1.2.3 The requirements for resting lymphocytes survival

An interesting common trait of resting B and T lymphocytes (henceforth referred to as B and T cells) is their dependence for survival on a basal level of stimulation of the B cell receptor (BCR) and T cell receptor (TCR) respectively. It is possible to speculate that, in order to avoid anergy, a condition of irreversible unresponsiveness to stimuli, B and T cells must keep their receptors constantly stimulated below threshold in order to prevent them from becoming unresponsive (Hamilton and Jameson, 2012). Tonic BCR and TCR signalling activates the transcriptional factor NF-κB, which promotes the transcription of genes required for survival, stimulation and development (Li and Verma, 2002).

IL-4 is normally produced by T cells during B cell activation but has also been shown to be required for the survival of B cells (Zamorano et al., 2001). Naïve CD8+ T cells, instead, require stimulation of their IL-7 receptor to remain alive and responsive (Schluns et al., 2000). This cytokine is also important for naïve CD4+ T cells in culture and in lymphopoenic hosts, although, to date, the extent of cytokine-dependence of naïve CD4+ T cells is unknown (Seddon et al., 2003).

The Kruppel-like factors (KLFs) and Forkhead box class O transcription factors (FoxO) are two groups of transcription factors that play key roles specifically in the onset and maintenance of the quiescent programme in resting B and T cells (see section 1.4.1). KLF2 is essential for T cell quiescence and
survival and its expression peaks in resting T cells and decreases following activation (Schober et al., 1999). KLF2 conditional deletion results in a dramatic reduction in the number of mature T cells in peripheral lymphoid organs (Kuo et al., 1997). It has been proposed that KLF2 induces quiescence by indirectly silencing Myc expression. Myc is a proto-oncogene upregulated in several tumours and promotes proliferation in normal cells (Johnston et al., 1999). Myc promotes cell proliferation by enhancing the expression of cell cycle-regulating genes (Kim et al., 1994) (Fig. 1.1).

**Figure 1.1: Schematic representation of the role of KLF2/LKLF in resting and activated T cells.**

KLF2-mediated growth arrest has been attributed mostly to its inhibitory effect on Myc. Myc promotes cell growth and proliferation by stimulating the transcription of CDC25 (see section 1.2.4) and repressing Gadd45 (growth arrest and DNA damage gene-induced gene 45, a cell cycle blocker). Other targets of Myc in activated T cells are CD30 and CD71, important for T cell activation. CD1a, also relevant for T cell activation is regulated by factors other than Myc and is also repressed by KLF2. TCR stimulation (above threshold) inhibits KLF2 and releases Myc from its negative regulation, leading to cell proliferation (reproduced with permission from Yusuf and Fruman, 2003, Trends in Immunology, copyright (2003) Elsevier).
The knockout of *Myc* in quiescent B cells renders them unresponsive to activating stimuli (de Alboran et al., 2001). On the other hand, *Myc* overexpression increases the size of B cells as one of its morphological phenotypes (Iritani and Eisenman, 1999). Overexpression of *KLF2* in leukaemia cell lines results in downregulation of *Myc* and in a quiescent phenotype (Buckley et al., 2001).

Another member of the KLF family, KLF4, has a prominent role in the regulation of quiescence in resting B cells. *Klf4* is highly expressed in quiescent but not in activated B cells, where its overexpression leads to cell cycle block (Yusuf et al., 2008). Interestingly, *Klf4* is, together with *Pou5f1*, *Sox2* and *Myc*, one of the genes whose ectopic expression confers pluripotency to differentiated fibroblasts (Wernig et al., 2007). This is particularly interesting in light of the observation that quiescent cells are resistant to differentiation (Coller et al., 2006). Serum starved and contact inhibited fibroblasts downregulate the expression of myogenic genes when this differentiation programme is stimulated. The authors of this study propose that, by inhibiting terminal differentiation, quiescence is maintained as a reversible exit from the cell cycle (Coller et al., 2006).

FoxO transcription factors (FoxO1, 3a, 4 and 6) are transcriptional activators that play roles in apoptosis, cell cycle arrest, cell stress and DNA repair (Kops et al., 2002). FoxO transcription factors are negatively regulated through phosphorylation by Akt, which is activated in response to growth factors (Brunet et al., 1999). Upon phosphorylation, FoxO transcription factors bind to 14-3-3 proteins and migrate from the nucleus to the cytoplasm, where they become inactive (Brunet et al., 2002) (Fig. 1.2).
Figure 1.2: Schematic representation of the regulation of FoxO transcription factors in resting and activated B cell.

In quiescent cells, unphosphorylated FoxO resides in the nucleus where it promotes the expression of Gadd45, p130 and p27, which together promote exit from the cell cycle before the restriction point (see section 1.2.4). Dyrk1 might work as a co-factor but its role is still debated. Antigenic stimulation of the BCR (above threshold) activates the PI3K/Akt pathway. Akt phosphorylates FoxO transcription factors at three residues. This triggers the displacement of the protein from its binding sequence and its relocalization to the cytoplasm, where 14-3-3 proteins sequester it. FoxO target genes are no longer activated and this leads to cell cycle entry (reproduced with permission from Yusuf and Fruman, 2003 Trends in Immunology, copyright (2003) Elsevier).

FoxO1 and 3a are expressed in quiescent B and T cells and their ectopic expression in B cells blocks their proliferation (Yusuf et al., 2004). FoxO directly promotes the transcription of Klf4 and p130 in quiescent B cells (Yusuf et al., 2008) and the conditional knockout of FoxO1 in T cells leads to auto-activation and reduces the viability of quiescent T cells by directly affecting the IL-7 survival pathway (Ouyang et al., 2009). Another target of FoxO’s is p27 (see below), which indicates that they might operate upstream of KLFs in the quiescent
programme by directly promoting the exit from the cell cycle of immature B cells (Medema et al., 2000).

Additionally, a different Forkhead transcription factor, FoxP1, promotes the maintenance of a quiescent state in resting T cells as opposed to a memory T cell state (characterised by slow but detectable proliferation) (Feng et al., 2011). The knockout of FoxP1 enhances the responsiveness of resting T cells to IL-7, turning them into effector CD8+ cells.

1.2.4 The molecular mechanisms of cell cycle progression and exit

Cyclins, Cyclin-dependent kinases (CDKs) and CDK kinase inhibitors (CKIs) have been identified as key players in the regulation of the different stages of the cell cycle and in the mechanisms that trigger quiescence (Fig. 1.3). Cyclins and CDKs form stable and active complexes that in turn regulate cell cycle decisions through phosphorylation of target proteins. In particular, CyclinD/CDK4-6 and CyclinE/CDK2 regulate progression through G1 or exit from the cell cycle before the transition to S (Blomen and Boonstra, 2007).
Figure 1.3: Schematic representation of the cell cycle-dependent expression of Cyclins and Cyclin/CDKs complexes that regulate progression through the cell cycle.

The expression of Cyclin D is triggered by mitogens in early G1 and is preserved until cytokinesis. Cyclin E is transiently expressed across the G1/S border. Cyclin A is expressed from early S to early mitosis (prometaphase). Cyclin B is expressed in G2 and its degradation at the onset of anaphase is required for mitotic progression. The Cyclin D/CDK4-6 complex operates in early G1 (before the restriction point). The Cyclin E/CDK2 regulates the entry into S. The Cyclin A/CDK2 is active at the onset of DNA replication. The Cyclin A/CDK1-2 complex is active in controlling DNA replication and repair in mid/late S phase. The Cyclin A/CDK1 complex regulates the start of mitosis. CDK1 regulates key steps during mitosis by interacting with Cyclin A and B, which are both degraded before metaphase and anaphase respectively.

Cyclin D expression begins early in G1 and reaches the plateau phase before the transition into S-phase (Musgrove, 2006). The expression of Cyclin E, instead, peaks at the G1/S border and rapidly decreases following the beginning of DNA duplication. Importantly, the promoter of Cyclin E is a target of the E2F1
transcriptional factor and, therefore, Cyclin D regulates the expression of Cyclin E (see below) (Ohtani et al., 1995). The rapid degradation of Cyclin E in early S-phase is accompanied by an increase in the expression of Cyclin A, whose levels are maintained through S, G2 and M (Fung and Poon, 2005). Cyclin B expression strongly correlates with mitosis and its degradation is necessary for correct chromosomal segregation in anaphase (Zhang et al., 1998). Despite the fact that the same E3 ligase (the Anaphase Promoting Complex, see section 1.5.3) regulates the degradation of both Cyclin A and B, Cyclin A is degraded during prometaphase and Cyclin B at the onset of anaphase. The mechanisms dictating this differential timing are still poorly understood (den Elzen and Pines, 2001).

Cyclin D expression is triggered in response to mitogens in early G1 and is the endpoint of the RAS/RAF/MAPK pathway through the binding of ERK (extracellular signal-receptor kinase) to the Cyclin D promoter (Albanese et al., 1995). Ras also stimulates the activation of the PI3K/Akt pathway, which promotes the translation and reduces the degradation of Cyclin D1 via two mechanisms. Firstly, Akt phosphorylates and activates mTor (mammalian target of rapamycin), which, through phosphorylation of downstream targets, promotes the translation of the Cyclin D mRNA (Koziczak and Hynes, 2004). Secondly, the kinase GSK-3 phosphorylates and promotes the degradation of Cyclin D. The phosphorylation of GSK-3 by Akt inhibits this kinase and, indirectly, favours the accumulation of Cyclin D in G1 (Diehl et al., 1998). The dependence on external mitogens makes Cyclin D a sensor of the environmental conditions by interacting with and activating CDK4 or 6 (depending on the tissue) (Matsushime et al., 1994).

The binding of Cyclins is necessary to fully activate CDKs, serine-threonine kinases whose main targets are retinoblastoma family (RBs) proteins Rb, p130 and p107 (Ren and Rollins, 2004). This leads to the phosphorylation of Rb, which
can bind to E2F transcription factors and sequester them, thus preventing the transcription of E2F-mediated transcription of genes required for S-phase entry (Stevaux and Dyson, 2002). The interaction between Rb and E2Fs also induces expression of Cyclin E in late G1, which associates with and activates CDK2. The CyclinE/CDK2 complex further phosphorylates Rb, an event that marks the restriction point, the turning point in the G1-phase after which proliferation is ensured even in the absence of mitogens (Cooper, 2003). In its hyperphosphorylated form, Rb no longer binds to E2Fs, which transcribe genes required for S-phase entry (Stevaux and Dyson, 2002) (Fig. 1.4).

Figure 1.4: The role of RB proteins in G1 regulation.
Unphosphorylated Rb is unable to bind E2F TFs. p130 binds and represses E2F TFs in quiescence (here termed G0). Phosphorylation of Rb by Cyclin D/CDK4-6 allows it to bind to E2F and promotes the transcription of Cyclin E. At the same time, this binding prevents E2Fs from activating the transcription of genes that promote entry into S. The Cyclin E/CDK2 complex hyperphosphorylates Rb, releasing E2Fs, which can now activate the transcription of genes that lead to entry into S. p21 and p27 CKIs (also known as WAF1 and KIP1) repress the CyclinE/CDK2 complex but can be sequestered by the CyclinD/CDK4-6 complex. INK4 CKIs (p16, p15, p18 and p19) negatively regulate the CyclinD/CDK4-6 complex (reproduced with permission from Malumbres and Barbacid, 2001, Nature Reviews Cancer, copyright (2001) Nature Publishing Group).
CKIs inhibit the activity of CDKs by disrupting their association with Cyclins (Sherr and Roberts, 1999). The WAF/KIP CKIs (p21 and p27) antagonize the Cyclin E/CDK2 complex and bind to Cyclin D/CDK4-6 preventing them from binding to CDK2. The INK4 CKIs (p16, p15, p18 and p19) target CDK4-6. INK4 CKIs displace WAF/KIP CKIs from the Cyclin D/CDK4-6 complex, thus making them available for Cyclin E/CDK2 inhibition (Sherr and Roberts, 1999).

Quiescent cells exhibit reduced levels of Cyclin D as a result of reduced mitogenic stimulation (Ladha et al., 1998). As a consequence, one of the hallmarks of quiescence is the presence of high levels of unphosphorylated Rb (Ezhevsky et al., 2001). Another RB family protein, p130, plays a pivotal role in quiescent cells. p130 forms a complex with the E2F transcription factor E2F4, which actively prevents the transcription of genes required for S-phase entry (Smith et al., 1996) (Fig. 1.5).
Figure 1.5: Proposed model for *in vitro* induced quiescence.

Under normal mitogenic stimulation, the MEK/MAPK pathway stimulates Cyclin D production and prevents p21 and p27 inhibition of Cyclin D/CDK4-6. This complex hyposphorylates Rb, which can bind to E2F. Lack of mitogens leads to accumulation of unphosphorylated Rb and allows p130 and p107 to bind to E2F and completely repress them. Contact inhibition inhibits the formation and the activity of the Cyclin D/CDK4-6 complex. Loss of adhesion (for adherent cells) might prevent proliferation by allowing p21 and p27 to exert their inhibitory function on the Cyclin E/CDK2 before the restriction point (reproduced with permission from Blomen and Boonstra, 2007, Cellular and Molecular Life Sciences, copyright (2007) Springer).

1.2.5 Irreversible exit from the cell cycle: senescence and terminal differentiation

Senescence is an irreversible process and disruptions in the senescence-programme are linked to cancer (Campisi, 2001). As the term implies, senescence is linked to the age of the cell. Unlike immortalized cells, primary cells have a finite number of doublings (Hayflick, 1965). Once a cell has exhausted its reproductive capability it exits the cell cycle but, unlike apoptosis,
which is a programmed cell death, senescence does not lead to cell death. Senescent cells are larger and have higher metabolic activities than quiescent cells, which is likely to result from the fact that senescent cells exit the cell cycle close the G1/S border (Demidenko and Blagosklonny, 2008).

Mechanistically, high expression of the CKIs p21 and p16 constitutes the hallmark of the senescent state. p21 expression increases towards the last cell cycles in the lifespan of a cell, whereas p16 expression peaks in senescent cells (Mirzayans et al., 2010). This suggests that p21 triggers entry into senescence before the cell can progress through the restriction point and p16 reinforces this state. Ectopic expression of p16 can trigger cell cycle exit and senescence in proliferating cancer cells (Dai and Enders, 2000).

p16 inhibits CDK4/6-mediated phosphorylation of Rb (Takahashi et al., 2006). p21 is upregulated following p53 activation, which is triggered by DNA damage and telomere depletion, a hallmark of cellular aging (Beliveau et al., 2007). p53 is a tumour suppressor that regulates growth arrest, apoptosis and DNA repair (Reinhardt and Schumacher, 2012). p53 is activated by DNA damage, hypoxia, oncogenes and other stressful conditions. Although Cyclin D expression is dependent on mitogens, expression of CKIs is also enhanced by MAPK kinases (Sewing et al., 1997).

Above a certain threshold, stimulation can cause senescence by promoting the expression of p21, a concept known as mitogen hyperstimulation (Blagosklonny, 2003). Moreover, mitogen hyperstimulation leads to the accumulation of reactive oxygen species (ROS), which causes DNA damage and is observed in senescent cells (Macip et al., 2002). Interestingly, accumulation of ROS is observed even in cultured cells over time (Hagen et al., 1997). DNA damage triggers p53 activation, which in turn leads to the expression of p21 (Fig. 1.6).
Figure 1.6: Proposed mechanism for p16 and p53 induced senescence.

Hypermitogenic stimulation is a form of stress that can lead to the activation of p53 with or without the intermediate step of the generation of ROS and ROS-induced DNA damage. p53 activation leads to the upregulation of p21 and p21-mediated senescence entry. Sustained MEK/MAPK stimulation by mitogens can also lead to the upregulation of p16, which, as a CKI, is involved in the maintenance of senescence (reproduced with permission from Blomen and Boonstra, 2007, Cellular and Molecular Life Sciences, copyright (2007) Springer).

Differentiated cells represent another type of viable and metabolically active non-proliferating cell. Although initially thought to be irreversibly post-mitotic in vivo, dedifferentiation and cell cycle re-entry has been documented, for example in Schwann cells (Mirsky et al., 2008). However, for the majority of fully differentiated cells, cell cycle exit is a one-way process and cells do not normally re-enter the cell cycle. Examples of terminally differentiated cells include plasma cells, which constitute the last stage in the differentiation of B cells and whose function is to secrete very large amounts of soluble immunoglobulin (Ig) molecules (Lisowska-Bernstein et al., 1970).

The CKIs p27 and p21 seem to play key roles in the establishment of differentiation programmes. Both proteins are generally upregulated in differentiated cells and this has led to the formulation of a general model in which p27 increases over the CDK4-6 saturation point and progressively inhibits CDK2 (Hsieh et al., 2000). In addition, p21 has been proposed to induce the transcriptional programme required for differentiation of neuroblastoma cells.
(Poluha et al., 1996). According to this model, p21 would exert its function after p27-mediated cell cycle arrest (Zezula et al., 2001). Rb is also implicated in differentiation in some cell types. For example, in muscle cells it binds to the differentiation repressor HBP1 and represses it (Shih et al., 1998). Rb also binds, in its phosphorylated form, to the transcription factor MyoD activating its myogenic activity (Gu et al., 1993). Another example is the interaction of phosphorylated Rb with NF-IL6, which enhances the capacity of NF-IL6 to promote monocyte and macrophage differentiation (Chen et al., 1996).

1.3 The development of mouse B and T lymphocytes

1.3.1 From Hematopoietic stem cell to lymphoid progenitors

In a developing mouse embryo, the first step in the formation of the hematopoietic system is the differentiation of pluripotent stem cells into haematopoietic stem cells (HSCs). HSCs are multipotent stem cells that are characterised by the ability to self-renew and differentiate into more than one lineage (Cumano et al., 1993). HSCs progressively lose part of their differentiation and self-renewal capacity as they develop into multipotent progenitors, which can then further differentiate into an early lymphoid progenitor (ELP) or a common myeloid progenitor (CMP) (Fig. 1.7).

ELPs constitute the first progenitors with strong lymphoid and weak myeloid potential and are therefore more likely to develop into natural killer (NK) cells, B or T lymphocytes (Igarashi et al., 2002). Conversely, CMPs differentiate preferentially into erythrocytes, platelets, basophils, mast cells, eosinophils, neutrophils, dendritic cells or macrophages (Fig. 1.7) (Akashi et al., 2000). These terminal products of HSC differentiation are relatively short-lived and therefore haematopoiesis is required throughout the lifespan of an adult organism.

HSCs are present in the bone marrow of adult mice and humans (Kiel et al.,
Adult HSCs are normally quiescent and fewer than 10% of them resume proliferation and differentiation every day (Weissman, 2000). This balanced equilibrium ensures that fresh leukocytes and erythrocytes are generated on a daily basis, without consuming all of the resources available to an adult organism.

Figure 1.7: Development of HSCs in the bone marrow and formation of oligopotent progenitors of the lymphoid (B, T and NK cells), myeloid (basophils, mast cells, eosinophils, neutrophils, dendritic cells and macrophages) and erythroid (erythrocytes and platelets) lineages.

HSCs generate different types of multipotent progenitors (MPPs), which further develop into early lymphoid progenitors (ELPs) or common myeloid progenitors (CMPs). ELPs lose part of their multipotency and differentiate into either common lymphoid progenitors (CLP) or granulocyte-myeloid progenitors (GMPs) but not megakaryocyte-erythroid progenitors (MEPs). CMPs can preferentially differentiate into GMPs or MEPs. CLPs further specialize into CLP-2, which mostly give rise to B lymphocytes. GMPs are oligopotent myeloid progenitors. MEPs are mostly erythroid progenitors (reproduced with permission from Bhandoola et al., 2007, Immunity, copyright (2007) Elsevier).

1.3.2 Early B cell development

In the bone marrow, ELPs further differentiate into common lymphoid progenitors (CLPs), which can give rise to the first B cell progenitor the pre-pro-B cell (also known as Fraction A), although they also retain the ability to differentiate into T cells (Welner et al., 2008). CLPs begin to synthesise the receptor for IL-7, a cytokine fundamental for B and T cells development and survival in adult mice (Vosshenrich et al., 2003). Pre-pro-B cells are
characterised by the presence of the surface marker B220, which is specific of the B cell lineage (Hardy et al., 1991).

Another key surface marker in the B (and T) cell lineage is CD43 (also known as leukosialin) (Gulley et al., 1988). This marker is present on early B cells but has an expression pattern that is the opposite to B220, decreasing as pre-pro-B cells differentiate into early pro-B (Fraction B) and late pro-B (Fraction C) (Hardy et al., 1991). Globally, pro-B cells exhibit a decreasing dependence on layers of feeder stromal cells and increased response to IL-7 for their proliferation (Hardy et al., 1991).

1.3.3 Immunoglobulin rearrangement

Immunoglobulin (Ig) rearrangement is a crucial process for the formation of an effective immune system (Jung et al., 2006). This process involves the random recombination of the different parts (V,D and J) of the Ig heavy, kappa and lambda loci and generates potentially infinite combinations (Li et al., 2004). This creates a degree of diversity in the population of lymphocytes that guarantees the recognition of virtually any antigen (Jung et al., 2006).

Ig rearrangement begins in early and late pro-B cells, with D-J rearrangement of the heavy chain detected in both, whereas pre-pro-B cells express germ line Ig (Hardy et al., 1991). Large pre-B cells are characterised by the complete rearrangement (VDJ) of the heavy chain genes but still have germ line light chain genes (Sakaguchi and Melchers, 1986). This leads to the formation of a pre-B cell complex (pre-BCR) on the surface, where a heterodimer of λ5 and VpreB (called surrogate light chain or SL chain) substitutes for the light chain (Pillai and Baltimore, 1987).

The formation of the pre-BCR complex is a fundamental checkpoint and failure to form this complex blocks B cell development (Melchers et al., 1993). The pre-BCR complex triggers the burst in proliferation observed for large pre-B
cells, thus ensuring enrichment of a population of cells capable of correctly presenting a rearranged and properly folded heavy chain (Hardy et al., 1991). Signalling through the pre-BCR allows the transition from large to small pre-B cells, where V-D rearrangement of the light chain takes place (Shapiro et al., 1993).

### 1.3.4 B cell maturation

Once small pre-B cells have completed V-D rearrangement, they become immature B cells and migrate from the bone marrow to the white pulp of the spleen (Allman et al., 1993). The spleen belongs to the group of secondary lymphoid organs together with Peyer’s patches, subcutaneous and mesenteric lymph nodes, and is the site of immature B cell maturation (Picker and Butcher, 1992) (Fig. 1.8).

**Figure 1.8: Hierarchical representation of B cell development.**

In the bone marrow, CLPs differentiate into pre-pro-B cells (here termed Committed progenitors). Pro-B cells begin to rearrange their heavy-chain and become pre-B cells once their pre-BCR is complete. Light chain rearrangement marks the end of the development of the BCR in the bone marrow, as immature B cells express a mature BCR and migrate towards the spleen. Here they develop into T1 and T2 B cells and mature into resting follicular and marginal zone B cells. Follicular zone B cells are not confined solely to the spleen and populate the other secondary lymphoid organs. B cell activation leads to the formation of terminally differentiated plasma cells and resting memory B cells (reproduced with permission from Bottero et al., 2006, Cell Death and Differentiation, copyright (2006) Nature Publishing Group).
Immature B cells have an estimated half-life of 3-4 days, are characterised by low level of B220 on their surface and constitute only 5-10% of the total B cells present in the spleen (Sprent, 1993). In this organ, immature B cells undergo full maturation by passing through up to 3 intermediate stages (termed T1, T2 and T3) (Allman et al., 2001).

1.3.5 Organisation and functions of the spleen

The spleen is composed of a red and a white pulp. The red pulp is the site of filtering of the blood and is highly enriched in macrophages, which engulf old erythrocytes and actively participate in the recycling of the iron present in haemoglobin (Kristiansen et al., 2001). This area also contains antibody-secreting B cells, which are thus conveniently positioned to release antibodies into the circulatory system (Sze et al., 2000).

The white pulp constitutes the largest lymph node in the body of both humans and mice (Cesta, 2006). From the inside to the outside, the white pulp is composed of a central arteriole, a T cell zone (also known as periarteriolar lymphoid sheath, PALS), follicles (also known as B cell zones) and an outer marginal zone (Mebius and Kraal, 2005) (Fig. 1.9). As implied by their name, the T and B cell zones are enriched for T and dendritic cells and B cells respectively, and are the sites of the T-cell-dependent activation and germinal centre formation (Jacob and Kelsoe, 1992).
The central arterioles originate from the afferent main splenic artery. The follicle contains quiescent follicular B cells and follicular dendritic cells and is the site of formation of germinal centres. The T-cell zone contains dendritic and T cells. The marginal zone is composed by endothelial cells, macrophages, marginal zone B cells, dendritic cells and memory B cell (reproduced with permission from Mebius and Kraal, 2005, Nature Reviews Immunology, copyright (2005) Nature Publishing Group).

The marginal zone is histologically more complex. Endothelial cells compose the area surrounding follicles and PALS (Balázs et al., 2001). Sialoadhesin-expressing macrophages form a layer close to the white pulp (Munday et al., 1999).

Marginal zone macrophages delimit the outermost border of the white pulp. These cells express SIGNR-1, which captures antigens present in the blood, and are therefore important for the T-independent activation of marginal zone B cells (see below) (Geijtenbeek et al., 2002). The area in between these two types of macrophages contains dendritic cells, MZ and memory B cells (Liu et al., 1991).

B cell maturation in the spleen gives rise to different subsets of mature B cells that are located in physically distinct areas of this organ. T2 cells mature into follicular type I and II B cells (Cariappa et al., 2007). Follicular B cells are mature B cells characterised by their ability to recirculate from the spleen and migrate to other lymph nodes (Allman and Pillai, 2008) (Fig. 1.10).
Figure 1.10: Model for splenic maturation of T1 cells into follicular and marginal zone B cells.

Rather than proceed through a hierarchical developmental model, T2 cells are likely to bypass the T3 stage and directly develop into type II (FOLII) cells, which have the potential of developing into marginal zone precursors (MZP) and marginal zone mature cells (MZ). Alternatively, T2 (and T3) cells can differentiate into type I follicular B cells (FOLI) (reproduced with permission from Allman and Pillai, 2008, Current Opinion in Immunology, copyright (2008) Elsevier).

T2 cells also develop into marginal zone precursor B-cells, which become marginal zone (MZ) B cells and lose the surface marker CD23 (Srivastava et al., 2005). MZ B cells remain largely in the marginal, outer layer of the spleen (Mebius and Kraal, 2005). The decision between follicular or MZ B cell development largely depends on the extent of the stimulation of the B cell receptor (BCR) on the surface of IM B cells (Cariappa et al., 2001). High BCR stimulation promotes the development of mature follicular B cells, whereas a weak BCR signalling leads to the formation of a MZ B cell. However, the BCR is required for the survival of all B cells in the periphery (Kraus et al., 2004). Follicular and MZ B cells differ in the markers that are expressed on the cell surface: CD23 and high IgD on the former and high HSA and CD1d on the latter (Allman and Pillai, 2008).

Both types of mature B cells normally survive in a quiescent state and wait for encounter with the antigenic stimulus that will active their proliferation and
activate the immune response (Paul et al., 1987). Follicular B cells play a key role in T-dependent activation, which is mediated by antigen-presenting cells (APCs) and T-helper (T_H) cells (see section 1.3.6) and represent, by far, the largest population of mature resting B cells in the spleen (Singer and Hodes, 1983). Activation of follicular B cells leads to formation of terminally differentiated long-lived plasma cells (PC) and memory B cells. Only 5% of the total pool of splenic resting B cells is composed of MZ B cells, which play an important role in T-independent activation following bacterial infection, leading to the formation of short-lived PCs (Andersson et al., 1972).

1.3.6 T-dependent B cell activation and development

Mature follicular B cells are located in the follicles of the spleen and peripheral lymph nodes. Physically, these regions are adjacent to T cell reservoirs known as T-cell zones (Pillai and Cariappa, 2009). The activation of T cells by APCs (see section 1.3.9) triggers the relocalization of T cells towards the edge of the follicles (Ansel et al., 1999). At the same time, antigens initiate a cascade of events that leads to a migration of B cells towards the T-cell zone (Garside et al., 1998). This generates a germinal centre (GC), which is characterised by B cell proliferation, rearrangement of the immunoglobulin variable (V) genes and clonal selection to ensure the proliferation of cells capable of recognizing the specific antigenic peptide (Berek et al., 1991). A proportion of clonally selected B cells exit the GC and become memory B cells (McHeyzer-Williams and Ahmed, 1999).

Memory B cells constitute a heterogeneous population of quiescent, selected, mature B cells that, upon re-encounter with the specific antigen that generated them, can rapidly mount an immune response by quickly resuming proliferation (Good-Jacobson and Shlomchik, 2010). Alternatively, GC B cells can further develop into plasmablasts that migrate to regions of the lymph nodes.
adjacent to blood capillaries (Sze et al., 2000). In these regions, termed junction zones, plasmablasts actively proliferate and terminally differentiate into long-lived PCs (García De Vinuesa et al., 1999) (Fig. 1.11).

![Image](image.png)

**Figure 1.11: Schematic representation of different developmental pathways for mature B cells.**

Upon encounter with blood-borne antigens, MZ B cells proliferate and develop into short-lived plasma cells, which rapidly become apoptotic. This pathway is also observed in splenic follicular B cells. Alternatively, follicular B cells can develop into memory B cells or plasmablasts. Upon second exposure to the antigen that generated them, memory B cells rapidly proliferate and directly form plasmablasts. These short-lived cells complete the developmental programme by becoming terminally differentiated long-lived plasma cells, which secrete immunoglobulins and are localized primarily in the bone marrow. Plasma cells are also present in secondary lymphoid organs and at sites of inflammation, where they eventually become apoptotic and die (reproduced with permission from Radbruch et al., 2006, Nature Reviews Immunology, copyright (2006) Nature Publishing Group).

Long-lived PCs develop in secondary lymphoid tissues and home mostly to the bone marrow (Manz et al., 1997). Terminal differentiation of a proliferating plasmablast into a terminally differentiated long-lived PC is marked by exit from the cell cycle in G1 (Vernino et al., 1992). PC will then go on exerting their sole function as Ig-secreting cells and will no longer resume proliferation (Tourigny et al., 2002).
1.3.7 *T-independent B cell activation and development*

MZ B cells are able to respond to blood-borne antigens and mount an effective immune response in the absence of T<sub>H</sub> cells (Balázs et al., 2002). Examples of T-independent antigens include lipopolysaccharide (LPS), present on the surface of bacteria (Martin and Kearney, 2002). The response of MZ B cells to antigens is a proliferative burst followed by formation of short-lived PCs (Martin and Kearney, 2002). MZ B cells develop into intermediate plasmablasts, which move from the marginal zone to the red pulp, in a region adjacent to blood vessels (O'Connor et al., 2002). These short-lived PC undergo apoptosis following a period of intensive production of Ig’s (Auner et al., 2010). Follicular and memory B cells are also capable of generating short-lived PC in response to sustained antigen exposure (Radbruch et al., 2006).

1.3.8 *T cell development*

The development of B and T cells overlaps in the earliest stages, until progenitors rapidly migrate from the bone marrow to the blood vessels before reaching the thymus (Wright et al., 2001). Indeed, all T cells precursors are detectable in the blood of adult mice (Umland et al., 2007) (Fig. 1.12).
Figure 1.12: Hierarchical representation of T cell development from HSCs in the bone marrow and blood, where the first T cell specific progenitor (common T cell progenitor, CTP) can be identified.

The thymus is the site of T cell maturation and TCR selection. Here blood-borne precursors enter in response to the cytokine CCR9 and become thymus-settling progenitors (TSPs). In the thymus they develop into double negative 1 (DN1) or early thymic progenitors (ETPs). ETPs develop into double negative 2 and then 3 (DN2 and 3) cells, whereas DN1 can directly lead to the formation of DN3. DN3 become double positive (DP) T cells, which express both CD4 and CD8 on their surface. Single positive (SP) mature, naïve T cells are the result of different stimulation of either receptor and migrate from the thymus to the secondary lymphoid organs (reproduced with permission from Bhandoola et al., 2007, Immunity, copyright (2007) Elsevier).

The first T cell-specific progenitor population is found in the blood and are called circulating T cell progenitors (CTPs) (Rodewald et al., 1994). These cells exhibit diminished B cell, myeloid and erythroid differentiation potential both in vivo and in vitro (Krueger and von Boehmer, 2007). ELPs, CLP1s, CLP2s and CTPs express the surface marker CCR9, which is important for their entry into the thymus (Schwarz et al., 2007). In the thymus, T cell progenitors are globally termed thymus-settling progenitors (TSPs) (Izon, 2008). TSPs receive strong
differentiation stimulation by thymic stromal cells through the Notch pathway (Radtke et al., 1999). TSPs develop into early thymic progenitors (ETPs) and double-negative 1 (DN1) cells, which are defined by their common lack of CD3, CD8 and CD25, the presence of CD44 and CD117 (also known as Kit) (Shortman and Wu, 1996).

ETPs are characterised by low levels of CD4 on their surface, whereas DN1 cells are completely devoid of this marker (Michie et al., 1998). DN1 cells can be further divided into subsets, based on the markers on their surface. DN1a and b are characterised by the absence (DN1a) or presence (DN1b) of CD24 on their surface (Porritt et al., 2004). DN1c cells have particularly high levels of CD24 but lower levels of Kit than DN1a and b. DN1d and e are defined by the presence of IL-7 receptor and lack of Kit. They differ for the presence (DN1d) or absence (DN1e) of CD24.

ETPs acquire CD25 and become double negative 2 (DN2) cells (Lind et al., 2001). These cells possess some multi-lineage potential as they can differentiate into NK and DC as well as DN3 T cells (Shen et al., 2003). Both DN1s and DN2 cells differentiate into DN3 cells, which are characterised by the reduction in the expression of CD44 and Kit as well as reduced DC and NK differentiation potential (Benz and Bleul, 2005). DN3s downregulate CD25 and CD44 expression and become DN4 T cells (Godfrey et al., 1993). Similar to the BCR receptor, T cells progressively rearrange the V, D and J regions to generate heterogeneous combinations of T cell receptors (TCRs) during their development in the thymus (Capone et al., 1998). There are 4 TCR loci (α, β, γ and δ) that are subject to rearrangement in T cells (von Boehmer, 1990).

The α and γ TCR loci contain V and J segments, the β locus V, D and J, the δ locus J and D. Most mature T cells have a rearranged TCR composed of a heterodimer of α and β (TCRαβ) but a distinct population of TCRγδ also exists (Pardoll et al., 1987). The choice is dictated by the intensity of signalling through
the Notch ligand in the thymus: high in the case of TCRαβ and low in that of TCRγδ (Ciofani et al., 2006). The rearrangement of the genes in the β locus begins in DN2 cells and this promotes the subsequent rearrangement of the α locus (Petrie et al., 1995). DN4s can either generate a TCR-bearing T cell or proliferate and express CD4 and CD8 surface markers and become double positive (DP) T cells (Ciofani et al., 2006). DP cells complete the maturation of their TCR receptors by rearranging the α locus (Guo et al., 2002).

DPs are immature T cells that present the receptors for major histocompatibility complex (MHC) class-I (CD8) and class-II (CD4) antigens (Germain, 1994). MHC antigens are peptides expressed on the surface of most cells as a response to the presence of cytosolic and extracellular antigens respectively. Class-I antigens are expressed on most cells, whereas class-II are synthesized by B cells, macrophages, dendritic cells, monocytes and endothelial cells.

The resolution of the double positive state to single positive (SP) CD4+ or CD8+ cells marks the maturation of T cells (Germain, 2002). The length of stimulation of CD4 or CD8 drives the developmental choice between CD4 versus CD8 differentiation (Yasutomo et al., 2000). SP CD4+ and CD8+ cells (henceforth referred to as CD4+ and CD8+ respectively) leave the thymus as quiescent mature cells (Feng et al., 2011) and populate secondary, peripheral, lymphoid organs: mostly subcutaneous lymph nodes but also the mesenteric lymph node, Peyer’s patches and, to a lesser extent, the spleen (Williams and Butcher, 1997).

Peripheral lymph nodes have a structure similar to the white pulp of the spleen (Fig. 1.13). The inner paracortex contains naïve T cells and DCs, while resting B and follicular DCs are located in the adjacent cortex (Drayton et al., 2006). Mature lymphocytes enter the lymph nodes via the high endothelial venules (HEVs), while DCs are transported by the afferent lymphatic vessels
Similar to the white pulp in the spleen, subcutaneous lymph nodes are divided into a T cell zone (paracortex) and follicular zone (cortex). Lymphocytes are introduced via the high endothelial venules (HEVs), while dendritic cells enter via the afferent lymphatic vessels. Cells in the cortex secrete CXCL13, which promotes the migration of B cells towards the paracortex. CCL19 and 21, instead, position DCs and T cells towards the B cell zone, thus promoting the formation of germinal centres in the cortex. The cortex is protected by an external capsule (reproduced with permission from Drayton et al., 2006, Nature Immunology, copyright (2006) Nature Publishing Group).

DCs activate T cells by means of their MHC antigens, which stimulate the TCR, and by co-stimulation of CD28 on the surface of T cells (King et al., 1996). As in the spleen, stromal and endothelial cells secrete different cytokines that help to position B and T cells in close proximity, in order to stimulate the formation of GCs in the cortex (Cyster, 2003). SP naïve (CD25-) T cells compose more than 80% of the pool of total T cells in peripheral lymph nodes, whereas the spleen contains naïve as well as memory T cells (Williams and Butcher, 1997).

**1.3.9 CD4+ activation and development**

The TCRs on the surface of CD4+ T cells recognize MHC class-II on the
surface of APCs, leading to activation of T cells (Abbas et al., 1996). Several types of activated, effector T-helper cells ($T_H$) have been identified: $T_H$1, 2, 9, 17 and follicular (Tfh) (Mosmann and Coffman, 1989).

**Figure 1.14: General scheme showing outcomes of CD4+ T cells activation.**
Exposure to pathogens triggers the activation of naïve CD4+ T cells and leads to formation of effector T helper ($T_H$1 and 2 only shown here) and regulatory T cells. Dendritic cells operate as antigen presenting cells and stimulate the TCR and CD28 receptor on naïve T cells through their MHC antigen and CD80/86 respectively. $T_H$1 cells secrete IFN-$\gamma$, which promotes cytotoxic activity of effector CD8+ T cells and macrophage activity. $T_H$2 cells secrete cytokines (IL-4, 5 and 6) that stimulate B cell activation and eosinophil recruitment. Regulatory T cells control the immune response by negatively regulating the activities of effector T cells and neutrophils
T\textsubscript{H}1 cells respond to the stimulation of their TCRs by secreting cytotoxic granule proteins and interferon-\(\gamma\) (IFN-\(\gamma\)), which activate macrophages and stimulate CD8\(^+\) T cells (Smeltz et al., 2002). They are implicated in mounting an immune response against intracellular bacteria, viruses, fungi and protozoa (Sadick et al., 1987). T\textsubscript{H}2 cells respond to extracellular pathogens and promote B cell activation by stimulating them through the BCR and CD40 receptor and by secreting IL-4 (Singer and Hodes, 1983) (Fig. 1.14).

T\textsubscript{H}9 cells are closely related to T\textsubscript{H}2 but secrete high levels of IL-9 (Veldhoen et al., 2008). T\textsubscript{H}17 cells share a spectrum of targets similar to T\textsubscript{H}1 and are characterised by the secretion of IL-17A, E and F (Weaver et al., 2006). T\textsubscript{FH} cells stimulate follicular B cells and are distinct from T\textsubscript{H}2 by the secretion of stimulating IL-21 rather than IL-4 and by a different transcriptional programme (Chtanova et al., 2004).

Activated CD4\(^+\) T cells can also become induced or adaptive regulatory T cells (Tregs), as opposed to the subset of naturally occurring, thymic Tregs generated in the thymus by clonally selecting the TCR via the interaction with a peripheral self-peptide (Sakaguchi et al., 1995). Natural Tregs play a pivotal role in the control of immunological responses and in the prevention of autoimmune diseases as they negatively regulate effector T cells and APS (Vignali et al., 2008) (Fig. 1.16). Natural and induced Tregs can inhibit effector T cells through secretion of immunosuppressive cytokines transforming growth factor-\(\beta\) (TGF-\(\beta\)) and IL-10 (Annacker et al., 2003).

Alternatively, natural Tregs secrete cytolytic cytokines such as granzyme A and B or perforin (Grossman et al., 2004). Treg cells are distinguishable from naïve SP T cells as they express high levels of the surface marker CD25 (a
marker acquired also by activated SP T cells) (Zelenay et al., 2005). CD25 is a component of the IL-2 receptor, a cytokine that promotes proliferation in activated T cells (De La Rosa et al., 2004). Therefore, it has been postulated that Tregs deprive activated SP T cells of IL-2, thus blocking their proliferation (Pandiyan et al., 2007). Lastly, Tregs can act upstream and inhibit DC activation of SP T cells. Tregs present the cytotoxic T-lymphocyte antigen 4 (CTLA4) on their surface and trigger the release by DCs of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) (Fallarino et al., 2003).

Figure 1.15: Proposed mechanisms of action of Treg cells.
(A) Tregs secrete immunosuppressive cytokines such as TGF-β, IL-10 and 35, which repress T cell proliferation. (B) Tregs release cytotoxic molecules (granzyme A and B and perforin) that trigger the apoptosis of effector T cells. (C) The CD25 on the surface of Tregs and activated T cells is a receptor for the stimulatory cytokine IL-2. Tregs can capture IL-2 and starve effector T cells. (D) Tregs express CTLA4 on their surface, which stimulates the CD80/86 receptor on
dendritic cells. This stimulates the release of immunosuppressive IDO from dendritic cells (reproduced with permission from Vignali et al., 2008, Nature Reviews Immunology, copyright (2008) Nature Publishing Group).

Following acute stimulation of the TCR, CD4+ cells exit quiescence and resume proliferation (Varga and Welsh, 1998). Most of these activated cells are short-lived, but a subset of them differentiate into long-lived CD4+ memory T cells (Harrington et al., 2008). Memory T cells provide a mechanism to trigger a stronger and faster immunological response upon re-encounter with the antigen that generated them (Zinkernagel et al., 1996).

Memory T cells proliferate rapidly following stimulation (Sallusto et al., 2004). They can either be central memory cells (characterised by the expression of CCR7, a surface marker required for homing in secondary lymphoid organs) or effector memory cells (which are negative for CCR7 and reside at peripheral sites) (Henao-Tamayo et al., 2010). Both types of memory cells are capable of producing IFN-γ upon restimulation but central memory T cells have a stronger proliferative tendency and are the only memory T cells capable of secreting IL-2 (Unsoeld et al., 2002).

1.3.10 Development of CD8+ T cells

Quiescent CD8+ cells can differentiate into short-lived effectors, long-lived memory T cells or Treg cells (Kim et al., 2010). A notable difference between CD4+ and CD8+ resting cells is the dramatic proliferative burst observed in the latter following stimulation (Murali-Krishna et al., 1998). Activated CD8+ cells produce and secrete high quantities of cytotoxic granule proteins and antiviral cytokines (Podack and Kupfer, 1991). The vast majority of these highly proliferating cytolytic effectors become apoptotic within 2-3 weeks of activation but a small fraction develop into memory T cells (Kaech and Ahmed, 2001).
Stimulation of resting CD8+ cells can also lead to the production of regulatory T cells (Kim et al., 2010). CD4+ and CD8+ Tregs inhibit different cell types, with the latter uniquely controlling Tfh cells (Kim et al., 2010).

1.4 Transcriptional regulation

One of the central aims of this thesis is to investigate the mechanisms that regulate the transcriptional programme of quiescent lymphocytes. In this section, I will cover some general concepts of eukaryotic transcription and review the recent literature on the role of chromatin and histone modifications in transcriptional control.

1.4.1 Regulatory elements

The mouse genome (mm9) encompasses 28,661 protein-encoding genes. With a few exceptions, these genes are present in their germline configuration in every mouse cell, from the pluripotent embryonic to the terminally differentiated ones. Therefore, what defines each cell type from the others is the regulation of gene expression. A transcriptional programme can be defined as a mechanism to regulate the differential expression of sets of genes. The regulation of gene expression can occur at different stages: transcription of the DNA sequence of a gene into an immature RNA, maturation (or processing) of immature RNA into a messenger RNA (mRNA), transport of RNA from the nucleus (site of the transcription) to the cytoplasm (site of translation into peptide sequences), stability of the RNA, translation and stability of the protein encoded by the mRNA. The regulation of RNA transcription is believed to be a major determinant of the expression of a gene, but regulation of RNA processing and stability can also have important roles (Maston et al., 2006).

Many genes have regulatory elements upstream or within their DNA
sequence. These regulatory elements can promote or repress transcription and are often regulated in a tissue-specific manner. Regulatory elements are often hypersensitive to the action of the enzyme DNase I (Wu, 1980) and DNase hypersensitivity is a widely exploited method for mapping regulatory regions across genomes (Crawford et al., 2006). Briefly, DNases cleave DNA regions not based on their sequence but on their accessibility, which is affected by the organisation of DNA into nucleosomes (Wu et al., 1979). Binding of transcription factors and the transcriptional machinery partially displaces nucleosomes leading to hypersensitivity relative to surrounding regions (Gross and Garrard, 1988).

DNase hypersensitive regions (DNase HS) can be either constitutive or inducible. Constitutive DNase HS regions are found both at transcriptionally positive elements (promoters and enhancers and locus control regions) and negative elements (silencers, insulators) (Gross and Garrard, 1988). DNase sensitivity can also be acquired as a previously silent gene becomes active (Elgin, 1988).

a. Core Promoters

Core promoters are generally located around the transcriptional start site (TSS) of eukaryotic genes and constitute the minimal regulatory element required for transcription (McKnight and Kingsbury, 1982). These regions define the TSS of a transcript as they recruit the components of the pre-initiation complex (PIC), which serves as a positioning mechanism for RNA Pol II (Safer et al., 1985). The PIC is composed of the general transcription factors (GTFs) TFIIA, B, D, E, F and H, RNA Pol II and the Mediator complex (Hahn, 2004).

Transcription factors bind to specific DNA sequences and promote or repress transcription (in which case they are termed silencers or repressors) (Ptashne and Gann, 1997). Transcription factors can directly interact with the PIC but often operate by recruiting other proteins that lack DNA binding domains (co-
activators or co-repressors), which interact with the PIC (Kadonaga, 2004). Moreover, transcription factors regulate transcription by recruiting chromatin-remodelling factors and histone-modifiers (discussed below).

According to the canonical model of transcriptional activation, the assembly of the PIC begins with the binding of TFIID to a DNA sequence within the core promoter known as the TATA box (Starr and Hawley, 1991) (Fig. 1.16). TFIID is a multi-protein complex composed by a TATA-binding protein (TBP) and several TBP associated factors (TAFs) (Reinberg et al., 1987). TBP is a universal eukaryotic transcription factor that binds to the TATA box at core promoters (Wu, 1985).

![Figure 1.16: Basic structure of core promoters.](image)

Consensus sequences are displayed below the names (reproduced with permission from Smale and Kadonaga, 2003, Annual Review of Biochemistry, copyright (2003) ANNUAL REVIEWS).

TAFs work as co-activators and govern the specificity of TBP binding (Dynlacht et al., 1991). TAFs also bind to other components of core promoters: TAF1 and 2 bind to the Initiator (Inr), TAF6 and 9 to the Downstream Promoter Element (DPE) (Smale and Kadonaga, 2003) (Fig. 1.16). TFIIA stabilizes the interaction between the TATA box and TFIID (Liu et al., 1999). TFIIIB binds to another element of core promoters called the TFIIIB-recognition element (BRE) and bridges between TFIID and RNA Pol II, thus establishing the direction of transcription (Lagrange et al., 1998).
There is evidence that RNA Pol II binds to the core promoter as a preformed holoenzyme, which contains the Mediator complex together with TFIIE, TFIIF and TFIIH (Myer and Young, 1998). The Mediator complex is a large multi-protein complex, which is required for optimal activation of transcription in eukaryotes in vivo (Jiang et al., 1998). TFIIE and H are important for transcriptional initiation through the phosphorylation of the carboxyl-terminal domain (CTD) of RNA Pol II and will be discussed later.

b. Proximal promoters

Proximal promoters are located within 100 bp upstream of core promoters (La Volpe et al., 1983). Proximal promoters often coincide with unmethylated CpG-rich regions called CpG islands (Bird, 1980). Current evidence suggests that CpG islands promote transcription of housekeeping, tissue-specific and developmental genes (Deaton and Bird, 2011). The distribution of CpG islands across the genome frequently overlaps with sites of transcriptional initiation and has helped with the identification of new TSSs (Carninci et al., 2006). CpG islands are often associated with an open DNA configuration but not necessarily with active transcription as RNA Pol II binding at CpG islands is observed even at silent genes (Guenther et al., 2007). Similarly, there is evidence that activating transcription factors might bind to CpG islands constitutively and independently of the transcriptional status of the gene (Cuadrado et al., 2001).

CpG islands are enriched for binding sites for the transcription factor Sp1 (G/T-GGGCGG-G/A-G/A-C/T) called the GC box element. Sp1 binds to these regions and recruits TBP even in the absence of a TATA box, thus allowing the formation of the PIC (Butler and Kadonaga, 2002). TATA-less promoters are quite frequent, particularly at CpG-rich promoters (Carninci et al., 2006). CpG islands also contain binding sites for other transcription factors such as E2F and ETS (Jaeger et al., 2010). However, not all CpG islands contain binding motifs for
activators and there is also evidence that they mediate the recruitment of repressors such as Polycomb group proteins (see section 1.6) (Mendenhall et al., 2010).

Cytosines in CpG islands can also become C5-methylated, a covalent modification that is thought to reinforce silencing (Stein et al., 1982). Historically, DNA methyltransferases (DNMT) 3A and B were termed de novo methyltransferases as they methylate unmodified cytosines, whereas DNMT1 preferentially methylates hemimethylated DNA and is therefore implicated in the copy of this mark to newly synthesised DNA strands during S phase (Jones and Liang, 2009). However, DNMT3A and B are also required for the maintenance of the mark and all three enzymes are required for the correct development of mammals (Okano et al., 1999). It is important to point out that DNA methylation does not always impair the binding of transcription factors (Hsieh, 2000).

At silent promoters, DNA methylation, however, frequently co-localizes with the repressive histone mark H3K9me3 (see section 1.4.3) and reinforces transcriptional repression through the binding of methyl-binding domain proteins (MBDs), which recruit repressive histone-modifiers (Bogdanović and Veenstra, 2009). Methylated cytosines are also bound by Tet1, which converts the methyl group into a hydroxymethyl group (Tahiliani et al., 2009). This phenomenon leads to different effects on promoters depending on the context. In ES cells conversion to hydroxy-methyl cytosine positively affects transcription at certain promoters, possibly by removing MBDs. However, it can also allow the recruitment of repressive complexes such as PRC2 (see section 1.6) (Wu et al., 2011a). Additionally, Tet1 promotes gene repression by directly recruiting co-repressors such as SIN3A (Williams et al., 2011).

c. Enhancers

Enhancers stimulate transcription of their target genes by promoting
formation of the PIC (Banerji et al., 1983). Unlike promoters, enhancers work independently of their orientation and show positional flexibility relative to the TSS (Banerji et al., 1981). Enhancers are often found upstream of proximal promoters but can also be located within or downstream from the gene they control (Gillies et al., 1983).

Similar to proximal promoters, enhancers feature binding sites for transcriptional activators (termed enhancer binding factors) (Atchison, 1988). Despite their distance from the TSS, factors that bind to enhancers can directly interact with the Mediator complex and TFIID through the recruitment of the Cohesin complex, which causes the DNA structure to loop out (Hadjur et al., 2009). The recruitment of histone modifiers and chromatin remodelling complexes by enhancer-bound factors is also crucial and, indeed, enhancers are characterised by the presence of particular histone variants (H3.3 and H2A.Z) and modifications (H3K27ac and H3K4me1/2) (see below) (Ong and Corces, 2012).

Furthermore, non-coding RNAs are transcribed directly at enhancers and have been shown to promote the interactions of enhancer-bound complexes to the promoter-bound PIC (Wang et al., 2011b). For some genes, enhancers could also be the site of initial recruitment of the PIC, as in the case of the E enhancer of the pD1 promoter in the TCRβ locus (Spicuglia et al., 2002).

d. Silencers

Silencers and enhancers share similar properties: both operate in a position- and orientation-independent fashion and both work through the binding of DNA binding proteins (Laimins et al., 1986). Silencers contain binding sites for repressors, which can directly impair the binding of the PIC or of activators (Harris et al., 2005). Alternatively, repressors can recruit histone-modifiers that cause chromatin to adopt an inaccessible conformation (heterochromatin), which
I will discuss later (Srinivasan and Atchison, 2004).

e. Insulators

Insulators define boundaries between DNA domains and block the effects of activators and silencers that regulate neighbouring genes (Chung et al., 1993). Insulators can restrain the effect of enhancers and proximal promoters by sequestering activators (Defossez et al., 2005). Insulators might block heterochromatic-spread by recruiting histone-modifiers that promote the formation of an open chromatin structure (euchromatin) (Litt et al., 2001).

f. Locus control regions

Locus control regions (LCRs) are regions capable of conferring position-insensitive and copy-dependent expression on a linked gene in a transgenic assay (Grosveld et al., 1987, Dillon et al., 1997). LCRs contain multiple clusters of transcription factor binding sites. These regions are bound by transcription factors and histone-modifiers, which generate an overall open conformation that promotes transcription (Tolhuis et al., 2002). LCRs operate independently of the position and orientation of the genes they control (Spilianakis et al., 2005).
Figure 1.17: Mechanism of action of regulatory DNA elements on gene expression.

(A) Enhancers are distal elements and promote transcription through the binding of activators that stimulate the recruitment of the PIC. (B) Silencers promote transcriptional repression through the binding of repressors, which in turn recruits chromatin-remodelers that lead to the formation of a compact structure. (C) Insulators confine the effects of enhancers to their target genes by sequestering activators (here depicted). At the same time, they restrict the activity of silencers by recruiting histone-modifiers that promote the formation of an open chromatin structure and antagonize silencer-induced chromatin compaction. (D) Locus control regions (LCRs) globally, promote the transcription of loci or groups of genes. This is achieved through the formation of an overall open chromatin structure and through direct contact between LCR sequences and their target promoters (reproduced with permission from Maston et al., 2006, Annual review of genomics and human genetics, copyright (2006) ANNUAL REVIEWS).

1.4.2 The regulation of RNA Pol II at promoters

a. RNA Pol II recruitment

Eukaryotic RNA Pol II is composed of 12 evolutionary conserved subunits
called Rbp1-12 (Allison et al., 1985). Rbp1 is the largest subunit of this complex and the one that mediates binding to the DNA (Kimura et al., 1997). The carboxyl-terminal domain (CTD) of Rpb1 contains multiple repeats of the peptide N-Tyr-Ser-Pro-Thr-Ser-Pro-Ser-C (Corden et al., 1985). There are 52 repeats of this sequence in the mammalian CTD, although the heptapeptide shows increased sequence variations towards the N-terminus (Egloff and Murphy, 2008). This long repeated region of the protein is subject to a dynamic cycle of post-translational modifications that play an essential role in regulating transcription and are involved in the recruitment of proteins implicated in the processing of immature RNAs (also known as pre-mRNAs) (Bentley, 2005) (Fig. 1.18).
Figure 1.18: The RNA Pol II cycle (clockwise from top-centre).

Unphosphorylated RNA Pol II is recruited to promoters by the PIC. The phosphorylation of the CTD by TFIIH on serine 5 disrupts the interaction between RNA Pol II and the Mediator complex (promoter clearance) and marks the beginning of transcription (initiation). RNA Pol II reaches the pausing site where a decision is made between transcriptional arrest and elongation. p-TEFb is implicated in RNA Pol II commitment to elongation at this stage through serine 2 phosphorylation and removal of negative elongation factors. The activity of p-TEFb is enhanced by the phosphorylation of serine 7 on the CTD, also mediated by TFIIH, which might occur after promoter clearance and during transcriptional pausing. Elongating RNA Pol II is therefore phosphorylated on serine 2, 5 and 7, although serine 5 is progressively lost toward the 3’ end of the gene. Possibly reflecting a role in transcriptional termination and 3’ end maturation of transcripts, both serine 2 and 7 marks persists after the transcriptional end site. Finally, the mRNA is released from the enzyme, which is dephosphorylated and removed from the DNA. Soluble RNA Pol II is recruited by the Mediator complex and can start another round of transcription (reproduced with permission from Brookes and Pombo, 2009, EMBO reports, copyright (2009) Nature Publishing Group).

Between recruitment of RNA Pol II and completion of a transcription cycle,
the CTD of RNA Pol II undergoes specific phosphorylations on different residues at specific stages of the transcriptional process (Komarnitsky et al., 2000).

b. Promoter escape and the phosphorylation of RNA Pol II on serine 5

As previously described, current evidence indicates that RNA Pol II is recruited to the DNA as part of a holoenzyme that features the Mediator complex and the GTFs TFIIE, F and H (see section 1.4.1). Mediator recruits the unphosphorylated form of RNA Pol II and any phosphorylation of the CTD disrupts this interaction and the recruitment of RNA Pol II to promoters (Chesnut et al., 1992).

Following recruitment of RNA Pol II, phosphorylation of serine 5 on the CTD (S5p Pol II) disrupts the interaction between Mediator and RNA Pol II, which can leave the promoter region and begin to move towards the 3'-end of the gene while transcribing a complementary RNA (Komarnitsky et al., 2000) (Fig. 1.18). Serine-5 phosphorylation is mediated by TFIIH, which is recruited to the PIC by TFIIE (Søgaard and Svejstrup, 2007). TFIIH is composed of 10 subunits that form a core and a kinase domain, with the former implicated in the denaturation of DNA during the beginning of transcription (Saunders et al., 2006). The kinase domain of TFIIH (also known as CAK: CDK-activating kinase) contains a Cyclin H/CDK7 complex (which phosphorylates the CTD) and a regulatory subunit (Madt1) (Shiekhattar et al., 1995).

S5p Pol II is also a mark recognized by enzymes responsible for the 5'-end methylguanosine capping of pre-mRNAs and by histone-modifiers that generate typical patterns of histone marks at active promoters (described below) (Fabrega et al., 2003). The loss of S5p Pol II has a stronger impact on nascent and precursor mRNAs than on fully spliced, polyadenylated mRNAs (Helenius et al., 2011). Indeed, the levels of S5p Pol II do not necessarily correlate with transcriptional levels in ES cells, as S5p Pol II is present at both active and
Polycomb-repressed genes, where little or no elongation and productive transcription is observed.

c. Phosphorylation of RNA Pol II on serine 7

TFIIH also phosphorylates the CTD on serine 7 (S7p Pol II) at core promoters (Akhtar et al., 2009). S5p and S7p Pol II are marks observed at core promoters and their localization decreases dramatically in the gene bodies (Brookes et al., 2012). S7p Pol II is always observed in conjunction with S5p Pol II at the promoter of actively transcribed genes, whereas S5p Pol II alone can be detected at some repressed genes (Brookes et al., 2012).

S7p Pol II is required for the expression of non-coding small nuclear RNAs (snRNAs) (Egloff et al., 2007). This activity is mediated through recruitment of the Integrator, a 12-subunit complex that regulates the 3' end formation of snRNAs (Baillat et al., 2005). Moreover, S7p Pol II recruits p-TEFb (positive transcriptional elongation factor-b), which phosphorylates RNA Pol II on serine 2 (S2p Pol II) thus triggering transcriptional elongation (see section 1.4.2d) (Czudnochowski et al., 2012).

d. Transcriptional pausing

Following initiation and release from the PIC, S5p Pol II frequently stops its 5’ to 3’ progression approximately 25-50 bp downstream of the TSS, a region termed the pausing site (Gariglio et al., 1981) (Fig. 1.19). Transcriptional pausing is observed at many housekeeping and inducible genes, regardless of their transcriptional status (Guenther et al., 2007). Between transcription of the first 25-50 nucleotides and full transcription of the gene, structural changes are likely to be required (Core and Lis, 2008).
Figure 1.19: Schematic representation of transcriptional pausing.

(A) The negative elongation factors DSIF and NELF stop the progression of S5p Pol II 20-50 bp downstream of the TSS. S5p Pol II helps to recruit factors implicated in RNA capping, which takes place at pausing sites. In some instances, a stalled RNA Pol II backtracks a variable distance from the actual pausing site and a now-unaligned 3' end transcript extrudes from the enzyme. To resume transcription this transcript has to be degraded. (B) TFIIS potentiates the RNA Pol II intrinsic RNase activity toward the extruding, unaligned transcript and limits transcriptional arrest. p-TEFb (and probably CDK12) phosphorylates the CTD on serine 2, which recruits elongation factors, histone modifiers, splicing and 3'-end maturation factors. p-TEFb binding also coincides with displacement of NELF from the pausing site (reproduced with permission from Saunders et al., 2006, Nature Reviews Molecular Cell Biology, copyright (2006) Nature Publishing Group).

mRNA capping occurs during this phase and it has been postulated that this pause serves as a checkpoint before further phosphorylation of the CTD recruits other enzymes to the CTD involved in splicing and 3' processing (Palangat et al., 2005). Pausing sites coincide with the localization of negative elongation factors: DSIF and NELF (Yamaguchi et al., 1999). DSIF is composed of the subunits Spt4 and Spt5 (Hartzog et al., 1998). Interestingly, Spt5 promotes RNA capping
in vitro, thus corroborating the hypothesis that transcriptional pausing is a key
class moment to ensure correct processing of pre-mRNAs (Wen and Shatkin, 1999).
NELF is composed of four subunits (A, B, C/D and E) and might bind directly to
the nascent transcript via its RNA-binding subunit NELF-E (Yamaguchi et al.,
1999). The release of RNA Pol II from pausing sites is a rate-limiting step in
transcription and is regulated by the general transcription factor TFIIS and by p-
TEFb.

The stalled RNA Pol II often backtracks from pausing sites and the now
unaligned 3’ end of the pre-mRNA extrudes from the enzyme (Pal and Luse,
2003). For transcription to resume, these unaligned 3’ end ribonucleotides need
to be cleaved. Backtracking constitutes a rate-limiting step and, depending on the
length of the sequence covered, pausing can become a transcriptional arrest,
which leads to the formation of abortive short and unprocessed transcripts
(Voliotis et al., 2008). RNA Pol II has a weak RNase activity and can therefore
degradate short unaligned 3’ end portions generated during pausing (Shirai and
Go, 1991). The GTF TFIIS enhances this RNase activity and reduces
transcriptional arrest after backtracking (Fig. 1.19B) (Adelman et al., 2005).

e. Transcriptional elongation and phosphorylation of RNA Pol II on serine 2

At the pausing site, RNA Pol II can either be S5p Pol II or S5p/S7p Pol II.
Phosphorylation of Serine 7 enhances the kinase activity of the p-TEFb complex
towards serine 2 of the CTD (S2p Pol II) (Czudnochowski et al., 2012) (Fig. 1.19).
p-TEFb is composed by Cyclin T and CDK9 and has been shown to promote
elongation in vivo (Ni et al., 2008). Other kinases, such as CDK12 in complex
with Cyclin K, have been shown to phosphorylate the CTD on serine 2 as well
(Bartkowiak et al., 2010). p-TEFb binding has also been shown to displace NELF
and, therefore, p-TEFb and CDK12 might cooperate to remove negative
regulators of elongation and switch the CTD to its elongating configuration
(Levine, 2011). S2p Pol II promotes the recruitment of enzymes implicated in mRNA splicing and 3' end maturation as well as histone-modifiers, which are responsible for the deposition of particular histone marks found on the bodies of transcribed genes (see below) (Buratowski, 2009).

S2p Pol II is also recognized by Spt6, which further promotes elongation (Yoh et al., 2007). Distribution of S2p Pol II across the body of a gene increases from the pausing site towards the transcriptional end site and peaks approximately 1kb downstream of the termination site, in antithesis to what is observed for S5p Pol II (Brookes et al., 2012) (Fig. 1.18). Serine-5 phosphorylation is progressively lost as the polymerase progresses towards the 3’ end of the gene through the action of the phosphatase Rtr1 (Mosley et al., 2009) and Ssu72 (Reyes-Reyes and Hampsey, 2007).

1.4.3 Chromatin conformation and transcriptional regulation

a. Spatial organization of the DNA in higher eukaryotes

In most eukaryotes, the DNA is organised in a beads-on-a-string structure called chromatin. The nucleosomes form the repetitive structural units of chromatin (Fig. 1.20). A nucleosome is composed of an octamer of highly basic histones around which the DNA is wound (Luger et al., 1997). The histone octamer is organised as dimers: histones H3 and H4 form two dimers that associate with two dimers of histone H2A and H2B. The DNA is wrapped around this structure (147 bp, in 1.65 superhelical turns) from which unstructured histone tails extrude (Andrews and Luger, 2011) (Fig. 1.20). A fifth histone (H1) can interact with the DNA between nucleosomes (linker DNA) and generate a tighter, higher-order chromatin structure.

The positioning and the density of nucleosomes have been shown to influence transcription. *In vitro* studies have demonstrated that nucleosomes
reduce transcription by 75% compared to naked DNA templates, which are similar to linker DNA. Nucleosomes affect the recruitment of RNA Pol II and the PIC, but elongation is not blocked by the presence of nucleosomes thanks to proteins that travel with S2p Pol II and remove nucleosomes ahead of the enzyme (Lorch et al., 1987). This observation is in agreement with the sensitivity to DNase that characterises regulatory elements (Crawford et al., 2004). Since nucleosomes protect DNA from the activity of DNases the DNase I sensitivity data provide evidence that regulatory elements are located in nucleosome-depleted or -poor regions.

Constitutive DNase sensitive regions possess distinguishing features that make them constitutively nucleosome-poor (Yuan et al., 2005). These constitutively open regions are normally localised at genes that are constitutively active in an organism (Schones et al., 2008). These regions usually lack a TATA box and present a high percentage of poly dA:dT in their sequence, which are harder to bend in order to position histones (Struhl, 1985). CpG islands are also common at these genes and these DNA sequences tend to generate unstable nucleosomes (Ramirez-Carrozzi et al., 2009).
Figure 1.20: Schematic representation of nucleosome assembly.

The DNA (black) is wrapped around an initial tetramer composed by two histone H3 (blue) and H4 (green) dimers. Subsequently, two dimers of histone H2A (yellow) and H2B (red) are incorporated (reproduced with permission from Cockerill, 2011, FEBS Journal, copyright (2011) John Wiley and Sons).

Pioneer factors are a class of transcriptional factors that initiate chromatin remodelling and allow other co-factors to be recruited to nucleosome-rich chromatin regions (Zaret and Carroll, 2011). Pioneer factors such as FoxA, FoxE, FoxO, TLE and GATA factors can bind to nucleosome rich regions through direct interactions with the nucleosome (Cirillo et al., 2002). Pioneer factors share domains with the linker histone H1, which avidly binds to nucleosomes (Sekiya et al., 2009).

Furthermore, FoxA factors have been shown to displace H1, possibly by competing with it for binding sites on nucleosomes (Taube et al., 2010). Like histone H1, the pioneer factor FoxL1 maintains its association with chromatin even during mitosis and might therefore flag promoters for activation or silencing.
in the daughter cell (Yan et al., 2006).

Two mechanisms for how pioneer transcription factors operate have been described. FoxO factors, for example, cause a direct perturbation of the interaction between the core nucleosome and the DNA, facilitating the opening of chromatin (Hatta and Cirillo, 2007). Alternatively, pioneer factors can bind to tissue-specific promoters and, if the correct stimulus is received, can serve as pre-formed scaffolding units for other factors (Levine, 2010). Following the initial binding of activators and repressors, these factors recruit chromatin-remodelers and histone-modifiers, which alter the local structure of chromatin and promote or repress the binding of the PIC (Fig. 1.21).

Figure 1.21: Schematic representation of nucleosome remodelling during the activation of a regulated gene.

Upon binding to its target sequence, a transcription factor can recruit chromatin-remodelers and/or histone-modifiers that alter the structure of neighbouring regions. This exposes additional binding sites and leads to recruitment of more activators and to the acquisition of an open chromatin structure that allows the binding of the PIC (reproduced with permission from Cairns, 2009, Nature, copyright (2009) Nature Publishing Group).
b. Chromatin remodelling

Chromatin-remodelers are co-factors that use the energy obtained from hydrolysis of ATP to assemble, eject and move nucleosomes (Cosma et al., 1999). Chromatin-remodelers interact with histone modifications, DNA-binding proteins and other co-factors. Based on their structural domains, they are classified into four families: SWI/SNF, ISWI, CHD and INO80 (Clapier and Cairns, 2009).

SWI/SNF family members (BAF and PBAF) contain a bromodomain and an HSA-domain (which functions as a DNA helicase) (Mohrmann and Verrijzer, 2005). Bromodomains recognize acetylated residues on histone tails, which are associated with active promoters (see section 1.4.3). SWI/SNF remodelers move and eject nucleosomes to open promoters and activate transcription (Lorch et al., 1999).

ISWI members (NURF, CHRAC and ACF) contain a SANT and a SLIDE domain that together bind to unmodified histone tails and DNA (Corona and Tamkun, 2004). CHRAC and ACF regulate nucleosomal spacing, creating a regular phasing and promoting repression, possibly by hiding activator binding sites inside nucleosomes (Längst et al., 1999). However, because NURF tends to randomize nucleosome phasing, this ISWI remodeler promotes activation of several essential genes (Deuring et al., 2000).

CHD members (CHD1, 2, 3, 4, 5, 9 and NuRD) feature two chromodomains, which recognize and promote direct binding to DNA, RNA and methylated histone tails (see section 1.4.3) (Marfella and Imbalzano, 2007). CHD members can both promote or repress transcription. In the first instance, they do so by sliding or ejecting nucleosomes, following recognition with transcriptionally positive histone methylation marks (see section 1.4.3). Alternatively, CHD members interact with histone deacetylases and methyl-CpG binders and therefore couple histone remodelling (sliding and spacing) with histone
deacetylation and DNA methylation. The INO80 family members (INO80 and SWR1) are structurally related but regulate chromatin remodelling via different pathways, as part of different multi-protein complexes (INO80.com and SWR1.com) (Bao and Shen, 2007). INO80.com can bind to DNA (like SWI/SNF members) and enhance transcription in vitro (Shen et al., 2000).

INO80 regulates nucleosome positioning by mobilizing octamers along the DNA (Udugama et al., 2011) This mechanism is similar to the one used by ISWI members and, like them, INO80 might both promote and repress gene expression, as its ablation in vivo leads to an equal number of genes up- and downregulated (Jónsson et al., 2004). SWR1 is the catalytic subunit of the SWR1 complex and replaces H2A-H2B with H2A.Z-H2B dimers (see below) (Kobor et al., 2004).

c. Histone variants

With the sole exception of histone H4, variants exist for all other canonical histones (H1, H3, H2A and H2B). Some of these variants differ by only one residue (i.e., histone H3 and its variant H3.2) (Marzluff et al., 2002). However, some are associated preferentially with specific histone modifications and, therefore, they might contribute to the differential recruitment of histone-modifiers (Hake and Allis, 2006). Other groups of non-canonical histone variants play specific roles in different processes such as gene regulation, X-chromosome inactivation and DNA damage repair (Talbert and Henikoff, 2010)(Fig. 1.22).
Figure 1.22: Schematic representation of the canonical histones and main histone variants.

For each sequence, the N-terminal tail and the globular core containing the conserved histone fold domain and the C-terminal tail are highlighted. Different shades of colour indicate highly divergent sequences, whereas single amino acids are shown. Circles, triangles, trapezoid and squares represents post-translational modification specific for each histone variant (circles stand for phosphorylation, triangles for acetylation, trapezoid for ubiquitination and squares for methylation). The macro-domains of the different types of macroH2A histone are not drawn to scale. which is responsible for intra-histone interactions, constitutes the conserved portion among variants. MacroH2A1.1 and 1.2 are splice variants and these differences are highlighted by using white and black triangles in the macro domains (reproduced with permission from Bernstein and Hake, 2006, Biochemistry and Cell Biology, copyright (2006) NRC Research Press).

Non-canonical variants usually replace canonical histones in a DNA replication independent manner and are transcribed throughout the cell cycle.
Some of these histones have a specific genomic location, such as the histone H3 variant CENP-A, which is found exclusively at centromeres and regulates kinetochore assembly during mitosis (Régnier et al., 2005). The histone H2A variant macroH2A is particularly enriched on the inactive X-chromosome (Costanzi and Pehrson, 1998).

Other variants are expressed in particular tissues, such as the testis-specific H2B variants H2B1A and H2AFWT (Churikov et al., 2004). Histone H1 variants exhibit both tissue-specific and cell cycle-specific patterns of expression within the same cell type (Izzo et al., 2008). The H2A variant H2A.Bbd (H2A.Lap in mouse) is expressed in the spermiogenic fraction of mammalian testis and specifically associates with acetylated H4-containing nucleosomes (Ishibashi et al., 2010).

Some non-canonical histones are ubiquitously expressed and play key functions. H2A.X, for example, is covalently modified (phosphorylated, acetylated and ubiquitinated) following double-stranded DNA breaks and, in this form, recruits the enzymes required for DNA repair (Ikura et al., 2007). Other variants are involved in gene regulation and will be discussed in greater depth.

i) Histone H3.3

The histone variant H3.3 differs from the canonical histone H3 by only four residues (Loyola and Almouzni, 2007). While canonical H3 (also known as H3.1) is deposited into nucleosomes only during S-phase, H3.3 is also deposited outside S (Ahmad and Henikoff, 2002). H3.3-containing nucleosomes are usually located in DNA hypersensitive regulatory elements, which display high turnover (Teves and Henikoff, 2011).

It has been shown that H3.3-containing nucleosomes are less stable than those containing H3.1 (Jin and Felsenfeld, 2007). This characteristic has the potential to allow H3.3-containing nucleosomes to alter the chromatin structure of
active promoters, where H3.3 is particularly enriched and presents histone modifications normally associated with transcription (Chow et al., 2005). Moreover, the deposition of H3.3 by the histone regulator A (HIRA) complex has been shown to antagonize the recruitment of histone-modifiers responsible for repressive histone marks (Sutcliffe et al., 2009).

ii) Histone H2A.Z

H3.3 is often found together with the H2A variant H2A.Z around the TSS of active genes in mammals or at enhancers (Henikoff, 2008). H2A.Z forms a less stable interaction with H2B than canonical H2A (Placek et al., 2005). H3.3-H2A.Z-containing nucleosomes assembled in vitro are particularly unstable and their presence at the TSS is likely to facilitate the recruitment of the PIC following their removal (Jin and Felsenfeld, 2007).

H2A.Z is conserved among species and is required for viability in *Tetrahymena*, *Drosophila* and mouse (Guillemette and Gaudreau, 2006). Positioning of H2A.Z by the SWR1 remodeler has been proved and genome-wide studies in human CD4+ T cells have shown that its localization is restricted to the first 3 nucleosomes upstream and downstream of the TSS (Schones et al., 2008). Interestingly, H2A.Z is found at both active and silent genes in budding yeast, although the latter group encompasses genes that are poised for activation (Raisner et al., 2005).

H2A.Z ablation in quiescent yeast strains results in a failure to activate inducible genes following growth restimulation (Li et al., 2005). One of the functions of H2A.Z is to oppose heterochromatin spreading and, at least in plants, H2A.Z deposition antagonizes DNA methylation (Zilberman et al., 2008). Similar to canonical H2A, H2A.Z is subject to post-translational modifications at its N- and C-terminal domain and these histone marks might regulate the stability of H2A.Z in the context of nucleosomes and determine the transcriptional activity
of genes that have this variant incorporated at their promoters (Draker et al., 2011).

Interestingly, the proximity of H2A.Z to the TSS is directly proportional to the transcriptional activity of active promoters in mice (Bargaje et al., 2012). In addition to its positive role in transcriptional initiation, the presence of H2A.Z downstream of the TSS at pausing sites positively regulates elongation in yeast (Santisteban et al., 2011). H2A.Z is also found at pericentric heterochromatin in mammalian cells, where it interacts with the components of the Chromosomal Passenger Complex (see section 1.5) (Rangasamy et al., 2003).

Deposition of H2A.Z is required for centromere silencing and chromosome segregation during mitosis in yeast (Hou et al., 2010). Interestingly, centromeric histones can be divided into those that contain H2A.Z and those that contain CENP-A as these two variants are mutually exclusive (Greaves et al., 2007). During mouse spermatogenesis, H2A.Lap1 is specifically inserted at the -1 nucleosome (relative to the TSS) of active genes instead of H2A.Z and its deposition partly unfolds chromatin (Soboleva et al., 2012).

d. Post-translational modification of histones

One of the ways in which co-factors can regulate transcription is to alter the stability of core nucleosomes by introducing post-translational modifications of histone residues. The histone tails are regions of the proteins, generally at the N-termini, that protrude from the nucleosome and are the principal, albeit not exclusive, sites of post-translational modification associated with transcriptional regulation (Luger et al., 1997). Histone tails can be acetylated, methylated, phosphorylated and monoubiquitinated (Fig. 1.23).
Figure 1.23: Schematic representation of the position of the major post-translational modifications of the tails of canonical histones.

The following modification are shown: acetylation (ac), methylation (me), phosphorylation (ph) and monoubiquitination (ub1) (reproduced with permission from Bhaumik et al., 2007, Nature Structural and Molecular Biology, copyright (2007) Nature Publishing Group).

Multiple modifications occur on different histone tails and on different residues within the same nucleosome. This generates a layer of transcriptional regulation referred to as the histone code (Strahl and Allis, 2000). Histone modifications are implicated in several mechanisms such as DNA repair, mitosis and transcriptional regulation. For the purpose of this thesis, I will now focus on the role of histone modifications in transcriptional regulation. The role of histone modifications in mitosis will be covered in section 1.5.
i) **Histone acetylation**

Nucleosomes are assembled by enzymes called chaperones, which recognize acetylated histones and load them onto the DNA (Verreault, 2000). After being translated in the cytoplasm, histone acetyltransferases (HATs) acetylate the lysine residues of cytoplasmic histones by using acetyl coenzyme A as a source of acetyl moieties. After chaperones have recognized and deposited the acetylated histone on the nucleosome, this mark is rapidly erased by histone deacetylases (HDACs) (Shahbazian and Grunstein, 2007).

Once they form part of nucleosomal DNA, several residues on all histones can be re-acetylated by HATs. Globally speaking, histone acetylation loosens the nucleosomal structure, favours DNA accessibility and therefore promotes transcription (Mutskov et al., 1998). The acetylation of lysine 16 on histone H4 (H4K16ac) is particularly important, as it is required to maintain an open chromatin conformation (euchromatin) and is found on the promoters and gene body of active genes (Wang et al., 2008).

Acetylation of other residues is also required for proper gene activation (Kuo et al., 1998). Two major HAT complexes have been identified: SAGA and NuA4. Gcn5, the catalytic subunit of SAGA, acetylates preferentially histones H3 and H2B, whereas Esa1, which belongs to NuA4, targets H4 and H2A (Suka et al., 2001). These complexes are recruited by activators and other histone modifications that are often found on active genes, such as trimethylation of lysine 4 and 36 on histone H3 (H3K4me3 and H3K36me3) (Ng et al., 2003). H3K4me3 is present on the promoters, whereas H3K36me3 is present on the coding region (Krogan et al., 2003). Histone trimethylation constitutes a mark, which is recognized by the chromodomain present in different classes of proteins mostly implicated in silencing (discussed later) as well as members of SAGA and NuA4.

The chromatin loosening initiated (and maintained) by H4K16ac is
enhanced by further histone acetylation, which in turn promotes recruitment of activators at promoters (Lee et al., 1993). However, H4K16ac has a paramount role in antagonizing the formation of higher-order chromatin compaction and nucleosome remodelling that would impede access to chromatin (Shogren-Knaak et al., 2006). The acetylated histone marks generated by HATs are read by cofactors such as the SWI/SNF remodelling complex, which bind to them through their bromodomains (Barbaric et al., 2001).

The Elongator complex, which interacts with the hyperphosphorylated RNA Pol II during elongation, contains a HAT domain and might directly mediate histone acetylation on gene bodies, loosening nucleosomal DNA and facilitating the passage of RNA Pol II (Close et al., 2006). Conversely, HDACs repress transcription by removing the acetyl mark (Robert et al., 2004). HDACs also promote the binding of repressors, such as SANT domain-containing proteins, which recognize unmodified histone tails and promote silencing through chromatin remodelling (Hartman et al., 2005).

**ii) Histone methylation**

Unlike histone acetylation, which is a general mark for active or poised genes, histone methylation can serve as a mark for active as well as silent genes depending on the residue (Saunders et al., 2006). Lysines can be mono-, di- or trimethylated. Arginines are monomethylated, or they can be symmetrically or asymmetrically dimethylated. Histidines are monomethylated (Greer and Shi, 2012). Lysine-methyltransferases include SET-domain-containing and DOT1-like protein families, arginines are methylated by protein arginine N-methyltransferases (PRMTs) (Greer and Shi, 2012).

Lysine-demethylases belong to the jumonji C (JmjC)-domain-containing dioxygenases and amine oxidases families (Greer and Shi, 2012). Some methyltransferases and demethylases are directly recruited to specific DNA
sequences (Woo et al., 2010). Additionally, non-coding RNAs (ncRNAs) have been shown to participate in the recruitment of these histone-modifiers (Tsai et al., 2010).

Interestingly, although they are mediated by different families of enzymes, DNA and histone methylation are linked. Some histone methyltransferases contain methyl-CpG-binding domains and their recruitment and activity is augmented by DNA methylation (Rajakumara et al., 2011). At the same time, DNMTs are recruited by histone marks such as H3K9me3 and thus participate in reinforcing silencing (Hsieh, 2000).

As previously mentioned, H3K4me3 is generated by several methyltransferases (such as SETD1A/B and MLLs) and usually marks active promoters. H3K4me3 also promotes recruitment of HATs (Santos-Rosa et al., 2002). In addition, methyltransferases responsible for generating H3K4me3 can form complexes with demethylases specific for the trimethylation of lysine 27 on histone H3 (H3K27me3), a well-characterised repressive mark established by the Polycomb repressive complex 2 (PRC2) (see section 1.6) (Miller et al., 2008). However, H3K4me3 and H3K27me3 can be present on the same promoter in a group of genes in ES cells (termed bivalent). These genes are repressed but poised for activation following differentiation (Stock et al., 2007).

H3K36me3 marks the body of active genes, where it recruits HATs and might regulate alternative splicing, as its level is higher in constitutively rather than in alternatively spliced exons and introns (Kolasinska-Zwierz et al., 2009). H3K36me2 peaks at the promoters of active genes and, together with H3K36me3, antagonises the repressive functions of PRC2-mediated H3K27me3 (Schmitges et al., 2011). While H3K4me3 marks promoters, H3K4me1 is usually detected at enhancers of active genes (Heintzman et al., 2007). H3K9me3 is found at inactive promoters, where its chromodomain is bound by heterochromatin protein 1 (HP1) (Fischle et al., 2005).
The site-specificity of each histone methylation means that it is difficult to make global assumptions about the effects of the methyl-motif on the stability of nucleosomes. This heterogeneity is likely to stem from the wide spectrum of proteins that recognize the different methyl marks on histone tails.

**iii) Histone phosphorylation**

All histones are subject to phosphorylation by a large number of kinases and dephosphorylation by phosphatases (Nowak and Corces, 2000). Histone phosphorylation is generally associated with transcriptional activation (Kouzarides, 2007). This comes primarily from observations in cells where the RAS/ERK/MAPK signalling has been activated (Barratt et al., 1994). The ultimate effectors of this signalling cascade are the mitogen and stress-activated kinases 1 and 2 (MSK1 and 2), which phosphorylate serine 10 and 28 on histone H3 (H3S10ph and H3S28ph) at the promoters of immediate-early genes, such as c-fos and c-jun, in response to mitogenic stimulation (Drobic et al., 2010). This is thought to facilitate transient activation of these genes, before the phospho-mark is removed by protein phosphatase 2A (PP2A) (Simboeck et al., 2010).

The H3S10ph mark is read by 14-3-3 proteins, a large family of co-factors that are involved in several signalling pathways (Macdonald et al., 2005). 14-3-3 proteins recruit the SAGA complex, leading to the acetylation of lysine 14 on histone H3 (H3K14ac) (Drobic et al., 2010). This phospho-acetyl motif (H3S10ph/K14ac) constitutes an even better docking site for 14-3-3 proteins, whose binding shields H3S10ph from the phosphatase activity of PP2A (Simboeck et al., 2010). 14-3-3 proteins can also recruit the SWI/SNF remodelling complex, which allows recruitment of the PIC and transcriptional initiation (Drobic et al., 2010) (Fig. 1.24).
Figure 1.24: Representation of transcriptional activation following activation of the Ras/ERK/MAPK pathway.

(A) Silent promoters are marked by the repressive H3K9me3 modification, which serves as a binding site for HP1 proteins (such as HP1γ). Following stimulation, MSK1 phosphorylates serine 10 on histone H3, which triggers the displacement of HP1γ and the binding of 14-3-3 proteins. 14-3-3 proteins loosely bind to H3S10ph but recruit HATs that acetylate lysine 14 on histone H3. In this phospho-acetyl configuration, 14-3-3 proteins can bind more stably to promoters and facilitate recruitment of other co-activators. (B) Repressors such as Polycomb group proteins bind to lysine 27 on histone H3 and deposit the repressive H3K27me3 mark on the promoter of silent genes. MSK1 phosphorylation of nearby serine 28 triggers the displacement of Polycomb repressors (but not the removal of H3K27me3) and constitutes on its own an excellent binding motif for 14-3-3 proteins.

H3S10ph and 14-3-3 proteins stimulate transcriptional elongation at pausing sites by recruiting HATs. The resulting acetyl-mark is read by the bromodomain protein BRD4, which then recruits p-TEFb (Zippo et al., 2009). 14-3-3 proteins display the same binding affinity for H3S10ph/K14ac and H3S28ph alone in vitro (Macdonald et al., 2005). H3S28ph antagonizes PRC2 recruitment and the subsequent trimethylation of lysine 27 (Lau and Cheung, 2011). Interestingly, H3S28ph but not H3S10ph has been shown to physically interact
with S5p Pol II \textit{in vivo}, suggesting a non-redundant role for these marks in transcriptional activation (Lau and Cheung, 2011).

Histone H3 is also phosphorylated on threonine 11 (H3T11ph) by the protein kinase C-related kinase 1 (PRK1) (Metzger et al., 2008). This mark is read by the H3K9 demethylase JMJD2C, which contributes to the removal of the repressive marks H3K9me2/me3 from the promoters of androgen receptor-responsive genes in response to stimulation. However, phosphorylation and methylation on the same histone tail are not always mutually exclusive, and histone phosphorylation can even be a mechanism to reinforce histone methylation when it constitutes a positive mark. The protein kinase C beta 1 (PKC1) phosphorylates threonine 6 on histone H3 (H3T6ph) following androgen stimulation (Metzger et al., 2010). This mark blocks the activity of the H3K4me3 demethylase LDS1 and promotes transcription.

Finally, histone H1 can be phosphorylated at multiple residues. It has been postulated that the number rather than the position of the phosphorylated residues is important for the role of this post-translational modification on gene regulation. Indeed, in its hyperphosphorylated form, histone H1 is displaced from chromatin, in turn revealing transcriptional factors binding sites and promoting transcription (Zheng et al., 2010).

\textit{iv) Histone ubiquitination}

Polyubiquitination is a general mechanism for targeting proteins for proteasomal degradation. On the other hand, monoubiquitination of histones is a reversible post-translational modification acquired by histones H2A, H2A.Z and H2B, which is associated with transcriptional silencing (H2A and H2A.Z) or activation (H2B) (Komander and Rape, 2012). Ubiquitination is a multi-step process that involves a ubiquitin-activating enzyme (E1), which activates ubiquitin and transfers it to a ubiquitin-conjugating enzyme (E2) (Fig. 1.25).
Figure 1.25: Schematic representation of the ubiquitin-conjugating reaction.

Ubiquitin binds in an ATP-dependent reaction to a ubiquitin-activating enzyme (E1). Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2) that lacks substrate recognition capacity. The ubiquitin-protein-isopeptide ligase (E3) mediates the recognition and binding of ubiquitin to the lysine of a specific substrate. Monoubiquitination is a reversible process implicated in signal transmission. Polyubiquitination, in contrast, marks substrate proteins for proteosomal degradation (reproduced with permission from Osley, 2006, Briefings in Functional Genomics, copyright (2006) Oxford University Press).

Ubiquitin is transferred from E2 to the substrate by a ubiquitin-protein-isopeptide ligase (E3). In mammals, H2A is monoubiquitinated on lysine 119 (H2Aubq), H2A.Z on lysine 120 or 121 (H2A.Zubq) and H2B on lysine 120 (H2Bubq). H2Aubq is associated with transcriptional silencing through inhibition of elongation (Zhou et al., 2008). The H2Aubq mark can be generated by two enzymes: Ring1B (which will be discussed in session 1.6) and 2A-HUB, which is part of the N-CoR/HDAC complex. Ubiquitination of H2A by 2A-HUB has been shown to repress the transcription of a subset of chemokine genes in macrophages (Zhou et al., 2008). H2Aubq blocks recruitment of the Spt16 subunit of the FACT complex. This complex is responsible for displacing H2A-
H2B dimers from nucleosomes allowing RNA Pol II to transit (Zhou et al., 2008).

In addition, there is evidence that H2Aubq promotes chromatin compaction through recruitment of H1 (Jason et al., 2005). H2A.Z is ubiquitinated by Ring1B and removal of this mark by the deubiquitinase USP10 results in activation of androgen receptor-dependent genes (Draker et al., 2011). H2Aubq deubiquitination is also associated with transcriptional derepression (Higashi et al., 2010). USP16 regulates expression of Hox genes by removing the repressive Ring1B-mediated H2Aubq mark (Joo et al., 2007). MYSM-1 preferentially deubiquitinates acetylated H2A and leads to increased binding of transcription factors (Jiang et al., 2011). Deubiquitination by USP21 counterbalances H2Aubq-mediated inhibition of H3K4 trimethylation and promotes transcriptional initiation (Nakagawa et al., 2008).

Unlike H2Aubq, ubiquitination of H2B is required for trimethylation of H3K4 at promoters and plays a positive role in transcriptional initiation (Lee et al., 2007). H2Bubq facilitates elongation probably by cooperating in FACT-mediated H2A-H2B removal (Pavri et al., 2006). Bre1, the mammalian E3 ligase for H2B, is recruited by activators to promoters before transcriptional initiation but H2Bubq can only be formed following S5p Pol II formation and promoter escape in vivo, as ablation of TFIIH eliminates this mark (Xiao et al., 2005).

In yeast, the DUB Ubp8 removes H2Bubq and, surprisingly, is required for transcription (Wyce et al., 2007). Ubp8 is required for S2p Pol II formation and for elongation at pausing sites. The other yeast H2Bubq DUB, Ubp10, is, on the other hand, implicated in promoting silencing at telomeres (Osley, 2006).

1.5 The Aurora B kinase

The Aurora kinases are serine/threonine kinases, originally identified in *Drosophila* in a screen to identify regulators of the mitotic spindle function (Glover et al., 1995). Subsequent work identified Aurora kinase homologues in *S.*
**cerevisiae** (named Ipl1) and *S. pombe* (Ark1) (Brown et al., 2004). In mammals, three Aurora kinases have evolved: Aurora A, B and C.

The mammalian Aurora kinases share a conserved catalytic domain in the central portion of the proteins and differ mostly at the N-terminal domain. The latter is implicated in protein-protein interactions (Fig. 1.26) (Brown et al., 2004). Similar to their *Drosophila* counterpart, the Aurora kinases orchestrate mitosis in mammalian cells. However, as a result of their different N-termini, Aurora A and B interact with different sets of proteins and exhibit different localizations during mitosis, which also translates into different functions (Carmena and Earnshaw, 2003).

![Figure 1.26: Sequence comparison of human Aurora kinases.](image)

The A- and D-Boxes (at the amino and carboxy-termini respectively) are implicated in recognition of these kinases by the enzymes that regulate their degradation. The catalytic domain resides within the highly conserved kinase domain and is termed the T-Box. Percentages represent the sequence homology between human Aurora kinases (reproduced with permission from Keen and Taylor, 2004, Nature Reviews Cancer, copyright (2004) Nature Publishing Group).

Aurora C is closely related to Aurora B and the two kinases share INCENP as a partner (Sasai et al., 2004). Moreover, a switch in roles between Aurora B and C is observed early during embryogenesis (Fernández-Miranda et al., 2011). Aurora C expression is tissue-specific and, after late blastocyst formation, is restricted to the testis. In meiotic cells, the gene that encodes Aurora C (*Aurkc*) is under the control of the transcription factor Testis Zinc Finger Protein (Tzfp).
Aurora A and B are both ubiquitously expressed and are subject to regulation during the cell cycle with expression peaking in G2. Expression of the two kinases is controlled by transcription factors such as E2F1, E2F4 and FoxM1 (Kimura et al., 2004). For the purposes of this work, I will first describe the regulation and functions of Aurora B during mitosis and I will then discuss the documented roles for the kinase outside mitosis.

1.5.1 Regulation of Aurora B

Activation of the kinase activity of Aurora B is a multistep process that begins with the autophosphorylation of its T-loop (Sessa et al., 2005). Although this is sufficient for substrate-specific phosphorylation in vitro, activation of Aurora B in vivo requires binding to the Inner Centromere Protein (INCENP) (Yang et al., 2009). INCENP is a microtubule binding protein that contains a sequence (the IN-box) at its C-terminal domain, which interacts with the N-terminus of Aurora B and induces a conformational change in the active site of Aurora B (Sessa et al., 2005). An additional conformational change is required for full activation of Aurora B in vivo and is triggered by the phosphorylation of a region on INCENP (called the TSS motif). Aurora B itself is responsible for this phosphorylation and current evidence suggests that this is accomplished via an in trans-activation mechanism, mediated by a nearby, fully active Aurora B (Kelly et al., 2007). Support for this idea comes from the observation that Aurora B-INCENP complexes tend to form small clusters.

Additionally, INCENP mediates the interaction of Aurora B with Survivin, a protein originally identified as a mediator of apoptosis (Ambrosini et al., 1997). Survivin interacts both with the N-terminus of INCENP and with the active site of Aurora B and stimulates its kinase activity (Chen et al., 2003). Survivin is also required for efficient localization of Aurora B at centromeres late in prophase.
(Wang et al., 2010a) together with another protein, Borealin, which interacts with the N-terminal domain of INCENP but not directly with Aurora B (Klein et al., 2006). Survivin and Borealin are required for the proper localization of Aurora B and INCENP at centromeres from late prophase until metaphase (Jeyaprakash et al., 2007). The recruitment of both Survivin and Borealin is regulated by histone modifications. Survivin binds to the phosphorylated threonine 3 on histone H3, which is generated by the kinase Haspin (Kelly et al., 2010). Aurora B accumulation at these regions is favoured by Aurora B phosphorylation of Haspin, thus generating a positive loop for the recruitment of both kinases to centromeres (Wang et al., 2011a).

Borealin interacts with the Shugoshin proteins (Sgo1 and 2), which bind to the phosphorylated threonine 120 on histone H2A (H2AT120) (Yamagishi et al., 2010). This mark is generated by the kinase Bub1, which plays also a role in the spindle assembly checkpoint together with Aurora B (see below) (Kawashima et al., 2010). Borealin is also subject to phosphorylation by CDK1, which favours the binding to Shugoshin proteins (Tsukahara et al., 2010). Similar to its role in recruiting Haspin, Aurora B promotes recruitment of Bub1 at centromeres, therefore positively regulating the binding of Borealin and, in turn, its own localization to centromeres (Ricke and van Deursen, 2011).

Aurora B, INCENP, Survivin and Borealin together form a complex called the Chromosomal Passenger Complex (CPC) whose components travel together throughout mitosis (Carmena and Earnshaw, 2003). Interestingly, Aurora C can substitute for Aurora B as the catalytic subunit of this complex when exogenously overexpressed and is therefore also considered to be a member of the CPC (Slattery et al., 2009). Other proteins interact with Aurora B during mitosis but are not part of the CPC.

In *C. elegans*, the Tousled-like kinase-1 (TLK-1) activates Aurora B in an INCENP-dependent manner, but this mechanism, to date, has not been reported
in mammals (Han et al., 2005). The Polo-like kinase 1 (Plk1) also promotes Aurora B activation by phosphorylating Survivin (Chu et al., 2011). Moreover, Plk1 cooperates with Aurora B in regulating the bipolar orientation of sister chromatids (see below) (Salimian et al., 2011). The DNA-damage kinase Chk1 phosphorylates Aurora B and promotes Aurora B in trans-activation of INCENP (Petsalaki et al., 2011). Mps1 phosphorylates Borealin and is implicated in the regulation of kinetochore attachments with Aurora B (see section 1.5.3) (Jelluma et al., 2008). TD-60 plays a role in localizing Aurora B at centromeres and might operate in a similar manner to Chk1 by creating a cluster of Aurora B-INCENP complexes at centromeres (Rosasco-Nitcher et al., 2008). Aurora B and Survivin have also been reported to interact with centromeric minor satellite RNAs, which would help to recruit the CPC and stimulate Aurora B kinase activity towards CENP-A (Ferri et al., 2009).

Towards the end of cytokinesis Aurora B is polyubiquitinated by the Anaphase Promoting Complex/Cyclosome (APC/C), which recognizes the D-box of Aurora B and targets the kinase for proteosomal degradation (Stewart and Fang, 2005). Additionally, monoubiquitination by the Cul3-KLHL21 E3 ligase triggers the displacement of Aurora B from centromeres at the onset of anaphase (Maerki et al., 2009). Aurora B is also targeted by the SUMO (small ubiquitin-related modifier) E3 ligase PIAS3 during mitosis (Ban et al., 2011). This post-translational modification, which is also found on Borealin, regulates the localization of Aurora B on centromeres but not its activity (Fernández-Miranda et al., 2010).

Aurora B is subject to negative regulation by the protein phosphatases 1 and 2A (PP1 and 2A) (Murnion et al., 2001). These phosphatases operate in conjunction with other proteins that regulate their target specificity. The Sds22/PP1 and EB1/PP2A complexes directly dephosphorylate Aurora B at its catalytic site (Barr et al., 2011). RepoMan/PP1 targets histone H3 and negatively
affects Survivin-mediated Aurora B recruitment to centromeres by removing the H3T3ph mark (Vagnarelli et al., 2011). Moreover, RepoMan/PP1 counterbalances Aurora B activity by dephosphorylating H3S10ph/S28ph. The KNL1/PP1 complex dephosphorylates Aurora B targets at kinetochores (Liu et al., 2010) (see section 1.5.3).

1.5.2 Aurora B and chromosome compaction

At the onset of mitosis the replicated DNA begins to condense into the compact structure of the mitotic chromosomes. During late G2 and early prophase, Aurora B localizes with the other members of the CPC on the chromosome arms (Hayashi-Takanaka et al., 2009). This correlates with increasing phosphorylation of serine 10 and 28 on histone H3 along the chromosomes (Hendzel et al., 1997).

Unlike the promoter-localized H3S10ph or H3S28ph that is involved promoter opening and transcriptional activation (see section 1.4.3), Aurora B-mediated mitotic H3S10ph/S28ph spreads along the chromosome arms and is accompanied by a global compaction of the DNA (Hayashi-Takanaka et al., 2009). These events are also associated with displacement of binding of the HP1 proteins from the H3K9me3 mark (Hirota et al., 2005). It has been suggested that HP1 proteins could be involved in recruiting the CPC to the chromosome arms through interaction with INCENP, which has been shown to directly interact with HP1α in vitro (Ainsztein et al., 1998). However, in vivo the recruitment of the CPC is likely to be mediated by an additional partner of INCENP, POGZ, which is required for displacement of the HP1α and activation and localization on chromosome arms of Aurora B (Nozawa et al., 2010). The deposition of the H3S10ph mark requires prior deubiquitination of histone H2A by the histone deubiquitinase USP16 (Joo et al., 2007). This deubiquitinase requires phosphorylation to become active and move from the cytoplasm to the nucleus at
the onset of mitosis, where it resides until cytokinesis (Cai et al., 1999).

Compaction of the mitotic chromosome requires the presence of ring-like protein complexes called Condensin I and II (Samoshkin et al., 2009). Recruitment and maintenance of Condensin I on chromosomes requires Aurora B activity throughout mitosis (Nakazawa et al., 2011). Aurora B also regulates the concentration of the Cohesin complex on centromeres during late prophase, where the CPC relocalizes, driven by Survivin and Borealin (Klein et al., 2006). The maintenance of Cohesin at centromeres by Aurora B might be due to its ability to keep Sgo1 at centromeres (Dai et al., 2006). Sgo1 keeps sister chromatids together until the onset of anaphase when, following degradation of Cohesin by Separase, sister chromatids migrate to opposite sides of the mitotic spindle (Kitajima et al., 2006).

1.5.3 Aurora B and sister chromatid segregation

From late prophase until the end of metaphase, the CPC localizes at centromeres where it promotes correct attachment of kinetochores to the microtubules of the mitotic spindle (Fig. 1.27) (Cimini et al., 2006). To ensure that the sister chromatids segregate their DNA equally between the two daughter cells, each chromatid pair must bind to two microtubules originating from opposite sites of the mitotic spindle (Cleveland et al., 2003). This type of attachment is called bipolar or amphitelic.
Figure 1.27: Localization of Aurora B during mitosis.

The levels of Aurora B (red) peak at the G2/M border, before prophase. At this stage the protein is diffused on the DNA (stained with the DAPI dye, blue), where it promotes its compaction. Later in prophase and prometaphase, Aurora B is concentrated at the centromeres. In metaphase, all chromosomes are lined up at the centre of the cell and Aurora B regulates the correct attachment of microtubules to kinetochores. After sister chromatid separation, Aurora B is retained in the central part of the spindle, where it helps the formation of the contractile actinomyosin ring required for daughter cells separation and assesses correct chromatid segregation before cytokinesis (reproduced with permission from Hake et al., 2005, Proceedings of the National Academy of Sciences of the United States of America, copyright (2005) National Academy of Sciences, U.S.A.).

Incorrect attachments (e.g. syntelic and merotelic attachments) lead to unequal distribution of chromatids between the two daughter cells (Fig. 1.28) (Lampson et al., 2004). Syntelic attachments are characterised by two
kinetochores bound to a microtubule. Merotelic attachments are generated upon binding of one kinetochore to two microtubules.

Figure 1.28: Different types of microtubule attachments.
Bipolar attachments (here termed bi-oriented and amphitelic) lead to correct segregation of sister chromatids towards opposite poles of the mitotic spindle (represented here by centrosomes). Incorrect attachments can be syntelic (two kinetochores bound to the same microtubule), monotelic (only one kinetochore bound to one microtubule) or merotelic (one kinetochore bound to two microtubules) (reproduced with permission from Keen and Taylor, 2004, Nature Reviews Cancer, copyright (2004) Nature Publishing Group).

Aurora B ensures that the correct chromosome attachment is in place by destabilizing the kinetochore-microtubule interaction in the event of syntelic and merotelic attachments (Cimini et al., 2006). Bipolar attachments generate a physical tension that dissociates Aurora B (which resides at the centromeres) from the KMN network (Dsn1/Mis13, KNL1/Blinkin and Ndc80/Hec1) located at the outer kinetochores (Liu et al., 2009). This allows dephosphorylation of the components of the KMN network by the KNL1/PP1 complex, which further
stabilizes the attachment by recruiting other complexes such as Astrin, the small kinetochore-associated protein (SKAP) and the Ska complex members Sk1, 2 and 3 (Welburn et al., 2010).

Incorrect attachments, on the other hand, fail to generate such physical tension and Aurora B can continue to phosphorylate Ncd80/Hec1 and other substrates such as the microtubule-depolymerising kinesin MCAK (Knowlton et al., 2006). Phosphorylation of these proteins releases the microtubules from the kinetochore and triggers activation of the spindle assembly checkpoint (SAC) (Lampson and Cheeseman, 2011). A multi-protein complex (composed of Mad2, Mad3/BubR1, Bub3 and CDC20) blocks progression of mitosis, preventing incorrect chromatid segregation (Schmidt et al., 2010).

This is achieved by preventing activation of the APC/C^{CDC20} E3 ligase and subsequent degradation of Cyclin B and Securin (Musacchio and Salmon, 2007). The latter protein inhibits Separase, the protease that removes Cohesin rings from the centromeres and permits separation of sister chromatids. Aurora B also activates the SAC via phosphorylation of the Ataxia telangiectasia mutated kinase (ATM) (Yang et al., 2011). This kinase, which is normally activated following DNA damage, is phosphorylated in mitosis by Aurora B in the absence of DNA damage. The phosphorylated ATM in turn phosphorylates Bub1, which is also required for SAC activation in addition to its role in Sgo1 and 2 recruitment at centromeres (Yang et al., 2011).

1.5.4 Aurora B and cytokinesis

During anaphase, the CPC relocates to the midzone microtubules of the mitotic spindle and regulates the formation of the central spindle while the sister chromatids are pulled towards the opposite poles of the mitotic spindle (Carmena and Earnshaw, 2003). Aurora B promotes the formation of a cluster of centralspindlin complexes by phosphorylating the homodimer of kinesin-6 motor
protein MKLP1 (Douglas et al., 2010). These centralspindlin complexes (formed by a homodimer of MKLP1 and a dimer of the GTPase MgcRacGAP) create a bundle of microtubules, which promotes recruitment of factors implicated in the formation of the actinomyosin contractile ring responsible for cytokinesis (Glotzer, 2009).

In the event of defects in either nuclear pore formation or sister chromatid separation, Aurora B can also block cytokinesis, possibly by phosphorylating CHMP4C (Carlton et al., 2012). CHMP4C is a subunit of the ESCRT-III complex, which provides constrictive force during the cell separation (Wollert et al., 2009). CHMP4C interacts with Borealin and its phosphorylation by Aurora B has been shown to block cytokinesis (Carlton et al., 2012). This checkpoint is known as the abscission checkpoint and constitutes a last safeguard that protects a cell from tetraploidization (Steigemann et al., 2009). Finally, Aurora B promotes the actual contraction of the actinomyosin ring that leads to the separation of the daughter cells by phosphorylating the Myosin Regulatory Light Chain-2 (Murata-Hori et al., 2000).

1.5.5 Aurora B and cell cycle regulation

The expression of Aurora B reaches its lowest level in cycling cells following re-entry of the daughter cells into G1, when the protein is polyubiquitinated and targeted for proteosomal degradation. Aurora B is however expressed at a basal level throughout the cell cycle. In CD28-deficient T cells, Aurora B interacts with Survivin and mammalian target of Rapamycin (mTor) (Song et al., 2007). These cells exhibit a block in the cell cycle progression at the G1/S border, which can be relieved by Aurora B ectopic expression. This leads to the phosphorylation of mTor and its downstream effectors Rb, Cyclin A and CDK1 and 2. Although these aforementioned proteins are known targets of mTor, it is also possible that Aurora B directly phosphorylates them.
Aurora B has also been shown to phosphorylate and negatively regulate the activity of the tumour suppressor p53 (Gully et al., 2012). Phosphorylation of p53 by Aurora B suppresses the inhibitory effect of p53 on cell proliferation by promoting its degradation and impairing its activity as a transcription factor. Interestingly, Aurora B interacts with p53 both in interphase (in the nucleus) and during mitosis (in the cytoplasm). This result was however obtained in transformed cell lines, where Aurora B is highly expressed throughout the cell cycle and localizes in the nucleus in interphase (Abdullah et al., 2005). The direct negative regulation of the tumour suppressor p53 is observed upon Aurora B ectopic overexpression and might relate to the role of Aurora B in cancer, where it is often overexpressed (see below). Aurora B and p53 have also been shown to share a common partner, NIR (novel INHAT repressor), which negatively regulates p53 functions (Wu et al., 2011b).

1.5.6 Aurora B and gene regulation

Aurora B expression persists outside mitosis in terminally differentiated plasma cells, where it displaces HP1\(\beta\) from H3K9me3 by phosphorylating the nearby serine 10 (Sabbattini et al., 2007). In this context, Aurora B might reinforce silencing by promoting the formation of a constitutively heterochromatic domain. Interestingly, Aurora B-mediated H3S10ph leads to opposite effects in pituitary cells, where it displaces HP1\(\beta\) and activates thyroid hormone receptor-regulated genes (Tardáguila et al., 2011). These genes normally have the thyroid receptor bound to their promoters, where it recruits co-repressors such as HDACs. Upon stimulation with thyroid hormone, a conformational change in the receptor converts it into an activator. The resulting transcriptional activation is accompanied by an increase in the levels of H3S10ph and a release of HP1\(\beta\) from chromatin. Aurora B acts as a co-activator at these promoters by phosphorylating histone H3 and in turn promotes transcription. This effect of
Aurora B on transcription is not related to mitosis as the cells are mostly in G1-phase (80-90%) (Tardáguila et al., 2011).

### 1.5.7 The role of Aurora B in cancer

Disruptions in the regulation of Aurora B functions can lead to uncontrolled proliferation, chromosomal abnormalities (aneuploidy and polyploidy), tumorigenesis and metastasis (Norton and Massagué, 2006). Aurora B overexpression is observed in several tumours, which has led to the hypothesis that *Aurkb* (the gene that encodes for Aurora B in mammals) can act as an oncogene (Fu et al., 2007). In tumour cells Aurora B overexpression is often associated with tetraploidy, which leads to genomic instability and tumorigenesis (Nguyen et al., 2009). However, polyploidy is observed even upon ectopic overexpression of a constitutively inactive mutant or ablation of the kinase, possibly as a consequence of inactivation of the spindle assembly and ablation checkpoint (Fu et al., 2007). This suggests that a certain amount of Aurora B activity is fundamental to guarantee the correct mitosis of non-tumoral cells.

In prostate cancer, Aurora B is subject to dynamic acetylation/deacetylation regulation during mitosis (Fadri-Moskwik et al., 2012). Acetylation of Aurora B leads to reduced activity and increased mitotic abnormalities. Aurora B overexpression is observed in metastatic melanoma and results from hyper-stimulation of FoxM1 by the ERK pathway (Bonet et al., 2012).

Specific chemical inhibitors of Aurora B such as AZD1152 are showing promising results in the treatment of several malignancies such as acute myelogenous leukaemia, multiple myeloma, colorectal and breast cancer. New inhibitors are currently being designed for use in other tumours (Oke et al., 2009). AZD1152 reduces cell proliferation by stimulating senescence and apoptosis in melanoma cells (Bonet et al., 2012). This compound has also proved successful in blocking the proliferation of cells isolated from patients with acute myeloid
leukaemia (Walsby et al., 2008).

1.6 Polycomb group proteins

Polycomb group (PcG) proteins were originally discovered in *Drosophila*, where the first member of the group (Polycomb or Pc, the gene for which was identified by P. Lewis in 1947), regulates body segmentation (Lewis, 1978). The mutations of other genes cause a similar phenotype to that observed in *Pc* mutants (Margueron and Reinberg, 2011). The proteins encoded by these genes were therefore named PcG proteins and for some of them mammalian homologues have been identified (Levine et al., 2002).

In *Drosophila*, PcG proteins are organized into two repressive complexes (PRC1 and 2), the PHO-repressive complex (PhoRC) and the Polycomb repressive deubiquitinase (PR-DUB) (Simon and Kingston, 2009). PhoRC regulates the recruitment of PRC2 in *Drosophila* and is composed by the DNA binding protein (PHO) and a methyl-lysine binder (dSfmbt), which binds to the H3K9me1/2 and H4K20me1/2 marks (Klymenko et al., 2006). PR-DUB is composed by the H2A deubiquitinase Calypso and the chromodomain containing protein Asx and this complex antagonizes PRC1-mediated gene silencing (Scheuermann et al., 2010). The core components of PRC1 and 2 are conserved from *Drosophila* to mammals as well as some of the components of PhoRC and PR-DUB. For the purpose of this work, I will focus on the role of mammalian PRC1 and 2 components.

By operating as co-factors in the regulation of a large number of genes, PRC1 and 2 regulate stem cell pluripotency, self-renewal, cell cycle progression, senescence, genomic imprinting and differentiation (Prezioso and Orlando, 2011). Moreover, many PcG proteins are overexpressed and are involved in tumourigenesis and tumour progression (Richly et al., 2010). The best-characterised mode of action of PRC1 and 2 is the post-translational modification
of histone H2A and H3 respectively. Nevertheless, both complexes might regulate the structure of chromatin by other as yet unidentified mechanisms.

PcG proteins have been mostly described as co-repressors that operate at promoters. Recently, it has become increasingly clear that the mechanisms for recruiting these complexes in mammals are more complicated and variable than was originally thought from comparisons with the mechanisms observed in Drosophila.

Figure 1.29: Hierarchical recruitment of PRCs to PREs in Drosophila.
PhoRC recognises the PHO/YY1 binding motif present in PREs. This leads to the recruitment of PRC2 and the deposition of the H3K27me3 mark. This post-translational modification is read by Pc in PRC1, which monoubiquitinates H2A (not shown) and blocks transcriptional initiation (reproduced with permission from Wang et al., 2004, Molecular Cell, copyright (2004) Elsevier).
Both PRC1 and PRC2 lack a subunit with a DNA binding domain. In *Drosophila*, Polycomb Response Elements (PREs) contain the binding motif for PHO (Chan et al., 1994). This in turn recruits PRC2 and leads to the trimethylation of lysine 27 on histone H3. *Drosophila* Pc and its mammalian homologues Cbx proteins read this mark and mediate the recruitment of the PRC1 component that reinforces silencing by means of the H2Aubq mark (Fig. 1.29) (Sing et al., 2009).

Binding motifs for YY1 (the mammalian homologue of PHO) do not, however, correlate well with PRC1 and 2 binding *in vivo* in mammals, which implies that other recruiting mechanisms might also be in place in mammals (Wilkinson et al., 2006). In this respect, genome-wide studies have uncovered the propensity of PcG proteins to colocalise with CpG islands devoid of binding sites for transcriptional factors (Ku et al., 2008). Indeed, GC-rich regions that do not contain such activating domains are sufficient to recruit PRC2 and initiate silencing in ES cells (Mendenhall et al., 2010).

PRC2 contains, among its ancillary subunits, Jarid2, a catalytically inactive histone demethylase that binds with PRC2 at GC-rich regions (Li et al., 2010). DNA methylation of GC-rich regions is associated with the reinforcement of a repressive state following the deposition of the H3K9me3 mark (Branco et al., 2012). The binding of PRC2 to DNA methylated GC-rich promoters is dependent on the prior binding of Tet1, which convert methyl-cytosines into hydroxymethyl-cytosines (Wu et al., 2011a).

ncRNAs generated by paused RNA Polymerases have also been shown to recruit and enhance the activity of PRC2 (Kanhere et al., 2010). PRC2 is itself recruited *in trans* by the H3K27me3 mark deposited by PRC2-mediated H3 methylation and this mechanism is implicated in the maintenance of repression (Margueron et al., 2009). It is important to stress that although the hierarchical model derived from *Drosophila* seems to be correct in some instances, many
examples of PRC2-independent recruitment of PRC1 have been described (Tavares et al., 2012).

### 1.6.1 Ezh1/2: the catalytic subunit of PRC2

The core of mammalian PRC2 is composed of Ezh1 or Ezh2, Suz12, Eed and RbAp46/48 (Fig. 1.30) (Margueron and Reinberg, 2011). Ezh1 and 2 are mutually exclusive in PRC2 complexes and contain a HMT domain called SET (named after the *Drosophila* proteins that contain it: Su(var), Enhancer of zeste, Trithorax) (Margueron et al., 2008). Ezh2 methylates both mono- and dimethylated lysine 27 on histone H3 thus generating two distinct repressive marks: H3K27me2 and H3K27me3 (Cao and Zhang, 2004). H3K27me2 is implicated in the maintenance of a repressed state at promoters by preventing acetylation of H3K27 (Simon and Kingston, 2009). The H3K27me3 histone mark is read by chromodomain-containing proteins (such as Cbx proteins, see below), which recruit PRC1 and in turn reinforce silencing (Fischle et al., 2003).

Ezh1 has a weaker HMT activity than Ezh2 and is more abundant in non-dividing cells than Ezh2, which is highly expressed in proliferating ES cells (Su et al., 2003). Binding of Ezh1 has also been observed at a small subset of active genes in ES cells, where it can deposit the H3K27me3 mark and compensate for Ezh2 in knockout cells (Shen et al., 2008). Ezh1 might also repress transcription via mechanisms other than histone methylation, such as the induction of higher-order chromatin compaction via an unknown mechanism (Margueron et al., 2008).
Ezh1 or Ezh2 act as the catalytic subunit of the complex through their SET domains, which possess HMT activity. Ezh1 and Ezh2 also have an RNA-binding motif that has been proposed to be involved in their recruitment. Eed is important for recruitment of the complex to previously trimethylated lysine 27 on histone H3. Suz12 also has an RNA-binding region and interacts with ncRNAs in vivo (not shown here). Suz12 allows the interaction between Ezh1/2 and RbAp46/48, which binds to the H3/H4 dimers. Ancillary subunits include Jarid2, AEBP2 and PCL1/2/3. There is evidence that Jarid2 might be involved in mediating the recruitment of PRC2 to CpG islands. There is also evidence for involvement of AEBP2 in mediating the interaction of PRC2 with DNA sequences important for its recruitment, PLC1/2/3 recruit PRC2 by reading histone modifications and potentiate the HMT activity of the complex (reproduced with permission from Margueron and Reinberg, 2011, Nature, copyright (2011) Nature Publishing Group).

Ezh2 contains an RNA binding domain and its association with long ncRNAs is enhanced by its phosphorylation on threonine 350 (Kaneko et al., 2010). The phosphorylation of serine 21 by Akt, on the other hand, blocks the binding of Ezh2 to nucleosomes and results in reduced levels of H3K27me3 and derepression of Hox genes (Cha et al., 2005). Ezh2 is required for lineage commitment in ES cells and for heavy chain rearrangement, which marks the development of pro B into pre B cells (Su et al., 2003). During muscle development, Ezh2 represses genes necessary for the development of
myoblasts into myotubes (Caretti et al., 2004). In the same cell type, however, Ezh1-containing PRC2 complexes bind to active promoters, which are devoid of the repressive H3K27me3 mark (Mousavi et al., 2011). In myoblasts, Ezh1-PRC2 interacts with RNA Pol II and promotes elongation. Knockdown of Ezh1 in myoblasts leads to impaired myogenic differentiation. It is possible that Ezh1- and Ezh2-containing complexes are differentially regulated at active and repressed promoters through interaction with different partners.

1.6.2 PRC2 core components and additional interactors

Eed plays a fundamental role in the recruitment of PRC2 to target genes by reading the H3K27me3 mark (Margueron et al., 2009). In Drosophila, mutations that disrupt this recognition impair the binding of PRC2 to PREs, reduce the global levels of H3K27me2/3 and disrupt embryonic development. In ES cells, knockout of Eed blocks their proliferation and differentiation (Shen et al., 2008).

Suz12 is required for ES cell differentiation but its function is still not fully understood (Pasini et al., 2007). Interaction of Suz12 with short ncRNAs generated by paused RNA Pol II has been described and this might provide an additional way to recruit PRC2 and to arrest RNA Pol II at the pausing stage (Kanhere et al., 2010). Suz12 knockout in ES cells is accompanied by a sharp reduction in H3K27me2/3 and an increase in H3K27ac and H3K36me3 (Jung et al., 2010). Interestingly, Suz12 seems to be required for the deposition of another repressive histone mark, H3K9me3, by another HMT: SUV39H1 (de la Cruz et al., 2007). Indeed, these proteins can directly interact and the presence of H3K9me3 together with H3K27me3 is probably a mechanism of reinforcement of gene silencing during development (Alder et al., 2010). Suz12 binds directly to Ezh1/2 and RbAp46 (or 48), a histone chaperone that binds to the H3-H4 dimer in the nucleosome and mediates Ezh1/2 binding to chromatin (Song et al., 2008).

Additional proteins associate with PRC2 but are not required for optimal
activity of the complex *in vivo*. Jarid2 belongs to the Jumonji family of histone demethylases but lacks enzymatic activity (Margueron and Reinberg, 2011). The role of Jarid2 in regulating Ezh2 activity is still unclear, as its knockdown does not significantly affect the levels of H3K27me3 *in vivo* (Shen et al., 2009). However Jarid2 enhances PRC2 activity *in vitro*. The presence of a GC-binding domain suggests that Jarid2 is responsible for the preferential binding of PRC2 to these regions in mammals (Li et al., 2010). Another candidate for involvement in PRC2 recruitment is the zinc finger protein AEBP2, which enhances the activity of PRC2 and contains a DNA binding domain (Kim et al., 2009).

PCL1, 2 and 3 are Ezh2 and Suz12 partners that are differentially expressed in a tissue-specific manner (Walker et al., 2010). Similar to the four core PRC2 members, depletion of PCL2/3 in ES cells enhances self-renewal and impairs differentiation. This is due to the loss of H3K27me3 observed at PLC2/3-PRC2 targets, which implies a positive role for these proteins in PRC2 activity (Hunkapiller et al., 2012).

**1.6.3 The PRC1 family of polycomb complexes**

In *Drosophila*, the core components of PRC1 are dRing (the catalytic subunit), Pc (Polycomb), Ph (Polyhomeotic) and Psc (Posterior sex combs) (Saurin et al., 2001). Mammalian homologues for these components have been identified and, both in *Drosophila* and humans, PRC1 can be co-purified with substoichiometric amounts of other PcG proteins and GTFs (Levine et al., 2002). PRC1 was found to block the activity of the SWI/SNF remodelling complex *in vitro* (Shao et al., 1999).

A large body of evidence is now accumulating that, instead of being a single core complex with ancillary subunits like PRC2, PRC1 represents a family of complexes whose only common denominators are the catalytic module subunits, Ring1A and Ring1B, which always associate with one of the six Psc homologues
PCGF proteins) identified in mammals (NSPC1, Mel18, PCGF3, Bmi1, PCGF5 and MBLR) (fig. 1.31) (Gao et al., 2012).

Figure 1.31: Schematic diagram of the different PRC1 complexes.

PRC1 complexes always feature Ring1A, Ring1B and one PCGF as the core catalytic subunit. All these complexes share histone H2A ubiquitination activity. Cbx proteins and RYBP/YAF2 generate mutually exclusive complexes and might influence differential requirements for Ring1B recruitment to chromatin (reproduced with permission from Gao et al., 2012, Molecular Cell, copyright (2012) Elsevier).

1.6.4 The role of Ring1A and B in PRC1 activity

Ring1A and B were identified as interactors with the mammalian homologue of Pc (M33) and represent the mammalian homologues of dRing (Schoorlemmer et al., 1997). Both Ring1A and B are E3 ligases that monoubiquitinate histone H2A in vitro, albeit with different efficiencies (Wang et al., 2004). It is likely that Ring1A enhances Ring1B activity in vivo and its knockout in mice results in anterior transformations and abnormalities in the axial skeleton (del Mar Lorente et al., 2000). Knockout of Ring1B is embryonically lethal (Vonckcn et al., 2003).
Therefore, unlike Ezh1 and 2, Ring1A and B are always co-purified as part of the same complex (Vidal, 2009).

Ring1B-mediated H2Aubq facilitates the binding of histone H1 to nucleosomes and might therefore provide a mechanism for enhancing gene silencing by promoting chromatin compaction (Jason et al., 2005). Knockdown of Ring1B is followed by a loss of H2Aubq and de-repression of target genes (Wang et al., 2004). Interestingly, a Ring1B mutant that is inactive as an E3 ligase can still repress transcription of *Hox* genes (Eskeland et al., 2010). However, it is possible that other repressive mechanisms could compensate for Ring1B-mediated silencing. For example, PRC2 could recruit SUV39H1 and promote the deposition of the H3K9me3 mark (de la Cruz et al., 2007). Indeed, SUV39H1 and Ring1B have been shown to be mutually exclusive in the regulation of silent genes, with the former implicated in a stronger repression of developmental genes (Alder et al., 2010). H2Aubq covers the inactive X-chromosome, where the binding and activity of Ring1B are independent from PRC2-mediated H3K27me3 (Schoeftner et al., 2006). Ring1B has been shown to ubiquitinate the histone variant H2A.Z (Sarcinella et al., 2007). This post-translational modification is also associated with the silencing of target promoters (Draker et al., 2011).

Ring1B and H2Aubq are believed to poise silent genes in ES cells for later activation (Stock et al., 2007). These genes, originally termed bivalent, are distinguished from fully silenced genes by the simultaneous presence of an activating (H3K4me3) and repressive (H3K27me3) mark on their promoters (Azuara et al., 2006, Bernstein et al., 2006a). In some cell types, particularly in the haematopoietic system, knockout or knockdown experiments of Ring1B (Yu et al., 2012), Bmi1 (Majewski et al., 2010) or Mel-18 (Jacob et al., 2011) result in the downregulation of some of genes bound by them. Rather surprisingly, PRC1 and PRC2 components associate with the promoters of active cytokines genes in activated CD4+ cells and the knockdown of members of both complexes leads to
transcriptional repression of these active Polycomb target genes (Jacob et al., 2011).

Additionally, the transcription factor ZRF1 is recruited to poised genes by Ring1B by means of the H2Aubq mark (Richly et al., 2010). Upon retinoic acid-induced differentiation, ZRF1 binds to H2Aubq and displaces Ring1B, resulting in partial loss of the H2Aubq, probably through recruitment of the histone deubiquitinase USP21 (Nakagawa et al., 2008). Therefore, H2Aubq can be a mark for recruitment of a transcriptional activator, which then displaces the ubiquitinating enzyme upon induction of gene expression.

At least five H2A deubiquitinases are known and, apart from USP21, two more are involved in transcriptional activation in vivo (Vidal, 2009). USP16 regulates chromosome compaction and the expression of Hox genes (Joo et al., 2007). In doing the latter, USP16 counteracts PRC1 by removing the H2Aubq mark from promoters but, interestingly, not displacing the complex (Joo et al., 2007). MYSM-1 plays a fundamental role in B cell development through derepression of the promoters of genes encoding key transcriptional factors (Jiang et al., 2011).

1.6.5 PCGF proteins regulate Ring1B activity

Ring1B can ubiquitinate nucleosomal H2A in vitro without any co-factors other than ubiquitin, the ubiquitin-activating enzyme E1 and the Ring1B-specific E2 ligase, UbcH5c (Buchwald et al., 2006). However, the efficiency of the reaction is greatly enhanced by the presence of Ring1A and Bmi1 (Cao et al., 2005). Upon interaction with Bmi1 (also known as PCGF4), UbcH5c can bind more efficiently to Ring1B (Buchwald et al., 2006). Moreover, Bmi1 facilitates the substrate recognition of Ring1B by stabilizing the interaction with the nucleosome (Fig. 1.32) (Li et al., 2006). The Ring1B-Bmi1 dimer benefits from conformational changes that the proteins induce in one another, which in turn stabilize the
interaction. To summarise, Bmi1 enhances Ring1B activity by promoting substrate recognition and by stabilizing the association between the E2 (UbcH5c) and the E3 ligase (Ring1B) (Li et al., 2006).

Figure 1.32: Schematic representation of the Ring1B-Bmi1 heterodimer, which promotes histone H2A monoubiquitination in vivo.

Ring1B interacts with Bmi1 through their RING domains. Furthermore, the N-terminus of Ring1B interacts with Bmi1, which in turn mediates the interaction between Ring1B and the nucleosomal substrate. The E2 ligase (UbcH5c) can therefore more stably interact with the E3 (Ring1B) (reproduced with permission from Li et al., 2006, Journal of Biological Chemistry, copyright (2006) American Society for Biochemistry and Molecular Biology).

Another PCGF protein, Mel18, interacts with and stimulates Ring1B E3 ligase activity towards histone H2A (Elderkin et al., 2007). Mel18 must be phosphorylated in order to confer Ring1B specificity towards nucleosomal substrates. In contrast, phosphorylation of Bmi1 by the MAPKAP kinase 3 (3pK) leads to its displacement from chromatin (Voncken et al., 2005). This event is cell cycle-regulated and seems to peak during G2 and mitosis (Voncken et al., 1999). Phosphorylation of Bmi1 by Akt, however, protects it from proteosomal degradation and favours its nuclear localization (Kim et al., 2011).

Ectopic expression of Mel18 in Bmi-deficient ES cells restores silencing of
some PRC1 target genes, which implies a certain degree of redundancy between the two proteins (Elderkin et al., 2007). Given the fundamental role of PCGFs in Ring1B activity \textit{in vivo}, it is not surprising that active Ring1B has always been copurified with one of them (Gao et al., 2012). Indeed, PCGF1 (also known as NSPC1) associates with Ring1A/B in the PRC1-related complex BCoR-Fbxl10 (see below) and promotes, at least \textit{in vitro}, the ubiquitination of histone H2A (Gearhart et al., 2006).

\section*{1.6.6 Cbx proteins define different PRC1 complexes}

Five Chromobox proteins (Cbx2, 4, 6, 7 and 8) represent the mammalian homologues of \textit{Drosophila} Pc (Tajul-Arifin et al., 2003). This definition is based on the affinity of their chromodomain for H3K27me3 (Pearce et al., 1992). Other Cbx proteins, such as HP1 proteins, contain a different chromodomain that allows them to bind with higher affinity to the H3K9me3 mark (Fischle et al., 2003). Because Cbx proteins are mutually exclusive and each one of them defines a different Ring1B-containing complex, their functions might be non-redundant \textit{in vivo} (Vandamme et al., 2011). Indeed, these proteins exhibit different patterns of nuclear localization, possibly due to different protein-protein interactions (Vincenz and Kerppola, 2008). The expression of Cbx2, 4 and 8 increases during differentiation of ES cells where they regulate different sets of genes as part of PRC1 complexes (Morey et al., 2012).

Cbx4 has been shown to interact with 14-3-3 proteins although this interaction has no effect on its binding to Ring1B (Vandamme et al., 2011). Cbx6 is expressed in ES cells but it does not associate with Ring1B on chromatin \textit{in vivo}, although its knockdown leads to gene derepression (Morey et al., 2012). Cbx6 and 7 are the only Pc homologues expressed in pluripotent ES cells and Cbx7 represses the expression of Cbx2, 4 and 8 as part of a PRC1 complex (O'loghlen et al., 2012).
During differentiation, the expression of Cbx7 decreases, which has led to the hypothesis that its expression is directly proportional to the degree of pluripotency of ES cells (O'loghlen et al., 2012). Interestingly, however, Cbx7 is highly expressed in some differentiated cell types such as GC lymphocytes (Scott et al., 2007). Notably, Cbx7 exerts an important role in the regulation of cellular senescence and proliferation (see section 1.2.5) by repressing the INK4b/ARF/INK4a locus (Gil et al., 2004). In this instance, Cbx7 is recruited not only by the H3K27me3 mark but also via a direct interaction with the long ncRNA ANRIL (Yap et al., 2010).

Cbx7 exhibits strong binding to both H3K9me3 and H3K27me3 in vitro (Bernstein et al., 2006b). In differentiated cells Cbx8 binds at several Cbx7 target genes, including the INK4b/ARF/INK4a locus (Dietrich et al., 2007). Cbx7 expression diminishes in in vitro differentiated ES cells as Cbx8 expression increases (O'loghlen et al., 2012). Two microRNAs (miR-125 and 181) have been shown to promote the repression of Cbx7 expression during ES cell differentiation (O'loghlen et al., 2012).

1.6.7 Recruitment and role of PRC1

The ability of Cbx proteins to recognize H3K27me3 provided evidence for the canonical model of PRC1 recruitment, discussed above. According to the hierarchical model, PRC1 binding requires the H3K27me3 to be read by the Cbx subunit. Nevertheless, PRC1 recruitment and the deposition of H2Aubq can occur in the absence of the H3K27me3 mark (Schoeftner et al., 2006). Cbx proteins exhibit different binding affinities to other methylated residues in addition to H3K27me3 and, thus, it is possible that different histone marks are used as a recruitment signal for PRC1 complexes in the absence of H3K27me3 (Bernstein et al., 2006b). Moreover, ncRNAs might play a bigger role under these circumstances (Yap et al., 2010). Lastly, it is possible to envisage a role for
transcription factors such as YY1, the mammalian homologue of the *Drosophila* DNA-binding PHO (Atchison et al., 2003).

RYBP and its homologue YAF2 bridge the interaction between YY1 and Ring1B (García et al., 1999). Indeed, RYBP has been shown to be present in active PRC1 complexes that bind to chromatin and ubiquitinate H2A in the absence of H3K27me3 (Tavares et al., 2012). Interestingly, RYBP-containing PRC1 exhibits a stronger E3 ligase activity *in vitro* than Cbx-containing PRC1 (Gao et al., 2012). RYBP and Cbx proteins are mutually exclusive in PRC1 complexes as they compete for the same binding site on Ring1B (Wang et al., 2010b). However, *in vivo* RYBP- and Cbx2-containing complexes are very often found in the vicinity of the same genes (Gao et al., 2012). The role of YY1 in the H3K27me3-independent recruitment of Ring1B has been questioned, as the protein is not co-immunoprecipitated with either Ring1B or RYBP *in vivo* (Gao et al., 2012).

Other transcriptional factors (Runx1 and CBFβ) have been shown to recruit Ring1B and Bmi1 to active genes in megakaryocytes and thymocytes (Yu et al., 2012). In these cells, PRC1 is recruited to active genes where it might fine-tune the expression of its target genes. Quite surprisingly, the knockdown of Ring1B leads to the upregulation of nearly a third of its target genes (Yu et al., 2012). Despite its known role as a silencer, therefore, the biological role of Ring1B might be affected by the factors that interact with it. It is important to bear in mind that *Drosophila* Pc blocks transcriptional initiation but does not impair the recruitment of the PIC to Polycomb-repressed genes (Dellino et al., 2004).

### 1.6.8 Non-canonical PRC1 complexes

Ring1A/B are also components of other non-canonical PRC1 complexes, which regulate transcription. The E2F6.com complex represses target genes in quiescent, serum-starved fibroblasts and is removed following cell cycle re-entry.

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(Ogawa et al., 2002). This complex contains the transcription factor E2F6, the Cbx protein HP1γ, YAF2, the PCGF homologue MBLR and L3MBT2, which binds to methylated histones (Ogawa et al., 2002).

Another stable complex with a role in transcriptional regulation is BCoR/Fbxl10 (Sánchez et al., 2007). This complex contains the co-repressor BCoR, the PCGF protein NSPC1, RYBP, the E3 ligase SKP1 and the H3K36 demethylase Fbxl10 (Gearhart et al., 2006). By coupling the monoubiquitination of H2A with the removal of the H3K36me3 mark, BCoR/Fbxl10 silences its target genes in B cells (Gearhart et al., 2006).

Finally, proteomics analysis of the Ring1A/B-interactome has revealed several proteins associated with transcriptional activation and elongation. In leukaemias, Ring1A/B have been shown to interact with the elongation factor p-TEFb and with DOT1L, which is responsible for the H3K79me3 mark on the body of transcribed genes (Mueller et al., 2007). Moreover, the methyltransferase responsible for H3K4me3 (MLL1) and the HAT responsible for H4K16ac (MOF) have also been co-purified together with Ring1A/B in human cells (Dou et al., 2005).

1.6.9 The role of PcG proteins in cancer

PcG proteins are overexpressed in several tumours and this usually correlates with the metastatic potential of the tumour (Suvà et al., 2009). It is possible that the tumourigenic activity of PcG proteins resides in their silencing of genes involved in differentiation which in turn leads to self-renewal and proliferation of cancer stem cells (Richly et al., 2011). This is likely to be the case for Ezh2, which is overexpressed in numerous tumours, such as bladder tumour, breast cancer, colorectal cancer, glioblastoma, oral squamous carcinoma and prostate cancer. However, PRC1 members, such as Bmi1, seem to operate differently. Bmi1 is overexpressed in bladder tumour, breast cancer, squamous
cell carcinoma, glioblastoma, leukaemias and neuroblastoma (Richly et al., 2011). In breast cancer, its overexpression correlates with good prognosis as opposed to the overexpression of Ezh2 (Pietersen et al., 2008).

In glioblastoma cancer cells, Bmi1 and Ezh2 seem to cooperate via two different mechanisms: Ezh2 promotes self-renewal and tumourigenesis of cancer stem cells, whereas Bmi1 positively regulates the proliferation of more differentiated glioma stem cells (Richly et al., 2011). Bmi1 is likely to favour proliferation through its inhibition of the INK4a locus (Jacobs et al., 1999). Overexpression of Ring1B is detected in lymphomas, gastric and colon tumours (Sánchez-Beato et al., 2006). However, Ring1B can also reduce proliferation through its inhibition of the Cyclin D gene (Calés et al., 2008). This regulation occurs in normal HSC and other early lymphoid progenitors in adults, conferring a regulatory role for Ring1B in the homeostasis of the hematopoietic system. In lymphomas and leukaemias, the misregulation of Ring1B expression disrupts this equilibrium and leads to uncontrolled proliferation of early, immature progenitors (Calés et al., 2008).

Interestingly, Cbx7 expression is lost in lung cancer, which has led to its classification as an oncosuppressor in this type of tumour (Forzati et al., 2012). A reason for this might be its negative role in the regulation of HDAC2, which promotes tumourigenesis in a number of cases through its downregulation of the E-cadherin gene (Federico et al., 2009). However, Cbx7 is overexpressed in GC-derived follicular carcinomas and plays a role in the generation of this malignancy through its repression of the INK4a locus (Scott et al., 2007).

1.7 Aim of the study

The core question of this paper is whether Aurora B plays a role in regulating gene expression aside from its mitotic functions. In particular, I focused my attention on the haematopoietic system, due to the prior results on
Aurora B in plasma cells published by our group. I have searched for new partners of Aurora B in non-mitotic mature lymphocytes and functionally characterised the functional interaction with Ring1B.

I have focused my study on resting lymphocytes as both proteins co-occupy the promoters of active genes and discovered that they cooperate to maintain the expression of these genes. These results identify a new role for both proteins during a critical stage in the development of the immune system.
Chapter 2 - Materials and methods

2.1 Cell culture

Primary murine lymphocytes and I.29+ cells were cultured in RPMI medium (Lonza) supplemented with 15% foetal calf serum (FCS, Sigma), 0.1 U/mL penicillin (Lonza), 0.1 μg/mL streptomycin (Lonza), 2 mM L-Glutamine (Lonza) and 50 μM beta-mercaptoethanol (Gibco). B cells were cultured in the presence of 6 ng/ml IL-4 (R&D). T cells were cultured in the presence of 10 ng/ml IL-7 (R&D). 293T cells were cultured in DMEM (Lonza) supplemented with 10% foetal calf serum (FCS, Sigma), 0.1 U/mL penicillin (Lonza), 0.1 μg/mL streptomycin (Lonza) and 2 mM L-Glutamine (Lonza).

2.1.1 B cell isolation

Resting B cells were isolated from the spleens of 6 to 8-week-old mice. The spleens were dissected and minced on sieves and the single-cell suspensions were centrifuged on a Ficoll-Paque (GE Healthcare) cushion to remove erythrocytes and dead cells. The resulting splenocytes were resuspended at a concentration of 10^7 cells/ml in 0.1% BSA/HBSS (both from Sigma) supplemented with 5% goat serum (Lonza) and anti-CD16 antibody (BD). The cells were firstly incubated with biotinylated anti-CD43 antibody (BD) and then with Dynabeads Biotin Binder beads (Invitrogen) to capture CD43+ cells. Bound CD43+ cells were removed by 4 sequential magnetic separations. The remaining population of CD43- cells was cultured at a concentration of 2-5x10^6 cells/ml.

For B cell activation experiments, lipopolysaccharide (LPS) (Sigma) was
resuspended at 10mg/ml in HBSS and used at a final concentration of 12.5 µg/ml. Anti-CD40 antibody (BD) was used at a final concentration of 5 µg/ml, together with anti-Igκ antibody (BD) at 0.5 µg/ml. After activation, B cells were kept in culture for a maximum of 3-4 days.

2.1.2 T cell isolation

Naïve CD4+ T cells were isolated from peripheral subcutaneous and mesenteric lymph nodes of 6 to 8-week-old mice. The lymph nodes were dissected and minced on sieves the single-cell suspensions were centrifuged on a Ficoll-Paque (GE Healthcare) cushion to remove dead cells. The purified cells were resuspended at a concentration of 10^8 cells/ml in 2% FCS/PBS (Invitrogen). Firstly, CD25+ positive cells were removed by negative selection using the CD25 Microbead kit mouse and LS columns (Miltenyi Biotec). In the resulting population of CD25- cells, CD4+ cells were positively selected using CD4 (L3T4) Microbeads mouse and LS columns (Miltenyi Biotec). These purified CD4+/CD25- T cells were cultured at a concentration of 2-5x10^6 cells/ml.

2.1.3 Conditional knockout mice

Aurora B conditional knockout (cKO) mice were generated by Dr. Monica Roman Trufero by crossing homozygous Rosa26-CreER<sup>T2</sup> mice with a strain of mice containing exons 2 and 6 of the Aurora B gene flanked by loxP sites for homologous recombination (a kind gift from Dr. Marcos Malumbres, CNIO, Madrid). The offspring generated from this cross will stably express CreER<sup>T2</sup>, as its gene is stably integrated in the ubiquitously expressed Rosa26 cassette. Following stimulation with 4-hydroxytamoxifen (4-OHT), CreER<sup>T2</sup> migrates inside the nucleus and the Cre recombinase targets the Aurora B gene for Cre-mediated recombination, effectively shutting down the expression of the Aurora B
gene. The Ring1B cKO/Rosa26-CreERT2 mice were a kind gift of Dr. Miguel Vidal (CNIO, Madrid).

2.2 Counterflow Centrifugal Elutriation

G1-fractions of activated B cells were isolated from unsynchronised populations by means of counterflow centrifugal elutriation in a Beckman Coulter Avanti J-26 XP Series centrifuge (Beckman Coulter), using a JE-5.0 Rotor (Beckman Coulter). After 3 days of \textit{in vitro} culture with LPS or anti-CD40, 4-8x10^8 activated B-cells were thoroughly resuspended in 50 ml of ice cold elutriation medium (RPMI supplemented with 1% FCS, 0.1 U/mL penicillin and 0.1μg/ml streptomycin), passed twice through an 18 gauge needle and loaded into a Sanderson chamber at constant flow of 6 ml/minute. The centrifuge was set at a constant speed of 2,300 rpm and 4°C temperature. Throughout the procedure, the elutriation medium was constantly pumped into the system using a peristaltic pump (SLS). The flow was increased to 7 ml/minute to remove the dead cells and 250 ml were discarded. Cell fractions (200 ml) were collected sequentially after increasing the flow-rate from 8 ml/minute. G1-enriched fractions were collected at a flow-rate between 9 and 12 ml/minute. The cells in each fraction were pelleted, resuspended in 5-10 ml of complete medium (RPMI with 15% FCS, 0.1 U/mL penicillin, 0.1 μg/mL streptomycin, 2 mM L-Glutamine and 50 μM beta-mercaptoethanol), counted and used for downstream applications. The complete medium was kept at room temperature to reduce cell stress. Cell cycle stage was determined by assessing the DNA content through propidium iodide (PI) staining of each fraction.
2.3 FACS analyses

2.3.1 Propidium iodide staining

For PI staining, 0.5-1x10⁶ cells were washed in PBS supplemented with 2% FCS. Fixation was carried out for 10 minutes in 70% ethanol at -20°C. After 2 washes with 2% FCS/PBS, cells were incubated for 30 minutes at room temperature in PBS containing 0.05 mg/ml PI (Sigma), 0.05% Igepal CA-630 and 1 μg/ml RNase A (Sigma). The samples were analysed for cell cycle distribution on a FACS Calibur machine (BD Pharmigen).

2.3.2 Cell surface markers staining

For determination of the expression of markers on the cell surface, 10⁶ cells were washed in PBS supplemented with 2% FCS. The unfixed cells were blocked with 5% goat serum and anti-CD16 antibody and incubated for 5 minutes. A 1:100 dilution was used for each antibody (see section 2.11). After 2 washes with 2% FCS/PBS, cells were resuspended in PBS and analysed within 30 minutes on a FACS Calibur machine.

2.4 Chromatin immunoprecipitation (ChIP)

2.4.1 Chromatin preparation

B or T cells were counted and their concentration was adjusted to 10⁶ cells/ml in complete medium. Proteins were crosslinked to the DNA by adding a 37% w/v formaldehyde solution (VWR) directly to the medium to a final concentration of 1% and incubating the cells at room temperature for 10 minutes with gentle tumbling. The reaction was quenched by adding to the medium a 1 M glycine solution (Sigma) to a final concentration of 0.125 M and incubating the cells for 10 minutes at room temperature with gentle tumbling.

The crosslinked cells were spun for 7 minutes at 780 g and 4°C. The
supernatant was removed and the cells were washed with 10 ml of ice cold PBS three times. Finally, the cells were resuspended in 1.5 ml of swelling buffer/10^7 cells (25 mM Hepes pH 7.9 (Sigma), 1.5 mM MgCl_2 (Sigma), 10 mM KCl (VWR), 0.1% Igepal CA-630 (Sigma)) supplemented with Complete MINI, EDTA-free protease inhibitors (Roche Diagnostics). The cells were incubated on ice for 10 minutes and then underwent a 50-stroke homogenization with a tight dounce homogeniser on ice.

The nuclei were pelleted by centrifugation at 3,000 g for 5 minutes at 4°C and snap-frozen. Prior to sonication, the nuclei were resuspended in sonication buffer (50 mM Hepes pH 7.5, 140 mM NaCl (Sigma), 0.1% sodium deoxycholate (Sigma), 0.1% SDS (Sigma), 1% Triton-X 100 (Sigma), 1 mM EDTA (VWR)) supplemented with Complete MINI, EDTA-free protease inhibitors (Roche Diagnostics). The final concentration of the cells was 1-3x10^6 cells/ml and 250 µl-aliquots were transferred into 1.5 ml low-binding DNA plastic tubes (Eppendorf). The tubes were placed in the carousel of a Bioruptor sonicator (Diagenode) connected to a water bath (Thermo Scientifics), which kept the temperature of the water in the sonicator basin constantly at 4°C. The nuclei were sonicated at 30-second on/off intervals for 2 hours at high intensity to obtain a fragment size of approximately 500 – 1,000 bp.

The insoluble fraction was removed from the soluble sonicated chromatin by two rounds of centrifugation at 10,000 g for 10 minutes at 4°C. The purified chromatin was quantified after a 5-minute alkaline lysis (1:10 dilution of chromatin in 0.1 M NaOH (Sigma)) and measuring the DNA concentration using an ND-1000 spectrophotometer (Nanodrop). A 2.5-5 µg aliquot of chromatin was saved as input material.

2.4.2 Immunoprecipitation

Antibodies were pre-bound to paramagnetic Protein G Dynabeads
(Invitrogen) into conventional 0.5 ml PCR tubes (SLS). For each immunoprecipitation (IP), 5 µg of antibody was incubated with 15 µl of beads in 200 µl of sonication buffer for at least 2 hours at 4°C with gentle rotation (8 rpm) on a rotating wheel. For ChIP experiments with IgM antibodies (see table 2.1), the beads were pre-incubated for 1 hour with IgG anti-IgM as a bridging antibody and subsequently incubated 4 hours with the IgM antibody of choice. The beads were finally washed once with 200 µl of sonication buffer by incubating them for 7 minutes at 4°C with gentle rotation (8 rpm) on a rotating wheel.

For each ChIP assay, 100 µg of chromatin was added to the antibody-bound beads and the chromatin concentration, when required, was adjusted to 0.5 µg/µl with sonication buffer. The samples were incubated over night at 4°C with gentle rotation (8 rpm) on a rotating wheel. The beads were sequentially washed with 200 µl of each of the following buffers: sonication buffer, high salt buffer (50 mM Hepes pH 7.5, 500 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton-X 100, 1 mM EDTA), LiCl buffer (20 mM Tris pH 8 (Sigma), 250 mM LiCl (VWR), 0.5% sodium deoxycholate 0.5% Igepal CA-630 (Sigma), 1 mM EDTA) and TE buffer (10 mM Tris pH 7.4, 1 mM EDTA).

Finally, the beads were washed one more time with 100 µl of TE, resuspended by gentle pipetting and transferred into a 1.5 ml low-binding DNA tube. To elute the chromatin from the beads and reverse the formaldehyde crosslink, the beads and the input DNA were resuspended in 150 µl of elution buffer (50 mM Tris pH 8, 50 mM NaCl, 1% SDS, 1 mM EDTA, 20 µg/ml DNase-free RNase (Roche Diagnostics)) and incubated at 68°C for 6-8 hours with vigorous agitation (1200 rpm) on a thermoshaker (Eppendorf). The supernatant was collected and the beads were further eluted for 1 hour with 150 µl of elution buffer at 68°C with vigorous agitation (1200 rpm). For input samples, 150 µl of elution buffer were added and crosslink reversal was carried out for an additional hour as well.
The resulting 300 µl of eluted DNA and protein mixture was incubated overnight at 42ºC with gentle shaking (300 rpm) in the presence of 200 µg/ml Proteinase K (Sigma) to remove proteins. The DNA was further purified using QIAQuick PCR purification kit (Qiagen), according to manufacturer’s instructions. The DNA from IP experiments was eluted in 30 µl of AnalaR grade water (VWR), while DNA from input samples was eluted in 60 µl of AnalaR water.

2.4.3 DNA quantification and quantitative real-time polymerase chain reaction

The concentration of DNA recovered from each sample was measured with a plate reader spectrofluorometer (Gemini) using the Picogreen Quanti-IT kit (Invitrogen). The fluorescent excitation and emission wavelengths were 480 and 520 nm, respectively. A standard curve was prepared using -phage DNA (NEB) at standard concentrations (12.8 – 6.4 – 3.2 – 1.6 – 0.8 – 0.4 – 0.2 ng/µl) and the DNA concentration of each sample was generated by line interpolation.

Quantitative real-time polymerase chain reactions (qRT-PCR) were used to determine the binding levels of each factor using the same amount of DNA (0.15 or 0.1 ng) for the immunoprecipitated and input sample. The amount of DNA produced in any PCR reaction can be described by the formula \( X = X_0 2^{Ct} \), where \( X \) and \( X_0 \) are respectively the final and initial DNA concentration of a sequence and \( Ct \) is the number of cycles. Since the same threshold value of DNA concentration is used, \( X \) becomes a constant. The number of cycles required to reach this value is inversely related to \( X_0 \) and, therefore, for a given sequence, \( X_0(IP) 2^{Ct(IP)} = X_0(input) 2^{Ct(input)} \), from which:

\[
\text{Fold enrichment} = \frac{X_0(IP)}{X_0(input)} = 2^{Ct(input)-Ct(IP)}
\]

For histone modifications, enrichment was calculated relative to
immunoprecipitation of the total histone instead of input DNA. qRT-PCR was performed either on a Chromo 4 (Biorad) or DNA Engine Opticon system (MJ research) under the following conditions: initial denaturation at 95°C for 10 minutes followed by 37 cycles at 95°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 15 seconds and final extension 72°C for 5 minutes. For each experiment the threshold was set to cross a point at which the amplification was linear (usually 0.025).

For each primer pair, the Ct values for input/total histone DNA were always determined in duplicate (technical duplicates), whereas IP samples were measured in duplicate only when a sufficient amount of DNA had been recovered. Anti-IgG control antibodies (negative control) always yielded 10-100 fold less DNA than specific antibodies and were diluted to 88 µl with AnalaR water. For each primer pair, each well on qRT-PCR plates (Biorad) was loaded with 5 µl of DNA and 20 µl of primer mix (5.5 µl of water, 1 µl of 100 µM forward primer, 1 µl of 100 µM reverse primer, 12.5 µl of SYBR Green JumpStart Taq Ready Mix (Sigma)).

2.4.4 Sequential ChIP (ReChIP)

For ReChIP analysis, the first ChIP was performed as previously described but the beads were eluted for 10 minutes at 68°C in 100 µl of ReChIP elution buffer (50 mM Tris pH 7.5, 1% SDS, 10 mM EDTA) with vigorous shaking (1400 rpm). One tenth of the eluted material was used to assess the first ChIP as previously described, while the remainder was diluted 1:10 with dilution buffer (16.7 mM Tris pH 8, 167 mM NaCl, 0.01% SDS, 1.1% Triton-X 100, 1.2 mM EDTA). The diluted eluate was divided into equal fractions and the second ChIP was performed as previously described.
2.5 ChIP-sequencing (ChIP-seq)

2.5.1 Library preparation

ChIP-seq libraries were prepared from at least 10 ng of DNA immunoprecipitated using the ChIP protocol described above. Multiple IPs from the same biological sample were performed in the case of low-DNA yield experiments in order to obtain a sufficient amount of DNA. The final DNA was first tested by qRT-PCR and then used to prepare ChIP-seq sequencing libraries with NEBNext ChIP-seq Library Prep Master Mix Set for Illumina (NEB) with minor variations on the manufacturer’s protocol. Specifically, the protocol was tailored to a lower amount of starting material (10 ng rather than the recommended 1 mg) with help from Dr. Carmelo Ferrai.

The quantity of DNA adaptors used was a 1:60 dilution of the recommended amount, in order to reduce the presence of unligated adaptors that would compete with and reduce the amount of sequenced specific DNA fragments. This is due to the fact that short fragments (such as those generated by unligated fragments) tend to be sequenced more than long ones. To sequence more than one library per lane on the Illumina HiSeq2000 (Illumina), DNA Illumina adapters and indexes from the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina) were used to allow sample multiplexing.

Following PCR enrichment of adaptor ligated DNA fragments, the library were resolved on a 2% agarose gel to remove primer dimers and gel purified with Qiaquick Gel extraction kit (Qiagen) according to manufacturer's guidelines. The purified libraries were analysed on the Bioanalyser (Agilent) with a High Sensitivity DNA kit (Agilent) for the determination of the average fragment size and concentration and to assess the integrity of the library. ChIP-seq libraries were given to the Genomics Laboratory at MRC CSC and run on the Illumina HiSeq2000 sequencer. Libraries were always subjected to single ended sequencing and the output size was either 50 or 100 bp. Gopuraja Dharmalingam
provided post-sequencing bioinformatic analysis of all the output files.

### 2.5.2 Data analysis

The overall quality of the sequencing reads including per base quality distribution, duplicate level, GC content and adapter contamination etc were assessed by 'fastqc' version 0.10.0. The reads were aligned to Mouse genome mm9 using Bowtie version 0.12.8 using '-S -n 2 -I 25 -m 1' parameters. Only reads aligned uniquely to the genome were retained for further analysis. The SAM files generated by Bowtie were converted to BAM files using Samtools version 0.1.18, sorted according to chromosomal co-ordinates and indexed. Reads with identical chromosomal co-ordinates and orientation (duplicates) were filtered using Picard version 1.65. To visualise the genomic coverage of samples in UCSC genome browser, the reads were extended to estimated fragment size and converted to BigWig format. The fragment size was estimated using SISSR method (Jothi et al., 2008).

### 2.5.3 Density plot generation

To visualise the ChIP-seq enrichment for each factor/modification around TSS, we divided TSS ± 3kb region in to 50 base bins (total 120 bins). Gene co-ordinates were retrieved from Ensembl version64 using 'biomRt' bioconductor package. The genes were ordered by FPKM values of 4 biological replicates of resting B cells mRNA-seq that were estimated using Cufflinks version v1.3.0 (see section 2.7). For each factor, number of reads overlapping with each bin was counted and Reads per Million per bin (RPM) was calculated. MeV version 4.8 was used to visualise the enrichment and generate the heat map images.
2.5.4 Line plot generation

FPKM values for 4 biological replicates of mRNA-seq in resting B-cells (see section 2.7) were estimated using Cufflinks version 1.3.0. Genes with at least 0.5 FPKM in any one of the biological replicates were classified as active genes (13264 genes). Gene co-ordinates were obtained from Ensembl version 64 and a window of TSS ± 3kb was constructed for all active genes. ChIP-seq datasets were aligned using Bowtie version 0.12.8 with default settings and only uniquely aligned reads were used for further analysis.

Fragment size was estimated using the SISSR method in 'chipseq' Biconductor package and reads were extended to fragment size. The number of reads overlapping with each base in TSS ± 3kb window was calculated and used for computing RPM (Reads Per Million of mapped reads). RPM values are averaged for all active genes and plotted using 'plot' function in R.

2.6 Transcriptional analysis

2.6.1 Spike-in RNA preparation

The generation and use of the spike-in RNA used to quantify transcription in this study has been previously described (Szutorisz et al., 2006). The sequence for the spike-in was chosen for its low homology with the mouse genome, in order to allow an accurate quantification. A pBluescript II SK (+) was linearised and in vitro transcribed using T7 RNA polymerase (Promega), according to the manufacturer’s instructions. The RNA generated in this reaction was treated with DNase I to remove the original DNA template and column purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s guidelines for RNA clean up. The spike-in RNA was eluted in 60 μl of DEPC-treated water (Ambion), quantified on an ND-1000 spectrophotometer and diluted to a final concentration of 0.1 ng/μl.
2.6.2 Total RNA extraction and cDNA preparation

Cell lysates were homogenized using QIAshredder spin columns (Qiagen). The spike-in RNA was added at this stage at a fixed ratio of 0.2 ng/10^6 starting cells. To obtain an accurate quantification, the number of cells was the average of 10 separate counts on a haemocytometer. Total RNA was purified with RNeasy Mini Kit (Qiagen), eluted in 30 µl of DEPC-treated water and quantified on an ND-1000 spectrophotometer. Using the least abundant sample as a limiting factor, the same amount of RNA (usually approximately 500 ng) from each sample was diluted to a final volume of 16 µl with DEPC-treated water and underwent DNase I (Invitrogen) digestion, according to manufacturer’s instructions. To remove traces of undigested DNA and DNase I that could impair downstream applications, the DNase treated RNA was further column purified by performing RNA cleanup with the RNeasy Mini Kit, according to the manufacturer’s guidelines.

The purified RNA was eluted in 30 µl of DEPC-treated water and quantified with an ND-1000 spectrophotometer and used for reverse transcription with Random Primers and SuperScript II (Invitrogen). Samples were prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>RT+</th>
<th>No RT (negative control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (ng)</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Random Primers (µl)</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>H2O</td>
<td>up to 44 µl</td>
<td>up to 22 µl</td>
</tr>
<tr>
<td>Incubate 10 minutes at 70°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5x buffer (µl)</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>0.1M DTT (µl)</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>10 µM dNTPs (µl)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>1/10 diluted SuperScript II (µl)</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

The reverse transcription was carried out as follows: 10 minutes at 20°C, 50
minutes at 42ºC, 10 minutes at 94ºC. Finally, the cDNA was column purified with QIAQuick PCR purification kit, according to manufacturer’s instructions, to remove potentially interfering reagents for the downstream qRT-PCR. Samples were diluted 1:100 and, for each primer pair, the Ct values for RT+ samples were always determined in duplicate (technical duplicates), whereas No RT controls were always measured in single. qRT-PCR were performed as previously described (section 2.4.3) with the sole difference of doing 40 cycles of amplification instead of 37.

2.6.3 RNA quantification

Specific primers were designed for the spike-in control and they were used to measure the abundance of the spike-in RNA in each sample. To calculate the relative expression level of each gene (x) with respect to the spike-in control, the following formula was used:

\[ \text{Gene X RNA expression} = 2^{\text{Ct(spike-in)} - \text{Ct(x)}} \]

2.7 mRNA-sequencing (mRNA-seq)

2.7.1 Library preparation

mRNA-seq libraries were prepared using 2 µg of total RNA purified as previously described. A total of four libraries were prepared for four independent biological replicates. Libraries were prepared using the TruSeq RNA Sample Preparation Kits v2 (Illumina) according to the manufacturer’s protocol. The libraries were resolved on a 2% agarose gel to remove primer dimers and gel purified with Qiaquick Gel extraction kit (Qiagen) according to manufacturer’s guidelines. The purified libraries were analysed on the Bioanalyser (Agilent) with DNA 1000 kit (Agilent) for the determination of the average fragment size and
concentration and to assess the integrity of the library. RNA-seq libraries were given to the Genomics Laboratory at MRC CSC and run on the Illumina HiSeq2000 sequencer. Libraries underwent paired-end sequencing and the output size was either 150 or 300 bp. Gopuraja Dharmalingam provided post-sequencing bioinformatic analysis of all of the output files.

2.7.2 Data analysis and quantification

RNA-seq sequencing reads were aligned to mm9 using Tophat using default parameters. Gene coordinates from Ensembl version 64 was used as known gene model. FPKM values for each sample was estimated using Cufflinks version 1.3.0 and correlation between replicates assessed using 'Spearman correlation'.

2.8 Proteomics

2.8.1 Protein extraction and western blotting

Cells were first washed 3 times in ice cold PBS (Gibco) and then resuspended in 10µl/10^6 cells of lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, 1% Triton-X 100, 1 mM EDTA) supplemented with Complete MINI, EDTA-free protease inhibitors (Roche Diagnostics) and Phosphatase Inhibitors Cocktail I and II (Sigma). Cells were placed at 4°C with vigorous shaking (1400 rpm) for 30 minutes. To improve the extraction of chromatin bound proteins, the lysate was frozen in liquid nitrogen and thawed by vigorously shaking (1400 rpm) at 37°C. This procedure was repeated three times. Finally the extract was cleared from the insoluble fraction by centrifugation at 10,000 g for 10 minutes at 4°C. The concentration of the protein extract was determined by Bradford assay (Biorad) using a Genesys spectrophotometer (Thermo). A standard curve was prepared using BSA
dissolved in lysis buffer (0.2 – 0.4 – 0.8 – 1.6 – 3.2 – 6.4 – 12.8 µg/µl).

For western blots, 20-50 µg of whole protein extract was loaded on acrylamide/bis-acrylamide gels and transferred onto nitrocellulose membranes (GE) using a transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS) supplemented with 20% methanol (VWR). The membranes were blocked over night at 4°C in 5% milk/ 0.1% TBS-Tween-20. The primary and secondary antibodies were both diluted in blocking solution and incubated with the membrane at room temperature for 60 and 45 minutes, respectively. All secondary antibodies were horseradish peroxidase (HRP)-conjugated and the chemiluminescent substrate used for visualisation of bands was Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer).

2.8.2 Protein co-immunoprecipitation

For co-immunoprecipitation (Co-IP) experiments, whole cell extracts were prepared by resuspending PBS-washed cells in 1% NP-40 lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1mM MgCl₂, 10% glycerol, 1% Triton-X 100, 5 mM EDTA, 1mM DTT), supplemented with Complete MINI, EDTA-free protease inhibitors and Phosphatase Inhibitors Cocktail I and II. Protein extracts were separated from genomic DNA and cell debris by centrifugation at 14,000 rpm at 4°C for 15 minutes. The extracts were then diluted with 4 volumes of dilution buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1mM MgCl₂, 10% glycerol, 0.5 mM EDTA) and quantified using the Bradford method.

1 mg of whole protein extract was incubated for 2 hours with 50 µL of protein G Dynabeads, pre-coupled to 2 µg of antibody. The beads were washed 6 times with 0.1% NP-40 buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1mM MgCl₂, 10% glycerol, 0.1% Triton X-100, 0.5 mM EDTA). Immunocomplexes were eluted by boiling the beads in 60 µL of 2X Loading buffer and 5X Reducing agent (both from Invitrogen), for 10 minutes at 99°C. For western blots, half or
one third of the eluted material was diluted 1:2 with water and loaded on acrylamide/bis-acrylamide gels.

2.8.3 Calcium phosphate transfection

For co-IP experiments with tagged proteins, 50% confluent 293T cells were co-transfected with plasmids expressing N-terminus FLAG-tagged mouse Aurora B and N-terminus HA-tagged mouse Ring1B. The FLAG-Aurora B construct was inserted in a pSPORT6 vector (Invitrogen), while the HA-Ring1B construct was inserted in a pSG5 vector (Invitrogen) and was a kind gift of Dr. Miguel Vidal (CNIO, Madrid). Both vectors (2 µg each) were resuspended in 500 µl of 0.4 M CaCl$_2$ (Sigma) to which a volume of 500 µl of HEBS solution (pH 7.05) (1.5 mM Na$_2$HPO$_4$.2H$_2$O (Sigma), 280 mM NaCl, 10 mM KCl, 12 mM glucose (Fischer), 50 mM Hepes) was added dropwise while vortexing the tube. This solution was incubated at 37ºC for 15 minutes and then added dropwise to the 9 ml of medium covering the cells in a 100mm Petri dish. The cells were returned to the cell incubator and, after 24 hours, the medium was changed. After an additional 24 hours the cells were harvested for protein extraction and co-IP experiments as described in section 2.8.2.

2.8.4 Cell labelling for SILAC

I.29+ cells were gradually adapted to grow in dialysed FBS (Biosera) by increasing the percentage of dialysed over normal FBS over 4 passages. The adapted cells were switched from normal RPMI to RPMI for SILAC (Thermo Scientifics) and supplemented with 0.1 mg/mL of $^{13}$C$_6$ $^{15}$N$_2$ L-Lysine-2HCl and $^{13}$C$_6$ $^{15}$N$_4$ L-Arginine-HCl (CKGas). After 4 doublings, a small number of cells ($\sim$10$^5$) were lysed and the heavy label incorporation was confirmed by mass spectrometry by Dr. Ambrosius Snijders.
2.8.5 Sample preparation for LC-MS/MS analysis

Co-immunoprecipitated proteins were spiked with 10 µg of heavy-labelled I.29+ whole cell extract. To drastically reduce the concentration of detergents in the samples, which could interfere with downstream applications, the samples were partly resolved by running them on a 10% acrylamide/bis-acrylamide gel. The proteins were fixed and stained with Simplyblue safe stain (Invitrogen), according to the manufacturer’s instructions. A total of 8 bands were excised per sample and transferred into clean plastic tubes. The slices were dehydrated by adding 250-500 µl of acetonitrile (VWR) and incubating the tubes at 37ºC for 5 minutes. The acetonitrile was removed and the samples left to air-dry. To increase the efficiency of the trypsin digestion, cysteine residues were first reduced by incubating the gel for 30 minutes at 55ºC with 100 µl of 100 mM DTT (Sigma) in 100mM ammonium bicarbonate (Sigma). After a second wash with acetonitrile, the gel was alkylated by incubating it in the dark for 20 minutes in the presence of 100 µl of 55mM iodoacetamide (Sigma) in 100mM ammonium bicarbonate.

The samples were washed with acetonitrile as before and destained for 30 minutes at 37ºC in 500 µl of 200 mM ammonium bicarbonate in 40% acetonitrile. In-gel trypsin digestion was performed overnight at 37ºC using 10 ng/µl Trypsin (Sigma) in the presence of 10 mM ammonium bicarbonate and 10% acetonitrile. The supernatant, containing the digested peptides, was collected and a second peptide extraction on the gel slice was carried out using 50 µl of 5% formic acid (Fischer) in 50% acetonitrile for 20 minutes. The peptides in the supernatants were dried by means of a vacuum centrifuge (Thermo). The dried samples were resuspended in 10 µl of 0.1% Trifluoroacetic acid (Sigma) using a sonicator water bath and subjected to mass spectrometry analysis by the Proteomics Facility at
the MRC CSC.

2.8.6 Mass spectrometry analysis and data processing

The analysis was performed by Dr. Ambrosius Snijders. Peptide mixtures were separated using a 50 cm analytical column on a 3 hour gradient from 3 to 50 % acetonitrile (0.1% fluoroacetic acid). The data was acquired on the LTQ-Orbitrap Mass Spectrometer using a top 6 MS/MS collision-induced dissociation (CID) method to ensure that each 3D peak is sampled multiple times thereby facilitating quantification. The number of MS/MS per cycle was extended to 20 to increase the number of peptide identifications. All raw data was analysed using MaxQuant.

Table 2.1 Antibodies

<table>
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<th>Antibody</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurora B (ab2254)</td>
<td>Abcam</td>
<td>Rabbit</td>
<td>ChIP, ChIP-seq</td>
</tr>
<tr>
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<td>Goat</td>
<td>Co-IP, Western Blot</td>
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<td>ChIP, ChIP-seq, Western blot</td>
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<td>B cell purification</td>
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**Table 2.2 Primers for ChIP (promoter region)**

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<tr>
<td>Gene</td>
<td>Forward Primer</td>
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<td>----------------</td>
</tr>
<tr>
<td>b-Actin</td>
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</tr>
<tr>
<td>Fcrl1</td>
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Table 2.3 Primers for transcriptional analysis

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Chapter 3 - Identification of new partners of Aurora B outside mitosis

3.1 Introduction

The Aurora B kinase (henceforth referred to as Aurora B) is a key mitotic regulator (Carmena and Earnshaw, 2003). Over the years, however, it has become linked to other functions such as regulation of the G1/S transition (Song et al., 2007), transcriptional regulation of pituitary genes (Tardáguila et al., 2011) and Xist RNA recruitment (Hall et al., 2009). The aim of this PhD project was to identify novel functions for Aurora B outside mitosis, in particular in gene regulation. The link between Aurora B overexpression and myeloid malignancies (Katayama et al., 2003), the relatively good efficacy of Aurora B inhibitors in leukaemias (Yang et al., 2007), its role in interphase in T-cells (Song et al., 2007) and its expression in terminally differentiated, post-mitotic plasma cells (Sabbattini et al., 2007) led to the choice of mature murine B and T cells as the cell models for the study. A further advantage of these cells is the fact that they are primary non-transformed cells that can be readily isolated and studied.

The initial strategy was to search for novel binding partners for Aurora B outside mitosis. The experimental approach used in this part of the project involved co-immunoprecipitation (co-IP) of Aurora B in G1 and control, unsynchronized LPS-activated B cells followed by quantitative mass spectroscopy. This strategy allowed me to quantify proteins that interact with Aurora B mainly in G1.
3.2 Isolation of G1 activated B cells by counter-flow centrifugal elutriation

The expression of Aurora B peaks at the onset of G2 and remains constant until the end of cytokinesis (see section 1.5). Therefore, most of the detectable Aurora B comes from the G2/M phase in an unsynchronized population of cycling cells. In LPS-activated B cells, for example, the levels of Aurora B in an asynchronous population are orders of magnitude higher than the amount of Aurora B present in the same cells when they are in G1 (Fig. 3.1) In order to study Aurora B outside mitosis it is therefore necessary to separate interphase from mitotic cells, as the contribution from the G2/M cells in a cycling population to the overall amount of Aurora B expressed is so large that it impairs the study of Aurora B in interphase.

![Figure 3.1: Differential expression of Aurora B in unsynchronized and G1-elutriated B-cells.](image)

Whole cell extracts from unsynchronized and G1-elutriated LPS-activated B cells were resolved on a 6% acrylamide/bis-acrylamide gel and probed with antibodies against Aurora B and RNA Polymerase II.

Several compounds, such as aphidicolin and nocodazole, have been shown to reversibly arrest the cell cycle by blocking critical steps during the cell cycle. However, attempts to use these drugs in primary, lipopolysaccharide (LPS)-activated B cells proved unsuccessful or unsatisfactory in terms of cell death.
(data not shown). I decided therefore to use counter-flow centrifugal elutriation (CEE) (Banfalvi, 2008) to obtain pure interphase B cells. This technique separates cells based on their size, which varies in direct proportion to their DNA content. CEE proved a reliable method for obtaining high numbers of cells in the G1 phase of the cell cycle (Figure 3.2 and section 2.2).

![Figure 3.2: Cell cycle distribution in unsynchronized and G1-elutriated (fraction 9ml/min) LPS-activated B cells.](image)

CD43-negative cells were isolated from 6- to 8-week old mice and stimulated with 12.5 µg/ml LPS for 72 hours.

### 3.3 Indirect SILAC-based determination of Aurora B partners in G1

#### 3.3.1 Introduction to SILAC

Stable isotopic labelling with amino acid in culture (SILAC) is a powerful tool to quantify protein interactions in different samples in vivo (Amanchy et al., 2005). Briefly, cells are growth in the presence of amino acids containing stable isotopic elements, such as $^{13}$C and $^{15}$N, so that every peptide will incorporate them over time and increase its mass. Heavy-labelled peptides differ from those containing natural (light) amino acids only for their higher mass, resulting in distinguishable spectra. The relative abundance of each protein is expressed as the ratio of relative intensity of heavy- to light-labelled peptides (Fig. 3.3).

A key requisite for SILAC is the full incorporation of the heavy amino acids. For this reason, cells are firstly adapted to grow in dialyzed serum, which is
devoid of the natural amino acids present in cell culture sera. Secondly, cells must go through at least 5-6 divisions (depending on protein turn-over) in medium supplemented with heavy amino acids before every peptide is fully labelled.

Figure 3.3: Schematic representation of the SILAC workflow from cell labelling to quantification of relative abundance (reproduced with permission from Amanchy et al., 2005, Science’s STKE 2005, pl2. Reprinted with permission from AAAS).

3.3.2 Use of an internal standard to perform quantitative mass spec analyses in primary cells

LPS-activated B cells can survive in culture for 3-4 days before entering
apoptosis or beginning to differentiate into plasmablasts and plasma cells. During this window of time no more than 3-4 divisions are completed, which is not enough to allow complete SILAC labelling. To circumvent this problem, unsynchronized I.29+ B cell lymphoma cells (I.29) were labelled with heavy amino acids. A protein extract from the heavy-labelled I.29 cells was mixed with the eluates obtained from co-IPs of Aurora B from primary G1-elutriated and unsynchronized LPS-activated B cells (Fig. 3.2). This method has been previously described for primary tissues (Ishihama et al., 2005) and allows for quantification of “light” samples by means of a heavy internal standard (Fig. 3.4). I.29 cells were used to provide the heavy internal standard because of their close resemblance to mature B cells (Alberini et al., 1987). This cell line has also used as a model to study proteosomal changes during B cell development (Romijn et al., 2005). In summary, I reasoned that the repertoire of proteins expressed in I.29 cells is likely to be similar to that of primary mature B cells. Therefore peptides from both the co-IPs of primary cells should be present in the heavy I.29 cells extract, making it possible to quantify them.

A fixed amount of heavy I.29 extract (1% of the input used for each co-IP) was added to both co-IP samples. The relative abundance of each heavy peptide was considered as a constant value in both the G1 and control (CTL) light sample. Hence, the relative abundance of each protein co-immunoprecipitated in both samples (G1 and unsynchronized control) could be directly compared and assigned a numerical value (ratio G1/CTL).
3.4 Quantitative analysis of the partners of Aurora B in G1-elutriated, LPS-activated B cells

My analysis focused on peptides that are present primarily in G1 and, as a first indication of the type of interactors that co-immunoprecipitate with Aurora B, I looked at the annotated function of peptides whose G1/CTL ratio was greater than 1. Gene expression, developmental process and chromatin organization
were represented among the terms displaying a statistically significant enrichment, (Fig. 3.5). This first analysis supported the original hypothesis that, outside mitosis, Aurora B interacts with different types of proteins implicated in biological processes such as gene regulation.

Figure 3.5: Functional annotation of proteins with G1/CTL ratio higher than 1.
Proteins are grouped according to their annotated biological process. (This analysis as carried out by Dr. Tom Carrol).

For the second phase of the analysis I looked at the specific proteins that interact with Aurora B mainly in G1. For this purpose, I considered proteins with a G1/CTL ratio greater than 0.2. The rationale for this value is based on the observation that 83.9% (80% by approximation) of the cells in the unsynchronised control sample are in G1 (Fig. 3.2, left panel) and, therefore, some of proteins with a G1/CTL ratio between 1 and 0.2 might still interact with Aurora B in G1 and, because of different efficiency of the co-IP in the G1 and CTL samples, be more represented in the CTL sample. Moreover, the SILAC determination was not performed in duplicate, as the aim of this experiment was to identify potential partners that would be validated by direct co-IP and western blot in at least two independent biological replicates. To validate this approach I selected a protein with a G1/CTL value slightly above the cut-off value, NIPP1 (G1/CTL = 0.28), and analysed its interaction with Aurora B by co-IP in the G1-elutriated and unelutriated LPS-activated B cells. NIPP1 interacts with PRC2 and
is required for its association with target genes (Van Dessel et al., 2010) and, to date, it has not been reported as a partner of Aurora B. Western blot detection with an anti-NIPP1 antibody confirms that this protein interacts with Aurora B and this interaction occurs in both samples (Fig. 3.6).

Figure 3.6: NIPP1 interacts with Aurora B in G1-elutriated cells despite being detected with a G1/CTL ratio of 0.2 in the SILAC-based screening for new potential partners of Aurora B outside mitosis.

Magnetic beads were pre-incubated with antibodies against goat IgG (negative control) or Aurora B. The input material for each co-IP was 500 µg of whole cell extract from unsynchronized (green, left) and G1-elutriated (red, right) B cells. Complexes were eluted by boiling the beads in 2x loading buffer and resolved on a 10% acrylamide/bis-acrylamide gel. Membranes were incubated with antibodies against NIPP1. Asterisks denote heavy and light chains of the IgG.

Table 3.1 lists some of the most interesting peptides with a G1/CTL ratio greater than 0.2. PcG proteins were identified including the PRC1 components Ring1B and RYBP and the DNA binding protein YY1. Since no PcG protein has been, to date, shown to directly or functionally interact with Aurora B or members of the CPC at any stage of the cell cycle, I decided to focus my attention on this possibility.

Other proteins identified in this screening as new partners of Aurora B were Dicer and Argonaute 2, members of the RISC complex. Interaction of short, centromeric non-coding RNAs with Aurora B has been described and minor satellite RNAs have been shown to enhance Aurora B kinase activity, hinting at the possibility of a role for short RNAs in Aurora B regulation (see section 1.5.1).
Furthermore, Two DNA methyltransferases (DNMT1 and 3A) were also found to co-immunoprecipitate with Aurora B (see section 1.4.1). DNA methylation has been shown to promote Aurora B recruitment and phosphorylation of serine 10 on histone H3 (H3S10ph) during the transition from S to G2 phase. Moreover, the knockdown of DNMT1 reduces H3S10ph on pericentromeric foci in G2 (Monier et al., 2007). However, these proteins have never been identified as potential interactors of Aurora B.

Other previously unknown interactors included members of the deadenylation complex CCR4-Not and Forkhead transcription factors. Specifically, the Cnot 2, 3, 6, 7, 8 and 10 subunits of the CCR4-Not complex immunoprecipitated with Aurora B and presented a G1/CTL value above 0.2. The Cnot 1 subunit was also co-immunoprecipitated with Aurora B but with a G1/CTL value below 0.2. Among the Forkhead transcription factors, FoxO1A, FoxK1 and FoxP1 were identified as interactors with a G1/CTL ratio above 0.2. These interactions were validated by other members of our group.

Table 3.1: Aurora B partners identified by SILAC screening as potential interactors during G1.

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The SILAC analysis confirmed that, as has been reported for T cells, Aurora B and mTOR interact with one another outside mitosis in B cells (see section 1.5.5). Aurora B displaces the heterochromatin protein HP1 from trimethylated lysine 9 on histone H3 (H3K9me3) at the onset of mitosis by phosphorylating the nearby serine 10 (see section 1.5.2). The histone methyltransferase SUV39H2, responsible for deposition of the H3K9me3 mark (O'Carroll et al., 2000) was detected, as well as HP1β. The CPC proteins INCENP and Borealin and several subunits of the APC complex were detected only in the CTL sample or with a G1/CTL ratio below the cut-off value of 0.2 (not shown in table 3.1). Interestingly, the PRC2 core component Suz12 was also identified in the CTL but not in the G1 sample, suggesting that this interaction might take place either during S or G2/M.

### 3.5 Validation of the Aurora B-Ring1B interaction

One of the major results of the SILAC analysis was the finding that Aurora B and Ring1B interact in G1 activated B cells. As this observation raised the
possibility that Aurora B might be involved in PRC1-mediated regulation of gene expression, additional experiments were performed to confirm the result. Co-IP was carried out on extracts from G1 activated B cells, followed by western blot analysis of immunoprecipitated proteins using an anti-Ring1B antibody (Fig. 3.7). The western blot detected a band at the predicted molecular weight of Ring1B both in the input and Aurora IP lanes but not in the negative IgG control (Fig. 3.7 bottom panel). A similar analysis carried out using an antibody that recognises the catalytic subunit of PRC2 Ezh2 found no evidence of interaction between Aurora B and Ezh2 (Fig. 3.7, top panel).

Figure 3.7: Aurora B interacts with Ring1B but not with Ezh2 in G1-elutriated LPS-activated B cells.

Magnetic beads were pre-incubated with antibodies against goat IgG (negative control) or Aurora B. The input material for each co-IP was 500 µg of whole cell extract from G1-elutriated B cells. Complexes were eluted by boiling the beads in 2x loading buffer and resolved on a 10% acrylamide/bis-acrylamide gel. Membranes were incubated with antibodies against Ezh2 (top panel) and Ring1B (bottom panel). Asterisks denote heavy and light chains of the IgG.

To further confirm this result, I transiently co-transfected 293T cells with vectors expressing tagged versions of Aurora B (Flag) and Ring1B (HA) (see
section 2.8.3). After confirming the efficiency of the co-transfection and the expression of the two proteins, I performed co-IP of HA (Ring1B) and assessed the presence of Flag-Aurora B among the proteins eluted (Fig. 3.8, top panel) and vice versa (Fig. 3.8, bottom panel). This approach confirmed the interaction between Aurora B and Ring1B, although it does not establish whether the interaction is direct or whether the DNA or other proteins mediate it.

![Figure 3.8: Co-immunoprecipitation of Aurora B and Ring1B in co-transfected 293T.](image)

Magnetic beads were pre-incubated with antibodies against IgG (negative control), HA (Ring1B) or Flag (Aurora B). The input material for each co-IP was 1 mg of whole cell extract from co-transfected 293T cells. Complexes were eluted by boiling the beads in 2x loading buffer and resolved on 8% acrylamide/bis-acrylamide gels. Membranes were incubated with antibodies against Flag-Aurora B (top panel) and HA-Ring1B (bottom panel). Asterisks denote heavy chains of the IgG.

### 3.6 Summary

In summary, I used centrifugal elutriation to purify G1 LPS-activated B cells from an actively proliferating population. Quantitative MS analysis of Aurora B-
containing complexes immunoprecipitated from G1 and unsynchronized cells demonstrated that Aurora B interacts with epigenetic regulators in interphase.
Chapter 4 - Aurora B and Ring1B bind to the regulatory elements of active genes in resting lymphocytes

4.1 Introduction

Co-IP and western blot confirmed that Aurora B interacts with Ring1B. Ring1B is an epigenetic regulator responsible for the monoubiquitination of histone H2A and Aurora B phosphorylates histone H3 to compact chromosomes in mitosis and to promote transcription in G0/G1 pituitary cells. Previous chromatin immunoprecipitation (ChIP) and ChIP-on-ChIP data generated in our lab by Dr. Marcela Sjoberg had shown that Aurora B, Ring1B and Cbx7 bind to promoters of active genes in resting B cells. PRC1 binding is independent of PRC2-mediated trimethylation of lysine 27 on histone H3, which decorates silent promoters in these cells. Moreover, Ring1B and the H2Aubq mark do not colocalise as the latter is detected at high levels only at silent genes.

On the basis of these findings, I firstly assessed the effects of different methods of B cell activation on the binding of Aurora B and Ring1B. Secondly, I expanded the analysis to resting CD4+ T lymphocytes. Finally, I used ChIP in conjunction with high-throughput DNA sequencing (ChIP-seq) to characterise the genome-wide localisation of Aurora B, PRC1 and PRC2 components.

4.2 Aurora B and Ring1B bind to the promoters of active genes in quiescent B cells

The spleen is the site of B cell maturation and the primary source of resting B cells (see 1.3.5). To obtain resting B cells, I followed a published methodology
routinely used in our group and described in detail in Section 2.1.1. Although the method relies solely on negative selection of CD43+ cells, analysis of the proliferation rate and B cell markers shows that more than 95% of the cells isolated with this method are mature B cells (B220+), which are also resting (CD69-) (Fig. 4.1).

**Figure 4.1: Characterization of CD43 negative cells isolated from the spleen of adult mice.**

After isolation from the spleen of adult mice, a proportion of the CD43- cells (typically 1-2x10⁶ cells) has been routinely used to assess purity (Resting B cells) and compared with activated cells from the very same preparation after 3-4 days (CD40- and LPS-activated B cells). Freshly isolated cells were left to recover and then stained with CD69 (activation marker) and B220 (mature B cells) (left panel) and propidium iodide (right panel) and analysed by FACS. CD40- and LPS-activated cells were stimulated as described in section 2.1.1 and analysed after 4 and 3 days in culture, respectively. The results obtained after one representative procedure are presented here.
Data from Dr. Marcela Sjoberg had shown that Aurora B and Ring1B are both bound to chromatin in resting CD43- splenocytes (henceforth referred to as, simply, resting B cells). These cells can be activated in culture with LPS to mimic a T-independent B cell activation (see section 1.3.7). The results shown in Fig. 4.2 demonstrate that Aurora B and Ring1B bind to the promoter regions of genes that are known to be active in LPS-activated cells (b-Actin, Fcrl1, Mapbpip, Gba, Cct3, Mtx1, Prcc, Pmf1) but not to genes that are silent (Nestin, InsrR, Bcan, Hapln2). In contrast, Suz12 exhibits opposite behaviour as it binds to the promoters of genes that are silent in activated B cells and not to promoters of active genes (Fig. 4.2).
Figure 4.2: Distribution of Aurora B, Ring1B and Suz12 on the promoters in quiescent B cells.

ChIP experiments were performed with antibodies recognizing Aurora B (red bars), Ring1B (blue bars), Suz12 (grey bars) and IgG (negative control, white bars). Chromatin binding was assessed using primer pairs covering the promoter region of the indicated genes. Genes were selected based on previous ChIP-on-ChIP results (Dr. Marcela Sjoberg). The genes that were analysed are located in a gene-dense region of mouse chromosome 3. Their transcriptional status (active or silent) had been previously assessed by qRT-PCR by Dr. Svetlana Nikic in LPS-activated B cells. The transcriptional status of the genes in quiescent cells was confirmed and is shown in Fig. 4.3. Binding is expressed as enrichment relative to input DNA using the same amount of DNA in each PCR reaction (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates) (For this picture, data obtained by Dr Marcela Sjoberg has been combined and averaged with data that I obtained).
4.3 Genes bound by Aurora B and Ring1B in quiescent B cells are transcribed rather than poised

It is known that some genes are transcribed selectively during quiescence but not in activated cells. To determine the transcriptional status of the Aurora B and Ring1B target genes shown in Figure 4.2, I compared their transcriptional status to that of quiescent and G1-elutriated, LPS-activated B cells by analysing total RNA from both stages. To prevent biases caused by genomic DNA, RNA was extensively treated with DNase I and four biological replicates were analysed (see section 2.6).

Since the transcriptional activity of quiescent cells is considerably reduced, it is impossible to choose a priori, a reference gene whose transcriptional levels are not affected by activation. When comparing transcriptional levels in resting and proliferating cells, a parameter that can be controlled and used as a reference is the number of cells. For this reason, a spike-in RNA was added to cell lysates prepared from equal numbers of cells and used as a reference to quantify the gene expression in each sample. Unlike a reference gene endogenously transcribed in the genome, the quantity of this reference will not be affected by the global transcriptional activity of the cell, because it has been exogenously added. By keeping the ratio of cells to spike-in constant and provided the spike-in RNA is added immediately after lysis of the cells, it is possible to quantify differentially expressed transcripts on a per-cell basis in cell types with intrinsically different characteristics such as resting versus proliferating.

The analysis of RNA isolated from quiescent and G1-elutriated, LPS-activated B cells provided two important pieces of information. Firstly, the results showed that genes that are bound by Aurora B and Ring1B produce detectable levels of transcripts in quiescent and activated cells and are therefore actively transcribed rather than being in a poised state (Fig. 4.3). At the same time, the
repressed genes that were analysed remained in a silent condition in both cell types. The second major observation was that all of the genes analysed (with the exception of Fcrl1 and Mtx1) showed substantial increases (ranging from 2- to 20-fold) in their absolute level of transcription in G1-LPS activated B cells.

Figure 4.3: Comparison of the transcriptional levels of active genes in resting (quiescent) and G1-elutriated LPS-activated B cells (G1 LPS-activated).

Resting B cells were isolated from the spleen and either immediately lysed or stimulated with LPS for 3 days and elutriated to obtain a pure G1-population. Total RNA was extracted from both cell types and used to produce cDNA (see section 2.6.2). Transcripts were quantitated by qRT-PCR and levels were calculated relative to a spike-in RNA (see section 2.6.1), which was added to the cell lysate prior to the RNA purification. The ratio of spike-in RNA to the starting number of cells was kept constant. This means that the transcript levels measured by qRT-PCR should be a measure of the absolute level of transcripts per cell, allowing direct comparison of the levels of each transcript in different cell types. Cell numbers were the average of 10 counts using a haemocytometer (see section 2.6.2). Error bars represent the standard deviation of the mean (n=2 biological replicates) for the qRT-PCR analysis.

4.4 Aurora B binding is lost upon B cell activation

If Aurora B binding is mainly a function of transcriptional activity, one might expect that during the activation-induced transcriptional burst, Aurora B binding will increase proportionally. To test this hypothesis, I measured the binding of
Aurora B to target promoters following activation in G1-elutriated cells. The presence of high levels of Aurora B on chromosome arms during G2/M made it necessary to carry out the analysis using cells in the G1-phase that had been purified by elutriation (see section 3.2).

Cells were activated in vitro by treatment with LPS or with a combination of anti-CD40 and anti-Igκ antibodies. The mitogen LPS triggers activation of the MAPK/ERK pathway, which has been shown to lead to binding of MSK1/2 to promoters, phosphorylation of serine 10 and 28 on histone H3 and consequent gene activation (see section 1.4.3). The anti-CD40 antibody, instead, mimics a T-dependent activation in culture and does not activate the MAPK/ERK pathway (see section 1.3.5), thus providing a test of whether any effects observed are specifically due to the action of mitogens. An additional consideration is the fact that MZ B cells are more sensitive to LPS-stimulation than follicular B cells, which are more responsive to anti-CD40 (see section 1.3.6). Therefore, by activating two different pathways that lead to B cell activation, it is possible to characterise the behaviour of Aurora B relative to activation per se in both types of quiescent cells, rather than the response to one particular stimulus in one subtype of resting B cells.

Following the addition of the stimulating agents, the cells were left in culture for three to four days (3 for LPS and 4 for anti-CD40 plus anti-Igκ) and then elutriated to obtain a purified G1-fraction (see section 2.2). The results of the analysis are shown in Figure 4.4 and reveal that both activation methods result in displacement of Aurora B from its target promoters as measured by its enrichment over input DNA. Indeed, with the possible exception of Fcrl1 in LPS-activated cells, Aurora B levels are reduced to background level (Fig. 4.4).
Figure 4.4: Binding of Aurora B to promoters is lost upon activation.

Binding of Aurora B kinase was measured in quiescent resting B cells (red bars), G1-elutriated LPS-activated B cells (orange bars) and G1-elutriated anti CD40-activated B cells (yellow bars). Resting B cells were isolated from the spleen and either immediately lysed or stimulated with LPS or anti-CD40 plus anti-Igκ for 3/4 days and elutriated to obtain a pure G1-population. Binding is expressed as enrichment relative to input DNA using equal amounts of DNA in the PCR reactions (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates).

4.5 Distribution of Ring1B in quiescent and activated B cells

Since Aurora B is removed from target promoters in these cells, I asked whether Ring1B follows Aurora B and is also removed from chromatin following activation. I performed ChIP experiments for Ring1B in G1-elutriated cells activated with either LPS or anti-CD40 plus anti-Igκ. Ring1B binding to active target genes is preserved following B cell activation, although on some genes (Fcrl1, Mapbpip and Pmf1) it is substantially reduced (Fig. 4.5). In addition to its continued presence on active genes, a low level of binding of Ring1B is detected on the promoters of the Nestin and Bcan genes following activation, as measured by levels of enrichment over input DNA. This hints at different roles for this
protein at different genes, possibly as part of different complexes (see section 1.6.5). In general, the results suggest that any effects that are observed on Ring1B binding are a consequence of activation per se, as opposed to a specific type of activation.

**Figure 4.5: Binding of Ring1B to promoters is maintained following activation.**

Binding of Ring1B was measured in quiescent (blue bars), G1-elutriated LPS-activated (cyan bars), G1-elutriated anti CD40-activated B cells (light blue bars). Resting B cells were isolated from the spleen and either immediately lysed or stimulated with LPS or anti-CD40 plus anti-Igκ for 3/4 days and elutriated to obtain a pure G1-population. Binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates).

4.6 Cbx7 and Bmi1 both colocalise with Ring1B in quiescent cells but exhibit divergent behaviours following activation

Ring1B associates with a wide spectrum of interactors to form different
PRC1 complexes. Preliminary data showed that the binding pattern of Cbx7 in resting B cells was identical to that of Ring1B (Dr. Marcela Sjoberg, data not shown). PCGF family members are core components of PRC1, which uniquely identify the different PRC1 complexes (see section 1.6.5). ChIP analysis showed that the PCGF family member Bmi1 binds to the same genes that bind Ring1B and Cbx7 in resting B cells. Since these proteins are involved in defining the functional properties of PRC1 complexes, I sought to characterise their behaviour following B cell activation and to compare it with the binding of Ring1B.

Following activation, Cbx7 remains bound to the active promoters that bind Ring1B and the two proteins display the same pattern at four out of the six active genes that were analysed (Fcrl1, Mapbpi, Gba, Cct3) (Fig. 4.6 top panel). However, the binding of Cbx7 is reduced on the b-Actin gene and unchanged on the Prcc gene, which is the opposite of what is observed for Ring1B. Silent genes show no increase in binding of Cbx7, which remains close to background.

In contrast, Bmi1 is completely removed from 5 out of 6 of the active genes analysed, with only Prcc, retaining a low level of binding (Fig. 4.6 bottom panel). This result was obtained in G1-elutriated cells and is therefore in agreement with the reported displacement of Bmi1 from chromatin during this phase of the cell cycle (Voncken et al., 1999). Moreover, these cells were activated using LPS. The MAPKAP kinase 3 (3pK), a downstream effector of the signalling cascade triggered by LPS, has been shown to remove Bmi1 from chromatin by phosphorylating the protein (see section 1.6.5).

Since the PRC1 complexes isolated to date all contain a PCGF family member, it is possible that a switch to another PCGF protein might occur upon activation. Further experiments will be required to determine whether this is the case and which (if any) PCGF protein substitutes for Bmi1 in G1-elutriated G1 activated B cells. Mel18 constitutes a good candidate for this role (see section 1.6.5). Binding of Mel18 was not detected on chromatin in quiescent cells (data
not shown). However, *in vitro* at least, this protein requires phosphorylation by an unknown kinase before it can confer substrate specificity to Ring1B. The question of whether a phosphorylation-dependent switch governs the association of Bmi1 and Mel18 with Ring1B/Cbx7 in this context will require further investigation.

**Figure 4.6:** Cbx7 binds to active promoters in quiescent and LPS-activated B cells, whereas Bmi-1 binds in quiescent cells but not in G1 activated cells. Binding of Cbx7 (top panel) and Bmi-1 (bottom panel) to promoters was measured in resting B cells (green bars) and G1-elutriated LPS-activated B cells (light green bars). Resting B cells were isolated from the spleen and either immediately lysed or stimulated with LPS for 3 days and elutriated to obtain a pure G1-population. Binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates) (The
top panel show the average generated by combining data obtained by Dr Marcela Sjoberg with data that I obtained).

4.7 H2A ubiquitination marks silent genes and does not co-localize with PRC1 components in quiescent and activated B cells

PCGF proteins allow Ring1B to monoubiquitinate histone H2AK119 (see section 1.6.5). However, despite the presence of Bmi1, H2AUbq was not detectable above background at the active Ring1B-bound genes but was instead present at silent genes, where Ring1B binding was not detected. ChIP experiments confirm that this pattern is conserved in activated cells, although a small increase in the presence of the mark is detected on active promoters (Fig. 4.7). The H2Aubq mark is also present in quiescent cells on active genes such as b-Actin and Fcrl1, albeit at much lower levels than on silent genes.

Following activation with LPS, H2Aubq increases above background also on active genes such as Mapbpip, Gba and Cct3. This reinforces the idea that Ring1B is part of a PRC1 complex with some ubiquitination activity in G1-elutriated, LPS-activated cells and again raises the possibility that Bmi1 is substituted by another PCGF protein following activation. A possible way of interpreting the acquisition of this repressive mark at the same time as the transcriptional burst that accompanies LPS-activation is that Ring1B is fine-tuning transcription.
Figure 4.7: H2A ubiquitination levels are higher on silent promoters than on active promoters in both quiescent and LPS activated cells.

ChIP experiments with antibodies against total histone H2A and against ubiquitinated lysine 119 on histone H2A were performed in quiescent and G1-elutriated LPS-activated B cells. Levels of ubiquitinated H2A were measured in quiescent resting B cells (dark green bars) and G1-elutriated LPS-activated B cells (light green bars). Resting B cells were isolated from the spleen and either immediately lysed or stimulated with LPS for 3 days and elutriated to obtain a G1-population. Levels of H2Aubq are expressed as enrichment relative to DNA immunoprecipitated by an anti H2A antibody that recognises total H2A using the same amount of DNA in the PCR. A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates).

4.8 The histone H2A deubiquitinase USP16 is present at Aurora B target genes and is displaced following B cell activation

The deubiquitinase USP16 removes the H2Aubq mark from chromosomes at the onset of mitosis thereby allowing Aurora B to bind and phosphorylate histone H3 (see section 1.5.2). Since H2Aubq is absent from Aurora B target genes...
genes in quiescent B cells, I asked whether USP16 is also present at these promoters. ChIP analysis of the distribution of USP16 shows that the deubiquitinase binds to promoters that are bound by Aurora B (b-Actin, Fcrl1, Mapbpip, Gba, Cct3) but not to active genes that are devoid of the kinase (Fdx1 and Atp5s) (Fig. 4.8, dark green bars). Binding of USP16 to active promoters is no longer observed in G1-elutriated, LPS-activated B cells (Fig 4.8 light green bars), consistent with its reported cytoplasmic localization in interphase (Cai et al., 1999). This observation raises the possibility that loss of the deubiquitinase allows Ring1B to ubiquitinate histone H2A at active promoters in G1 cells.

Figure 4.8: USP16 binds to promoters that bind Aurora B and Ring1B in quiescent B cells but not in G1 activated B cells.

The binding of USP16 to promoters was measured in quiescent (green bars) and G1-elutriated LPS-activated (light green bars) B cells. Resting B cells were isolated from the spleen and either immediately lysed or stimulated with LPS for 3 days and elutriated to obtain a pure G1-population. Binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates).
4.9 Aurora B and Ring1B co-occupy the same promoters

An important question is whether Aurora B and Ring1B are colocalised at the same active promoters within the same cell. Moreover, it is also possible that they occupy different alleles within the same cell. To test whether they physically co-occupy the same target promoter, I performed Re-ChIP (also known as sequential ChIP) for Aurora B and Ring1B in resting B cells isolated from mouse spleen.

Chromatin from resting B cells was first immunoprecipitated with anti-Aurora B antibody and the precipitated chromatin was then used as the input for ChIP with anti-Ring1B antibody (Fig. 4.9 top panel). The results confirmed that Aurora B and Ring1B co-occupy the same promoters in the same cell. To validate this result, I performed Re-ChIP for Aurora B using chromatin immunoprecipitated with anti-Ring1B as the input (Fig. 4.9 bottom).
Figure 4.9: Sequential ChIP demonstrates co-occupancy of Aurora B and Ring1B at active promoters in quiescent B cells.

Chromatin was immunoprecipitated with an anti-Aurora B antibody (red bars) and the precipitated chromatin was then re-immunoprecipitated with an anti-Ring1B antibody (blue bars) (top panel) and vice versa (bottom panel). These experiments show that Aurora B and Ring1B co-occupy the same DNA fragments. For the first ChIP (top and bottom left), binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). For the second ChIP (top and bottom right), the input DNA is obtained from the first ChIP. A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological replicates).
4.10 Aurora B and Ring1B bind to active genes in quiescent resting T cells

To test whether the co-localization of Aurora B and Ring1B at the promoters of active genes is a common trait of resting lymphocytes or, alternatively, is restricted to B cells, I looked at their distribution in quiescent T cells (CD4+/CD25-), purified from subcutaneous and mesenteric lymph nodes (see section 2.1.2). I chose to perform a double purification with a positive enrichment (CD4+) to avoid the risk of obtaining a cell population that was too heterogeneous, particularly due to the presence of follicular B cells in lymph nodes. The assessment of the purity of the population shows that 93.4% of these cells are positive for CD4 and negative for CD25 and only 0.5% result positive for both marks (Fig. 4.10, left panel). The analysis of the cell cycle distribution of the cells shows that 99.6% of the purified cells are in G0/G1 (Fig. 4.10, right panel). The isolated T cells present therefore a satisfactory level of purity similar to the CD43 negative resting cells used so far.

![Figure 4.10: Analysis of the composition of the cell population after CD25 negative selection and CD4 positive selection.](image)

CD4+/CD25- cells were isolated from the subcutaneous and mesenteric lymph nodes and stained with antibodies against CD4 and CD25 and analysed by FACS (left panel). The cells in the top left panel (CD4+/CD25-) represent resting T cells and constitute 93.4% of the total population. The cell cycle distribution of the isolated cell was also assessed by PI staining and FACS (right panel). This analysis shows that 99.6% of the cells are in G0/G1.
To assess the transcriptional status of these genes, I firstly measured the RNA levels of a group of genes largely overlapping with those that I had previously analysed in resting B cells (Fig. 4.11). Similar to the analysis of resting B cells, total RNA was analysed by qRT-PCR and levels of specific transcripts were compared with an exogenously added spike-in RNA control. This made it possible to identify active and silent promoters in resting CD4+ cells. Secondly, I looked for the presence of Aurora B and Ring1B on these promoters using ChIP. The results are shown in Fig. 4.12 and demonstrate that Aurora B and Ring1B bind to all active promoters tested and are absent from silent genes.

**Figure 4.11: Determination of the transcriptional levels of active genes in resting CD4+ cells.**

Total RNA was extracted from CD4+ T cells isolated from subcutaneous and mesenteric lymph nodes and was used to produce cDNA (see section 2.6.2). Gene expression was calculated as a percentage relative to a spike-in RNA (see section 2.6.1), which was added to the cell lysate prior to the RNA purification. The ratio of spike-in RNA to the starting number of cells was kept constant. Cell number was the average of 10 counts using a haemocytometer (see section 2.6.2). Error bars represent the standard deviation of the mean (n=2 biological replicates).
Figure 4.12: Distribution of Aurora B and Ring1B on the promoters of active and silent genes in quiescent resting T cells.

ChIP experiments were performed with antibodies recognizing Aurora B (red bars), Ring1B (blue bars) and IgG (negative control, white bars). Chromatin binding was assessed using primer pairs covering the promoter region of the indicated genes. The transcriptional status of the genes encompassed in that region (active or silent) had been previously assessed by qRT-PCR (Fig. 4.11). Binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological replicates).

4.11 Genome-wide analysis of the distribution of Aurora B and Ring1B in quiescent B cells

4.11.1 Quantification of absolute expression levels in resting and activated B cells

ChIP-seq has now replaced ChIP-on-ChIP in the study of the genomic
distribution of chromatin-bound factors as it yields a genome-wide view and also provides quantitative results. Therefore, the analysis of the distribution of Aurora B, Ring1B and other important factors in quiescent B cells was extended genome-wide by performing ChIP-seq. In order to be able to relate the presence of a factor with the transcriptional status mRNA sequencing (mRNA-seq) was first carried out on mRNA extracted from freshly purified CD43- mouse resting B cells.

Four independent biological samples were paired-end sequenced using a next-generation high throughput sequencer. Figure 4.13 shows that the four samples exhibit a good degree of correlation as measured by Spearman correlation (see section 2.7). A total of 37,158 genes were analysed based on the Ensembl annotation. Of these, for 19,749 of them, at least one copy of the mRNA was found, while for the remaining 17,409 genes no mRNA was detected in any of the samples. A value of 0.5 FPKM (fragments per kilobase of exon per million fragments mapped) in all four samples was chosen as the cut-off value, above which a gene was considered active. A total of 12,251 genes had FPKM values above this threshold with the most highly expressed gene (the H-2 class II histocompatibility antigen gamma gene CD74) exhibiting a value of 8,087 FPKM.
Figure 4.13: Correlation plot of the mRNA-seq samples.

Four biological samples were used to prepare a total of four mRNA sequencing libraries as described in section 2.7.1. The libraries were paired-end sequenced on a next-generation high throughput sequencer and Gopuraja Dharmalingam provided post-sequencing bioinformatic analysis of all the output files. FPKM values for each sample was estimated using Cufflinks version 1.3.0 and correlation between replicates assessed using Spearman correlation. This picture was generated by Gopuraja Dharmalingam.

4.11.2 Aurora B and Ring1B bind to the promoters and enhancers of active genes

Having obtained information regarding the transcriptional status of resting B cells, I performed ChIP followed by DNA sequencing (ChIP-seq) for Aurora B and Ring1B to study their distribution across the genome in quiescent B cells. Both proteins were subjected to single-strand sequencing on a next-generation high throughput sequencer (see section 2.5). The sequencing of the Aurora B library generated 86,082,547 reads with a Phred quality score of 94%. The Ring1B sequencing library generated 89,377,580 reads with a quality score of 94.95%. The results of the ChIP-seq are shown in Fig. 4.13 and Appendix A, (red and
blue tracks). Aurora B and Ring1B display a high degree of overlap on a genome-wide scale.

DNase hypersensitivity can be used to identify candidate regulatory elements (see section 1.4.1). Comparison of Aurora B and Ring1B tracks with the publicly available DNase I hypersensitivity dataset generated in CD43- B cells (Fig. 4.13 and Appendix A, top track) confirms that Aurora B and Ring1B bind almost exclusively to regulatory elements, such as promoters and enhancers. For example, both proteins bind strongly to the known enhancer of the CD19 gene, whose tight transcriptional control is fundamental during B cell development (Walter et al., 2008) (Fig. 4.13).
Figure 4.14: Genome-wide analysis in quiescent B cells.

Snapshots representative of ChIP-seq analysis of the distribution of Aurora B (red), Ring1B (blue), H2A.Z (purple), Ezh2 (dark grey), H3K27me3 (black), Cbx7 (green) and USP16 (orange). Data view scaling (y-axis) for each single library was selected based on the signal to noise ratio of each individual track and kept constant (25 for Aurora B and USP16; 50 for Ezh2, H3K27me3 and Cbx7; 100 for Ring1B and H2A.Z). RPKM values representing the expression of each gene are reported next to the gene name and were defined by RNA-seq analysis of mRNA in freshly isolated resting B cells (table 2). DNase I sensitivity data are shown through the publicly available dataset generated in CD43-, CD11b- B cells from 8-week old male mice (M. Bender’s laboratory). See also Appendix A for more examples. These pictures were generated in collaboration with Gopuraja Dharmalingam (see section 2.5.2 for details).

In collaboration with Gopuraja Dharmalingam, density maps were generated for Aurora B and Ring1B to assess the relationship between their genome-wide distribution and the amount of mRNA of genes in resting B cells (Fig. 4.15). The generation of these maps is described in detail in section 2.5.3. Briefly, active
genes were ranked in descending order based on the FPKM value obtained from the mRNA-seq analysis. In order to include silent genes (RPKM=0), 2,000 genes (PRC2-repressed) were selected based on the decreasing density of tags for H3K27me3 around the TSS ±3 kb region (see below) and another 2,000 genes (silent) were randomly selected among the silent genes with low H3K27me3 tag density.

The distributions of Aurora B and Ring1B were analysed in a window of ±3kb around the annotated TSS. By summarising the genome-wide distribution of Aurora B and Ring1B in these density maps it is possible to appreciate that the binding of both proteins clusters around the TSS of active genes and are absent from both PRC2-repressed and silent groups of genes. Moreover, the signal for both proteins is particularly strong at highly transcribed genes and lower at genes with FPKM values close to 0.5.
Figure 4.15: Heat maps showing the distribution of Aurora B (red), Ring1B (blue), H2A.Z (purple), Ezh2 (black), H3K27me3 (black), Cbx7 (green) and USP16 (orange) within 3kb of the transcriptional start site (TSS) in quiescent B cells.

Active genes were ranked based on their transcriptional level as assessed by mRNA-seq analysis (see section 2.7). A total of 2,000 genes (PRC2-repressed) were selected based on the highest density of tags for H3K27me3 around the TSS and ranked in decreasing order (top to bottom). Another 2,000 genes (silent) were randomly selected among the silent genes with low H3K27me3 tag density. This image was generated by Gopuraja Dharmalingam.

4.12 Genome-wide distribution of other key factors in quiescent B cells

4.12.1 Genome-wide analysis of the distribution of the histone variant H2A.Z

The histone variant H2A.Z has been shown to mark a wide range of promoters and enhancers in resting human cells (Barski et al., 2007). To further
analyse the extent to which Aurora B and Ring1B are localised to these types of elements in quiescent resting B cells from mouse, I analysed the distribution of the histone variant H2A.Z in these cells.

The pattern of H2A.Z deposition is shown in Fig. 4.14 (purple track). The distribution of H2A.Z was found to correspond closely to the locations of DNA HS regions. Moreover, the localization of H2A.Z across the genome is very similar to the binding pattern of Aurora B and Ring1B, with an abundant presence around the TSS of highly expressed Aurora B target genes (Fig. 4.15, purple heat map). Similar to Aurora B and Ring1B, the signal for H2A.Z disappears from PRC2-repressed and silent genes. This could simply stem from the absence of the histone variant around the TSS of these genes and/or from the monoubiquitination of the C-terminal tail that has been reported to impair the antibody from binding to the epitope, such as monoubiquitination (Sarcinella et al., 2007). Indeed, the monoubiquitination of H2A.Z has been shown to be a mechanism to silence some genes in mammals (Draker et al., 2011).

4.12.2 Genome-wide analysis of the distribution of Ezh2 and the histone mark H3K27me3

Since our previous ChIP analyses in resting B cells had suggested a mutually exclusive binding of PRC1 and PRC2 to active and silent promoters respectively, I expanded the analysis of the distribution of Ezh2 and H3K27me3 relative to Ring1B using ChIP-seq analysis. mRNA-seq data showed that, despite resting lymphocytes being non-dividing cells, the Ezh2 gene is transcribed to comparable levels with Ezh1. Nevertheless, the ChIP-seq data shows that Ezh2 binding is largely absent from the active genes that bind Ring1B and is mainly restricted to repressed genes. H3K27me3 marks broad domains across the whole body of silent genes such as the silent genes in the HoxA locus (Fig. 4.14, black track). This binding profile is completely different from the sharp peaks of Aurora B, Ring1B and H2A.Z binding that are observed around the TSS. Ezh2
and the H3K27me3 mark are present only at silent genes PRC2-repressed genes, such as the silent genes in the HoxA locus (Fig. 4.14). In agreement with previous reports in ES cells (Brookes et al., 2012) and Drosophila (Schwartz et al., 2010), some silent genes are regulated by other mechanisms and devoid of Ezh2 and H3K27me3, as well as the other analysed proteins (see Robo1 in Appendix A).

4.12.3 Genome-wide analysis of the distribution of Cbx7

Our ChIP data had shown that Cbx7 is present with Ring1B at active promoters in resting and activated cells. I decided to perform ChIP-seq in order to test the degree of association genome-wide between Ring1B and Cbx7 at active promoters in resting B cells. Cbx7 exhibits a striking overlap with Aurora B and Ring1B at the same targets genes and no overlap with H3K27me3 (Fig. 4.15, green heat map). This is particularly surprising as, among the different Cbx proteins that can be present in PRC1 complexes, Cbx7 shows the highest degree of overlap with H3K27me3 (Vincenz and Kerppola, 2008). Moreover, unlike other Cbx proteins, Cbx7 has been associated almost exclusively with the repression of developmental genes in ES cells (Morey et al., 2012).

Nevertheless, the recruitment of Cbx7 to promoters has also been shown to depend on ncRNAs and to be reduced by the inhibition of transcription obtained following treatment with α-amanitin (Yap et al., 2010). The observation that Cbx7 binds to the promoters of active genes together with Ring1B and Aurora B might therefore reflect an alternative recruitment mechanism of this protein where RNAs or other components play a major role and the H3K27me3 mark is not read as a recruiting signal for the protein. Finally, the correlation in the binding patterns of Ring1B and Cbx7 indicates that Ring1B binds to active promoters in these cells as part of a PRC1 complex. Other Ring1B-containing complexes (see section 1.6.8), such as E2F6.com-1, do not feature Cbx7 among their members.
4.12.4 Genome-wide analysis of the distribution of USP16

Finally, I examined the genome-wide distribution of the USP16 deubiquitinase (Fig. 4.14, orange track). The results show that USP16 binds together with Aurora B, Ring1B and Cbx7 exclusively at active genes. These regions also include the histone variant H2A.Z, a target for Ring1B-mediated monoubiquitination. USP16 is the most effective deubiquitinase for both H2A and H2A.Z in vitro (Draker et al., 2011) and the antibody used for the ChIP of H2A.Z does not recognize the epitope in its monoubiquitinated form (Sarcinella et al., 2007). The strong signal obtained for H2A.Z using this antibody provides indirect evidence that these regions are devoid not only of H2Aubq but also of H2A.Zubq, although the lack of a commercially available antibody for H2A.Zubq prevented me from directly testing this hypothesis.

Since Aurora B requires USP16 to remove the H2Aubq mark in order to bind to mitotic chromosomes, the colocalization of these proteins at active promoters (Fig. 4.15, orange heat map) might explain the binding of Aurora B in concomitance with Ring1B, as these regions have probably been deubiquitinated by USP16. It is possible therefore that USP16 recruitment constitutes a prerequisite for the recruitment of Aurora B to its target genes.

4.13 Summary

The results presented in this Chapter show that Aurora B binds to a high proportion of active promoters and to at least some enhancers in resting B cells. The highest levels of Aurora B binding are observed at highly transcribed genes and binding is absent from some genes that are transcribed at a low level. Aurora B binding is specific for quiescent cells as its binding is lost upon activation. Ring1B is present at active promoters in quiescent cells in a PRC1 complex that includes Cbx7 and Bmi1. Both Ring1B and Cbx7 are retained on active promoters following B cell activation, whereas Bmi1 binding is lost. In
resting B cells, PRC1 is recruited in a non-canonical manner as the PRC2 component Ezh2 and PRC2-mediated H3K27me3 are detected on silent genes only. The H2Aubq mark decorates silent promoters both in quiescent and LPS-activated cells. In quiescent cells, the histone deubiquitinase USP16 is present on Aurora B and Ring1B target genes and USP16 association with promoters is lost upon activation with LPS and cell cycle re-entry. Genome-wide analysis showed that regions that bind Aurora B and Ring1B are also characterised by the presence of the histone variant H2A.Z (a marker of promoters and enhancers in mammals), Cbx7 and USP16. The PRC2 component Ezh2 and the repressive histone mark H3K27me3 show mutual exclusivity with Aurora B and Ring1B at the majority of silent and active genes. However, at some low-expressing genes that lack Aurora B, there is a degree of overlap between Ring1B and PRC2 and it is possible that PRC1 and PRC2 cooperate at these genes to fine-tune gene expression.
Chapter 5 - Aurora B and Ring1B are involved in regulating transcription in quiescent lymphocytes

5.1 Introduction

The results described in Chapter 4 show that Aurora B and Ring1B co-occupy the regulatory elements of active genes in quiescent B and T cells. Aurora B binding is lost upon activation and cell cycle re-entry. In resting B cells, Ring1B is part of a PRC1 complex that contains Cbx7 and Bmi1 and is recruited to active target genes in the absence of PRC2 members and H3K27me3, which are detected (together with the H2Aubq mark) on silent genes. Since both Aurora B and Ring1B bind to regulatory elements and are known to regulate transcription, I studied their effect on gene expression. For Aurora B, I took advantage of a specific inhibitor of its kinase activity (AZD1152) and of a conditional knockout (cKO) mouse model.

5.2 Use of conditional knockout models to study of the functions of Aurora B and Ring1B in resting lymphocytes

5.2.1 Aurora B cKO system

To determine the role of Aurora B in gene expression in resting B cells, I investigated the effect of Aurora B ablation in quiescent lymphocytes using a knockout model. Germline knockout of Aurora B is embryonically lethal due to problems with chromosomal segregation (Fernández-Miranda et al., 2011). Therefore, I used an Aurora B cKO mouse model in which a floxed Aurora B allele (Aurkbfl) has been crossed with a tamoxifen-inducible cre-ERT2 gene under
the control of the constitutive Rosa26 promoter (see section 2.1.3). Resting B cells were isolated from mice that were homozygous for both the Aurkb^fl and cre-ER^{T2} alleles were treated with tamoxifen for 48 hours and the level of Aurora B was determined by western blotting. The results show a dramatic reduction in the level of Aurora B protein, indicating that the Aurora B gene (Aurkb) has undergone recombination, giving an efficient knockout of the Aurora B protein (Fig. 5.1).

**Figure 5.1: Analysis of Aurora B cKO resting B cells.**

CD43- B cells were extracted from the spleen of 6-8 weeks old WT (CreER^{T2} +/+ ) and AurB KO (CreER^{T2}/Aurkb flx +/+ ) mice. To induce the conditional knockout of the Aurora B gene, resting B cells were incubated for 48 hours in the presence of 250nM 4-OHT. Under these conditions, the level of Aurora B was drastically reduced (left panel) and resting B cells were not spontaneously activated (right panel). Equal amounts of whole protein extract from WT and AurkB KO resting B cells were resolved on a 5-20% acrylamide/bis-acrylamide gel and probed with antibodies against Aurora B and histone H3 (loading control). AurkB KO cells were stained for B220 (mature B cells marker) and CD69 (lymphocytes activation marker) by FACS. Numbers of stained cells are shown as mean ± standard deviation from the mean (n=2 biological duplicates). One representative experiment is shown.

**5.2.2 Ring1B cKO system**

To study the effect of the ablation of Ring1B in resting lymphocytes I employed the same strategy used to study the function of Aurora B: a conditional,
tamoxifen-dependent knockout model. The knockout mice were kindly provided by Dr. Miguel Vidal, CIB, Madrid. Since the knockout of the Rnf2 gene (which encodes for the protein Ring1B) is embryonically lethal (Voncken et al., 2003), a conditional knockout mouse model made it possible to remove Ring1B from purified resting B cells. A near complete knockout of Ring1B was achieved after 48 hours of 4-OHT treatment and staining for CD69 showed that the Rnf2 knockout does not trigger B cell activation (Fig. 5.2).

Figure 5.2: Analysis of Ring1B cKO resting B cells.
CD43- resting B cells were isolated from spleens of 6-8 weeks old WT (CreER\textsuperscript{T2} +/+ ) and Ring1B KO (CreER\textsuperscript{T2}/Rnf2 flx +/+ ) mice. To induce the nuclear relocalization of CreER\textsuperscript{T2} and homologous recombination, B cells were incubated for 48 hours in the presence of 250nM 4-OHT. Under these conditions, the presence of Ring1B was almost completely abrogated (left panel) and resting B cells were not spontaneously activated (right panel). Equal amounts of whole protein extract from WT and Ring1B KO B cells were resolved on a 5-20% acrylamide/bis-acrylamide gel and probed with antibodies against Ring1B and histone H3 (loading control). Ring1B KO cells were stained for B220 (mature B cells marker) and CD69 (lymphocytes activation marker) by FACS. Error represents the standard deviation from the mean (n=2 biological replicates). One representative experiment shown.

5.2.3 Validation of the Aurora B and Ring1B ChIP-seq libraries
The cKO systems were used to validate the ChIP-seq tracks presented in chapter 4. ChIP experiments were carried out on Aurora B and Ring1B cKO resting B cells and ChIP-seq libraries were prepared from the
immunoprecipitated DNA and subjected to high-throughput sequencing. The results are shown in figure 5.3 and clearly demonstrate that the respective knockouts result in strong reductions in binding of Aurora B and Ring1B to promoters and neighbouring sequences compared with the binding observed in wild-type resting B cells.
Figure 5.3: Genome-wide validation of the signal specificity of the Aurora B and Ring1B binding analysed by ChIP.

Aurora B and Ring1B were immunoprecipitated in their respective cKO resting B cells and the resulting ChIP-seq are lined up against the tracks obtained for freshly isolated WT resting B cells. Representative examples were selected for the data sets. To allow direct comparison of the intensities, the same y-axis scales were used for each WT and knockout pair. RPKM values representing the expression of each gene are reported next to the gene name and were defined.
by RNA-seq analysis of mRNA in freshly isolated CD43- B cells. TSS are derived from the UCSC dataset. Images in this figure were generated in collaboration with Gopuraja Dharmalingam (see section 2.5.2).

5.3 Aurora B promotes transcription of target genes in quiescent lymphocytes

The presence of Aurora B on regulatory elements prompted the question of whether this kinase has an activating or a silencing effect on transcription. Aurora B has been shown to promote transcription of thyroid hormone responsive genes by phosphorylating serine 10 on histone H3 following hormonal stimulation (see section 1.5.6). However, in resting B cells Aurora B is lost from promoters following B cell activation (Fig. 4.2), when transcriptional levels increase (Fig. 4.3). In addition, histone phosphorylation by Aurora B triggers chromosomal condensation at the onset of mitosis, although in this case Aurora B is distributed all over the chromosome arms and not localised at promoters (see section 1.5.2). Thus, it was equally possible that Aurora B might have an activating or a silencing effect on transcription in resting B cells.

The resting B cells used as the control for the experiment were isolated from wild type (WT) mice homozygous for CreER\textsuperscript{T2} but not containing any floxed allele. Use of the CreER\textsuperscript{T2} cells means that the experiment is controlled for possible effects caused by Cre in response to 4-OHT. Freshly isolated lymphocytes from both WT and AurkB KO mice were exposed to the same dose (250nM) of 4-OHT for the same amount of time (48 hours). In both WT and KO cells, 4-OHT induces nuclear localization of CreER\textsuperscript{T2} but only in the latter cells this promotes homologous recombination and knockout of the expression of the Aurkb gene. After 48 hours resting cells do not spontaneously activate, as measured by CD69 expression on their surface (Fig. 5.1).

As described in section 4.3, total RNA was analysed using primers pairs located within the same exon, in order to assess the levels of both primary and
processed transcript. Instead of selecting a gene within the cell, I used a spike-in RNA as the reference in each sample, since it was impossible to predict a priori which genes will be unaffected (directly or indirectly) by the inhibition. cDNA was prepared from total RNA extracted from equal numbers of WT and KO resting B cells and spike-in RNA was added in a standard proportion to the number of cells.

The results of the analysis are shown in Fig. 5.4 and demonstrate that Aurora B targets (b-Actin, Fcrl1, Gba, Cct3, Mtx1, Prcc, Pmf1) are reduced by approximately 50% in the KO cells. This transcriptional phenotype appears to be specific for Aurora B target genes, as it is not observed on Fdx1, Atp5s and Rnf2 (which encodes the Ring1B protein). qPCR-ChIP and ChIP-seq analysis showed that these genes lack strong binding of Aurora B to their promoters.

![Figure 5.4: Knockout of Aurora B in resting B cells results in a reduction of transcription from Aurora B-bound genes.](image)

Total RNA was extracted from WT (CreER\textsuperscript{T2}+/+) (blue bars) and AurkB KO (CreER\textsuperscript{T2}/Aurkb flx +/+ ) (red bars) resting B cells treated with 250nM 4-OHT for 48 hours. Gene expression was calculated relative to a spike-in RNA (see section 2.6.1), added to the cell lysate prior to the RNA purification. Cell number was the average of 10 counts using a haemocytometer. Differential expression (DE) was calculated relative to transcript levels in WT cells (DE=1). Error bars
represent the standard deviation of the mean (n=4 biological replicates).

To study the role of the kinase activity of Aurora B in this transcriptional phenotype, I treated freshly purified resting B cells with a specific Aurora B inhibitor, AZD1152, frequently used to study the partners and activity of Aurora B (Kettenbach et al., 2011). qRT-PCR analysis of the cDNA extracted from resting B cells treated for 24 hours with 200nM AZD1152 shows a reduction of the transcripts of Aurora B target genes (Fig. 5.5). The treatment results in a more dramatic reduction in the levels of transcript than the reduction observed in the cKO model. Treatment with AZD1152 also reduces transcription of non-Aurora B target genes such as *Fdx1* and *Atp5s*, albeit to a lesser extent. This could be due to more general effects of Aurora B inhibition on the transcriptional or RNA processing machinery or it could be caused by off-target effects of the drug, which cannot be completely excluded, despite the relatively high specificity of AZD1152 for Aurora B. In summary, these results show that Aurora B promotes the transcription of its target genes in resting B cells and demonstrate that its kinase activity is required for this role.
Figure 5.5: Inhibition of the kinase activity of Aurora B in resting B cells results in a dramatic reduction in the level of transcription of Aurora-bound genes.

Total RNA was extracted from resting B cells treated with 200nM AZD1152 (an inhibitor of the kinase activity of Aurora B, red bars) or DMSO (vehicle, blue bars) for 24 hours. Gene expression was calculated relative to a spike-in RNA (see section 2.6.1), added to the cell lysate prior to RNA purification. Cell number was the average of 10 counts using a haemocytometer. Differential expression was calculated relative to transcript levels in WT cells (DE=1). Error bars represent the standard deviation of the mean (n=4 biological replicates).

5.4 Ring1B cKO results in reduced transcription of target genes in quiescent B cells

Genome-wide data had shown that Ring1B is bound to a high proportion of the active promoters in resting B cells (Fig. 4.11). These promoters are however also devoid of detectable levels of H2Aubq, the canonical mark associated with PRC1 activity as a repressor. Nevertheless, a Ring1B point-mutant that cannot ubiquitinate histone H2A has been reported to be sufficient for transcriptional repression of Hox genes (Eskeland et al., 2010). I asked whether Ring1B is counterbalancing the stimulatory effect of Aurora B providing a mechanism to
keep the overall low transcriptional levels that characterise quiescent cells.

Total RNA was extracted from wild type (homozygous for Cre-ER\textsuperscript{T2}) and Ring1B knockout (homozygous for Cre-ER\textsuperscript{T2} and floxed\textsuperscript{Rnf2} allele) quiescent B cells and differential expression of genes was calculated by means of a spike-in reference RNA. Surprisingly, Ring1B knockout resulted in a substantial reduction in expression of the Aurora B target genes analysed. A small reduction in \textit{Fdx1} was observed but \textit{Atp5s} was not affected (Fig. 5.6). It should be noted that \textit{Fdx1} and \textit{Atp5s} are both transcribed at a low level and it is therefore necessary to be cautious about inferring a biological meaning to changes in their expression.

![Graph showing differential expression of genes in WT (blue) and Ring1B KO (red) B cells](image)

**Figure 5.6: Knockout of Ring1B in resting B cells leads to a substantial reduction in transcription of Aurora B-bound genes.**

Total RNA was extracted from resting B cells from WT (CreER\textsuperscript{T2} +/+) mice (blue bars) and Ring1B KO mice (CreER\textsuperscript{T2}/\textit{Rnf2} flx +/+) (red bars) treated with 250nM 4-OHT for 48 hours. Gene expression was calculated relative to a spike-in RNA (see section 2.6.1), added to the cell lysate prior to the RNA purification. Cell numbers were the average of 10 counts using a haemocytometer. Differential expression was calculated relative to WT (DE=1). Error bars represent the standard deviation of the mean (n=3 biological replicates).
The transcriptional phenotype following Ring1B cKO is stronger in terms of reduction, with genes such as \(b\text{-}\text{Actin}, \text{Gba}, \text{Cct3} \) and \(Pmf1 \) becoming almost silent. The promoter of the Aurora B gene itself (\text{Aurkb}) appears is bound by Aurora B and Ring1B and is downregulated following Ring1B cKO, which raises the possibility that the observed transcriptional reductions might stem from a combined effect of reducing Ring1B and Aurora B protein levels. The \text{Rnf2} \) gene, which encodes Ring1B, does not seem to be regulated by Aurora B and levels of expression of the gene are not reduced in Aurora B cKO cells (Fig. 5.4). This might explain why the effect of Aurora B cKO on transcription was less pronounced than that of the Ring1B cKO.

5.5 Aurora B and Ring1B cKO resting T cells display reduced transcription of target genes

As Aurora B and Ring1B bind to the promoter region of active genes in quiescent T cells as well as in B cells, I assessed the impact of knocking out each protein in resting CD4+ T cells. WT (CreER\(^{T2}++\)), Aurora B cKO (CreER\(^{T2}/\text{Aurkb}\) flx +/-) and Ring1B cKO (CreER\(^{T2}/\text{Rnf2}\) flx +/-) cells were incubated for 72 hours in 4-OHT and knockout was assessed by measuring the RNA levels of \text{Aurkb} \) and \text{Rnf2}. RNA was extracted from WT and both KO cells and differential expression of a set of active genes that bind Aurora B and Ring1B was quantified by qRT-PCR using the spike-in RNA as an internal standard. The knockout of both genes was confirmed by qRT-PCR, which showed that a 72-hour incubation with 4-OHT is necessary to achieve a satisfactory knockout of both proteins (Fig. 5.7).
Figure 5.7: The knockouts of Aurora and Ring1B in naïve T cells result in a reduction of total RNA produced from Aurora B-bound genes.

Total RNA was extracted from WT (CreER\textsuperscript{T2} +/+ (black bars), AurB KO (CreER\textsuperscript{T2}/Aurkb flx +/+) (red bars) and Ring1B KO (CreER\textsuperscript{T2}/Rnf2 flx +/+) (blue bars) mice treated with 500nM 4-OHT for 72 hours. Gene expression was calculated as relative to a spike-in RNA (see section 2.6.1), added to the cell lysate prior to the RNA purification. Cell number was the average of 10 counts using a haemocytometer. Differential expression is expressed as relative to the WT (DE=1). Error bars represent the standard deviation of the mean (n=2 biological replicates).

The results obtained from analysing the knockout cells are shown in Fig. 5.7. The Aurora B cKO resulted in a 30-50% reduction in the levels of total transcripts from a panel of active target genes (Fig. 5.7 red bars). Of the active genes tested, only Klf4 and FoxO4 were upregulated following the knockout of the kinase. It is notable that both of these are transcribed at relatively low levels in naïve CD4+ cells. Ring1B cKO resulted in a more pronounced reduction (50-80%) in expression of active genes, particularly at genes such as Gba (Fig. 5.6 blue bars). Moreover, both Klf4 and FoxO4 were downregulated. It is possible that, as in resting B cells, Aurora B and Ring1B differentially regulate a subset of active but poorly transcribed genes. Interestingly, both proteins have a positive effect on transcription of the KLF2 gene a master regulator of T cell quiescence and survival (see section 1.2.3).
5.6 Aurora B and Ring1B cKO's affect different steps in the RNA Pol II cycle at the promoter of active target genes

The finding that Aurora B and Ring1B bind to promoters and enhancers and that knockout of either protein leads to a reduction in transcription of their target genes, raises the possibility that they regulate binding of RNA Pol II to regulatory elements (see sections 1.4.2 and 1.4.3). To test this hypothesis, I quantified and compared the binding of the unphosphorylated and serine 5-phosphorylated RNA Pol II by ChIP in WT, Aurora B KO and Ring1B KO resting B cells. The unphosphorylated RNA Pol II binds to promoters as part of the PIC (see section 1.4.2). Phosphorylation of serine 5 of the RNA Pol II CTD allows promoter clearance and marks transcriptional initiation (see section 1.4.2). S5p Pol II is also often found at pausing sites and is progressively lost during elongation (see section 1.4.2).

5.6.1 Aurora B regulates the levels of S5p Pol II at target genes

I looked first at the levels of unphosphorylated (8WG16) and phospho-serine 5 RNA Pol II (S5p) at the promoters of active and silent genes following the knockout of Aurora B. The results are shown in Figure 5.8 and demonstrate that both forms of the polymerase are present at active promoters and completely absent from silent promoters in WT cells (Fig. 5.8, blue bars). In general, both types of RNA Pol II seemed to bind with the same affinity to each gene and the enrichments of both forms were particularly high at the promoters of two highly transcribed genes (b-Actin and Fcrl1). Interestingly, S5p Pol II was not detected at the promoter of any of the two silent genes. This indicates that these promoters are not in the poised state observed in ES cells, possibly due to a particularly compacted chromatin structure that blocked the recruitment of the PIC.
Figure 5.8: Aurora B cKO resting B cells exhibit reduced levels of S5p Pol II at promoters but the unphosphorylated RNA Pol II is less affected.

The binding of S5p Pol II and unphosphorylated RNA Pol II (8WG16) was measured in resting B cells isolated from mice that were homozygous Cre-ER<sup>T2</sup> (WT, blue bars) or homozygous Cre-ER<sup>T2</sup>/Aurkb floxed allele (AurkB KO, red bars). Cells were treated with 250nM 4-OHT for 48 hours. Binding is expressed as enrichment relative to input DNA using equal amounts of DNA in the PCR reactions (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological replicates for both experiments).

Following the knockout of Aurora B, however, S5p Pol II was strongly reduced and in some instances (*Mapbpip, Gba, Cct3*) the levels of enrichment are down to background levels (Fig. 5.8, top panel). The effect of the knockout on the levels of unphosphorylated RNA Pol II is milder and the signal is not reduced to background for any of the genes analysed (Fig. 5.8, bottom panel). These
results strongly suggest that Aurora B promotes transcription of its target genes by favouring promoter clearance and transcriptional initiation. To test whether the kinase activity of Aurora B is required for these activities, I looked at the effect of a 48-hour treatment of resting B cells with 200nM AZD1152 on the levels of S5p Pol II at promoters. The results shown in Fig. 5.9 show that Aurora B kinase activity is required for efficient binding of S5p Pol II as the levels of this form of the polymerase were dramatically reduced compared to cells treated with DMSO.

![Bar graph showing the effect of AZD1152 on S5p Pol II levels.](image)

**Figure 5.9: The kinase activity of Aurora B is required to maintain high levels of S5p Pol II at its target promoters.**

Binding of S5p Pol II was measured in resting B cells treated with 200nM AZD1152 (an inhibitor of the kinase activity of Aurora B, red bars) or DMSO (vehicle, blue bars) for 48 hours. Binding is expressed as enrichment relative to input DNA using equal amounts of DNA in the PCR reactions (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=3 biological replicates).

**5.6.2 Ring1B cKO resting B cells have reduced levels of RNA Pol II at target genes**

The analyses of the binding of the two types of RNA Pol II to promoters are
shown in Fig. 5.9. The results show a near complete loss of both forms of the polymerase in Ring1B KO resting B cells with the levels of S5p Pol II reduced to near-background values in 4 of the 5 Aurora B target genes analysed (Fig. 5.10, top panel). The results also show that binding of unphosphorylated RNA Pol II (8WG16) to promoters was abolished or drastically reduced at all of the promoters (Fig. 5.10, bottom panel).

Since RNA Pol II is unphosphorylated in the PIC, when it is recruited to
regulatory elements and therefore appears on promoters before S5p Pol II (see section 1.4.2), the observed reduction in the levels of S5p Pol II might be due to a reduced recruitment of the enzyme per se. Moreover, as the knockout of Ring1B reduces transcription of the Aurora B gene, this partial knockdown of Aurora B could directly affect the levels of S5p Pol II. Indeed, upon close inspection, the degree of reduction of S5p Pol II at promoters is almost identical in both knockouts.

5.6.3 Genome-wide effect of Aurora B and Ring1B cKO’s on binding of unphosphorylated and serine 5-phosphorylated RNA Pol II

In order to assess the scale of the effects of the Aurora B and Ring1B cKOs on RNA Pol II on the whole genome, DNA immunoprecipitated in the ChIP of S5p Pol II in WT and Aurora B cKO cells and the unphosphorylated RNA Pol II in WT and Ring1B KO cells was analysed by high-throughput sequencing (ChIP-seq). One of the advantages of ChIP-seq over ChIP-on-ChIP is that, like conventional ChIP-qRT-PCR, it allows quantification, but on a genome-wide scale. ChIP-seq type of analysis showed a global reduction in binding of both forms of RNA Pol II around the TSS of Aurora B and Ring1B target genes, particularly at highly transcribed genes (Fig. 5.11).
Figure 5.11: Genome-wide comparison of the distribution and binding intensities of S5p Pol II and 8WG16 by ChIP-seq in WT, Aurora B and Ring1B cKO cells.

Representative examples were selected for the data sets. To allow direct comparison of the intensities, the same y-axis scale was used for WT cells (blue) and KO cells (red) tracks. RPKM values representing the expression of each gene are reported next to the gene name and were defined by mRNA-seq analysis of freshly isolated resting B cells. Positions of the TSS are derived from the UCSC dataset. DNase I sensitivity data were obtained from the publicly available dataset generated using CD43-, CD11b- B cells from 8-week old male mice (M. Bender’s laboratory) This image was generated in collaboration with Gopura Dharmalingam (see section 2.5.2).
Figure 5.12 shows line plots that summarise the ChIP-seq results for the analysis of S5p Pol II in the Aurora B cKO and unphosphorylated RNA Pol II (8WG16) in the Ring1B cKO. The line plots show the average binding distribution each type of RNA Pol II in a window of ±3kb around the TSS. There is no change in the binding profile of either S5p Pol II or 8WG16 in the cKO’s, but the average amount of bound enzyme is affected. Average levels of binding of S5p Pol II are halved in the Aurora B KO (Fig. 5.12, left plot) and average levels of the unphosphorylated RNA Pol II showed a 2.6-fold reduction in the Ring1B cKO (Fig. 5.12, right plot).

Figure 5.12: Line plots depicting the average reduction in binding of S5p Pol II following the knockout Aurora B (left) or Ring1B (right).

Blue lines indicate the average signals for RNA Pol II in WT cells, while the red lines represent the average signal in the AurKB and Ring1B cKO samples. The binding is assessed in a window covering 6 kb and centered on the TSS (based on RefSeq data). This image was generated by Gopura Gopuraja Dharmalingam (see section 2.5.4 for details).

5.7 The knockouts of Aurora B or Ring1B reduce the viability but not the responsiveness of quiescent lymphocytes to activating stimuli

The analysis of the effect of Aurora B and Ring1B cKO in resting B and T
cells revealed global reductions in the binding of RNA Pol II and the transcription of highly expressed genes. These results raise the important question of what specific roles are played by Aurora B and Ring1B in generating and maintaining the quiescent phenotype. One possibility is that maintenance of transcription of many of the affected genes is required for survival of quiescent lymphocytes. To test whether this is the case, in collaboration with Dr. Monica Roman Trufero, I measured the viability of both resting B and T cells in both cKO models.

Freshly isolated resting B and T cells were incubated for 72 hours with 4-OHT and trypan blue exclusion was then used as a measure of lymphocyte viability. The percentage of viable wild type resting B and T cells after this period of culture was 84% ± 2.5% for B cells and 80.75% ± 2.5% for T cells. (Fig. 5.13, black lines). The value for B cells agrees well with published data (Dayal et al., 2011) At 72 hours, the Aurora B cKO resting B and T cell cultures had a lower percentage of viable cells (67% ± 4.2% for B cells and 69% ± 1.4% for T cells) (Fig. 5.13, red lines). The knockout of Ring1B has, on average, a slightly greater impact on cell viability (59% ± 1.4% for B cells and 61% ± 1.4% for T cells) (Fig. 5.13, blue lines). This difference mirrors the differences in transcriptional phenotype observed by the analysis of total RNA in between the two cKO’s in both B and T cells and might also stem from the possible dual reduction in Ring1B and Aurora B levels observed in Ring1B cKO cells (see section 5.4).
Figure 5.13: Resting lymphocytes display reduced viability following knockout of Aurora B or Ring1B.

Viable cells were scored as the percentage of cells not stained with Trypan Blue. WT (CreER\textsuperscript{T2} \text{+/+}) (black line), AurB KO (CreER\textsuperscript{T2}/Aurkb flx \text{+/+}) (red line) and Ring1B KO (CreER\textsuperscript{T2}/Rnf2 flx \text{+/+}) (blue line) naive cells were cultured for the indicated times in the presence of 250nM (B cells, top panel) or 500nM (CD4+ T cells, bottom panel) 4-OHT for 72 hours. Error bars represent the standard deviation of the mean (n=3 biological replicates for all experiments).

To test whether Aurora B and Ring1B are required for B cell activation, I stimulated homozygous CreER\textsuperscript{T2} (WT) and AurB and Ring1B cKO cells with LPS or anti-CD40 after 48 hours of incubation with 4-OHT. I then assessed the activation of these three cell types by measuring the levels of CD69 expressed on their surface, as this marker appears rapidly following activation. Because
Aurora B ablation in cycling cells results in mitotic disruption (see section 1.5), I looked at activation before entry into G2/M and, therefore, within a relatively short window of time (14 hours for LPS and 24 for anti-CD40).

Aurora B KO resting B cells exhibit no changes in their responsiveness to either LPS or anti-CD40, as the percentages of CD69 positive cells are identical to WT cells (Fig. 5.14, left and central panels). Ring1B KO resting B cells do not display an altered responsiveness to anti-CD40 and a slightly reduced response to LPS (approximately 10% less than WT cells). It is possible that such a small difference is merely a reflection of the reduced viability of resting B cells in the absence of Ring1B. Overall, however, the results of these experiments seem to suggest that both proteins are more important for the homeostasis rather than the responsiveness of resting B cells.

**Figure 5.14: Aurora B and Ring1B cKO does not affect activation of resting B cells.**
Analysis of the responsiveness to LPS and anti-CD40 of resting B cells isolated from WT (CreER\(^T^2\) +/+ ) (left panel), AurB KO (CreER\(^T^2\)/Aurkb flx +/+ ) (central panel) and Ring1B KO (CreER\(^T^2\)/Rnf2 flx +/+ ) (right panel) mice. Cells were treated for 48 hours with 250nM 4-OHT and then stimulated either for 14 hours with 12.5 µg/mL of LPS or 24 hours with 5 µg/mL anti-CD40 antibody. The cells were then stained with anti-B220 (mature B cell marker) and anti-CD69 (lymphocyte activation marker) and analysed by FACS. Values represent mean standard deviation from the mean (n=2 biological replicates). One representative experiment is shown.
5.8 Summary

The results presented in this Chapter show that Aurora B promotes transcription of a subset of highly transcribed genes in quiescent lymphocytes. Treatment of resting B cells with an Aurora B inhibitor results in reduced transcription of a high proportion of the genes analysed. Conditional knockout of Aurora B in quiescent B and T cells confirms that the kinase promotes transcription in both cell types.

In contrast to its repressive role in ES cells, Ring1B knockout results in a transcriptional reduction of active target gene in quiescent B cells. As Ring1B knockout reduces Aurora B expression (but not vice versa), this very strong effect could be due to the proteins acting in synergy to regulate transcription in quiescent lymphocytes. Ring1B also induces a stronger transcriptional phenotype than Aurora B in resting T cells.

qPCR-ChIP and ChIP-Seq analysis shows that ablation of Aurora B and Ring1B proteins reduces the presence of S5p Pol II and the unphosphorylated RNA Pol II around the TSS of active genes in resting B cells. Ring1B knockout affects both forms of the polymerase whereas Aurora B knockout has a stronger effect on the S5p Pol II form. This result matches the reductions observed by qRT-PCR analysis of total RNA. Finally, loss of either protein negatively impacts on the viability of resting lymphocytes but not on their responsiveness to activating stimuli.
Chapter 6 - Aurora B phosphorylates histone H3S28 and modulates Ring1B-mediated H2A ubiquitination at active promoters

6.1 Introduction

Aurora B and Ring1B promote transcription in resting lymphocytes as their conditional knockout leads to transcriptional repression of active genes and reduced recruitment to promoters of RNA Pol II (in its unphosphorylated form) and transcriptional initiation (serine 5-phosphorylated). In this section of the project, I sought to understand the molecular mechanism through which Aurora B exerts its function as a transcriptional co-activator. I examined whether Aurora B is responsible for phosphorylating H3S28 at active promoters since H3S28 phosphorylation is known to be a marker of transcriptional activation. I also tested whether Aurora B affects mono-ubiquitination of H2A at active promoters and binding of the USP16 deubiquitinase. Finally, I investigated the functional role of Aurora B in quiescent lymphocytes by testing the effect of the conditional knockout of Aurora B on resting B cell survival and activation.

6.2 Aurora B controls phosphorylation of histone H3S28 at active promoters

Aurora B phosphorylates both histone H3S10 and H3S28 at gene promoters and this post-translational modification has generally been associated with transcriptional activation at promoters (see section 1.4.3). Published ReChIP experiments have shown that the H3S28ph histone mark, but not H3S10ph,
physically co-occupies active promoters with S5p Pol II (Lau and Cheung, 2011). Therefore, I asked whether the levels of H3S28ph are also reduced in Aurora B cKO cells. For this purpose, I performed ChIP analysis for H3S28ph in resting B cells isolated from mice that were either WT (homozygous for Cre-ER\textsuperscript{T2}) or Aurora B cKO (homozygous for Cre-ER\textsuperscript{T2} and the floxed \textit{Aurkb} allele).

The results of this analysis are shown in Fig. 6.1. In the wild-type cells, the ChIP analysis detected the H3S28ph mark both at Aurora B target genes and at silent promoters (Fig. 6.1, blue bars). This is in agreement with unpublished data generated in our lab that describe the presence of H3S10ph and H3S28ph in conjunction with H3K9me3 and H3K27me3 at silent genes in resting B cells (Sabbattini et al., manuscript in revision). However, the conditional knockout of Aurora B only affects the levels of H3S28ph on active target genes (Fig. 6.1). This result indicates that the presence of Aurora B is required to maintain the levels H3S28ph modification at active promoters, whereas its loss has little impact on the levels of H3S28ph at repressed genes in quiescent B cells.

**Figure 6.1:** Aurora B cKO resting B cells have reduced levels of H3S28ph at the promoters of Aurora B target genes.

Enrichment of H3S28ph over the total histone H3 at promoters was measured in homozygous Cre-ER\textsuperscript{T2} (WT, blue bars) and homozygous Cre-ER\textsuperscript{T2}/\textit{Aurkb} floxed allele (AurK KO, red bars) resting B cells after incubation with 4-OHT for 48 hours. Levels of H3S28ph are expressed as enrichment relative to DNA immunoprecipitated by the anti-total H3 antibody using equal amounts.
of DNA in the PCR reactions (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological duplicates).

H3S28 phosphorylation by MSK1/2 has been shown to be involved in displacing PRC2 from repressed promoters (Lau and Cheung, 2011) and (Gehani et al., 2010). Since Aurora B targets are devoid of Ezh2 and H3K27me3, I tested whether Aurora B prevents the deposition of the H3K27me3 mark via phosphorylation of H3S28. However, although the conditional knockout of Aurora B reduces the levels of H3S28ph, ChIP experiments show that this event does not coincide with an increase of H3K27me3 above the background value of 1 and does not affect the high levels of this mark at silent promoters (Fig. 6.2). Therefore, PRC2-mediated silencing seems to be regulated by a different mechanism in resting B cells.

Figure 6.2: Reduction of the levels of H3S28ph is not accompanied by an increase in the levels of PRC2-mediated H3K27me3 in Aurora B cKO resting B cells.

Enrichment of H3K27me3 over total histone H3 at promoters was measured in homozygous Cre-ER\textsuperscript{T2} (WT, blue bars) and homozygous Cre-ER\textsuperscript{T2}/\textit{Aurkb} floxed allele (Aurb KO, red bars) resting
B cells. Levels of H3K27me3 are expressed as enrichment relative to DNA immunoprecipitated by anti-total H3 antibody using equal amounts of DNA in the PCR reactions (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent standard deviation of the mean (n=2 biological replicates).

6.3 Aurora B regulates the levels of H2A ubiquitination at active promoters

6.3.1 Loss of Aurora B is accompanied by acquisition of the H2Aubq mark at promoters

Since Aurora B and PRC1 co-occupy the same promoters, I assessed whether Aurora B influences deposition of the H2Aubq mark. This mark is detectable at silent promoters but almost completely absent from active promoters (Fig. 4.7, green bars). In cKO cells, the levels of H2Aubq (as measured by enrichment over total histone H2A) increase dramatically at Aurora B target genes and to a level similar to what observed on silent genes (Fig. 6.3). In the latter group, little increase in H2Aubq is observed, on 3 out of 4 promoters with only the Bcan gene showing any increase. Moreover, Fdx1, which is not bound by either Aurora B or Ring1B, maintains background levels of H2Aubq.
Figure 6.3: The histone mark H2Aubq marks Aurora B target genes in Aurora B cKO resting B cells.

In Aurora B cKO resting B cells the levels of H2Aubq at Aurora B/Ring1B-bound promoters increase to the same level observed on silent genes. Enrichment of H2Aubq over total histone H2A was measured in homozygous Cre-ERT2 (WT, blue bars) and homozygous Cre-ERT2/Aurkb floxed allele (Aurb KO, red bars) resting B cells following a 48-hour treatment with 4-OHT. Levels of H2Aubq are expressed as enrichment relative to DNA immunoprecipitated by the anti-total H2A antibody using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=4 biological replicates).

6.3.2 USP16 binding to promoters is lost following knockout of Aurora B

Aurora B binding to mitotic chromosomes requires H2A deubiquitination by USP16 (Joo et al., 2007). Moreover, ChIP-seq analysis revealed a high degree of overlap of Aurora B and USP16 at target genes (Chapter 4, Figs. 4.11 and 4.12). In addition, USP16 association with promoters is lost in G1-elutriated, LPS-activated cells (Chapter 4, Fig. 4.8), where levels of H2Aubq slightly increase (Chapter 4, Fig. 4.7). qPCR-ChIP was used to analyse binding of Aurora B in resting B cells following conditional knockout of Aurora B. The results of the ChIP experiments show that binding of USP16 to active promoters is severely reduced.
and in some instances is almost completely lost from promoters following the knockout of Aurora B (Fig. 6.4).

![Graph showing USP16 binding in Aurora B cKO resting B cells](image)

**Figure 6.4: USP16 no longer associates with active promoters in Aurora B cKO resting B cells.**

The binding of USP16 was measured in homozygous Cre-ER\(^T2\) (WT, blue bars) and homozygous Cre-ER\(^T2\)/Aurkb floxed allele (Aurb KO, red bars) resting B cells. Binding is expressed as enrichment relative to input DNA using equal amounts of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological replicates).

These results suggest that Aurora B and USP16 co-operate to regulate the levels of H2Aubq at promoters. Moreover, binding of both proteins is lost from the active promoters that I have analysed following activation (see Chapter 4). However, Aurora B and USP16 both associate with chromatin at the onset of mitosis, with USP16 required to deubiquitinate H2A to allow the binding and activity of Aurora B (see section 1.5.2). It would therefore be interesting to test whether USP16 has a role in Aurora B recruitment at active gene promoters, similar to its role during mitosis.
6.3.3 Binding and composition of PRC1 at active promoters is independent of the presence of Aurora B or USP16

USP16 has been shown to promote transcription of the HoxD10 gene in HeLa cells via deubiquitination of H2A at the promoter but without affecting binding of Bmi1 (Joo et al., 2007). When Aurora B is knocked out in resting B cells, more PRC1 (or another complex) could bind to active promoters and monoubiquitinate histone H2A. Alternatively, PRC1 could be recruited by an Aurora B-dependent pathway and, in cKO cells, another complex with E3 ligase activity could substitute for PRC1. To test whether any of these hypotheses is correct or whether Aurora B and USP16 simply control the activity, but not the recruitment of PRC1, I compared the binding of Ring1B and Bmi1 (the core components of PRC1 that are responsible for the E3 ligase activity) in WT and Aurora B cKO cells.
Figure 6.5: In the absence of Aurora B, Ring1B binding increases at some active promoters, whereas Bmi1 and Cbx7 binding is largely unaltered.

The binding of Ring1B, Bmi1 and Cbx7 were measured in homozygous Cre-ER<sup>T2</sup> (WT, blue bars) and homozygous Cre-ER<sup>T2</sup>/Aurkb floxed allele (Aurkb KO, red bars) resting B cells. Binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological duplicates, for all experiments).

The results of the analysis are shown in Fig. 6.7 The level of enrichment over input of Ring1B increases substantially at three promoters (Gba, Cct3 and Mtx1) out of eight active genes analysed by qRT-PCR, whereas it remains unaltered on the remaining ones. On the other hand, Bmi1 and Cbx7 association with promoters appears unchanged following the removal of Aurora B, with the
exception of *Mapbpip* where a 3-fold decrease is observed in the Cbx7 ChIP (Fig. 6.5).

While recruitment of other PRC1 components cannot be excluded, the analysis shown here does not support the idea that the increased ubiquitination is due to a change in the composition of the PRC1 complex at promoters that bind Aurora B. Therefore, the main change in protein binding that can be related to the change in ubiquitination levels in Aurora B knockout cells is decreased binding of USP16, which would be expected to lead to increased H2A ubiquitination.

Data generated by Dr. Monica Roman Trufero supports the hypothesis that, rather than regulating the binding of PRC1 components to promoters, Aurora B counteracts Ring1B-mediated monoubiquitination of histone H2A. Firstly, Aurora B phosphorylates USP16 *in vitro* and this increases the activity of the deubiquitinase towards nucleosomal substrates *in vitro*. Secondly, Aurora B phosphorylates *in vivo* the E2 ligase specific for Ring1B, UbcH5c, and this reduces the ubiquitin ligase of PRC1 *in vitro* (data not shown).

### 6.3 MSK1 replaces Aurora B on active promoters after B cell activation

#### 6.3.1 B cell-activation induces a rapid exchange of Aurora B with MSK1 at transcriptionally active promoters

Finally, I sought to understand the role played by Aurora B in resting B cells and B cell activation. The binding of Aurora B to promoters is lost in B cell activated with LPS or anti-CD40 (Fig. 4.3) and Aurora B cKO B and T cells activate normally (Fig. 5.14). These observations suggest that Aurora B does not play a major role in B cell activation pathways.

The mitogen LPS induces B cell activation and initiates the Ras/ERK/MAPK signalling pathway. One of the downstream effectors of this signalling cascade is MSK1, which activates transcription through histone H3 phosphorylation on
serine 10 and 28 (see section 1.4.3). I decided to test whether Aurora B is replaced at its target promoters by MSK1 in activated B cells. ChIP analysis of Msk1 binding was carried out on resting B cells and ChIP G1-elutriated LPS-activated B cells. The results show that MSK1 does not bind to active promoters in resting B cells whereas enrichment for the kinase was observed at these promoters in G1 activated b cells. These results indicate that that MSK1 replaces Aurora B on gene promoters during B cell activation (Fig. 6.6).

Figure 6.6: MSK1 replaces Aurora B at active target genes following LPS activation.

Binding of MSK1 to promoters was measured in quiescent (dark green bars) and G1-elutriated LPS-activated (light green bars) B cells. A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological replicates).

MSK1 has been shown to bind rapidly to the promoters of its target genes and phosphorylate histone H3S10 and S28 (Drobic et al., 2010) (see section 1.4.3). These phosphorylations can occur within less than 30 minutes, depending on the stimulus used to trigger MSK1 activation and the type of cell. I decided to analyse the kinetics of binding of MSK1 and Aurora B to the promoters of active
genes during LPS activation of resting B cells.

I selected three promoters (Fcrl1, Mapbpi and Gba) and measured the binding to their promoter at 30, 60 and 120 minutes after the addition of LPS (see section 2.1.1). MSK1 binds rapidly to both Fcrl1 and Mapbpi, with respective 4- and 2-fold increases observed within 30 minutes of adding of LPS to the cells (Fig. 6.7, top and middle graphs, blue lines). A similar type of kinetics has been described for immediate early response genes in LPS-stimulated fibroblasts (Drobic et al., 2010).
Figure 6.7: MSK1 and Aurora B rapidly exchange at active promoters following LPS stimulation of resting B cells.

The binding dynamics of exchange of MSK1 (blue line) and Aurora B (red line) were measured on the promoters of Fcrl1 (top panel), Mapbpip (middle panel) and Gba (bottom panel). Naive resting B cells were freshly purified from the spleen of adult (6-8 weeks old) mice analysed at 0, 30, 60 and 120 minutes after the addition to the culture of 25 µg/ml LPS as an activating stimulus. Binding is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). Error bars represent the standard deviation of the mean (n=3 biological
replicates for both MSK1 and Aurora B).

Aurora B shows, instead, a slower but progressive removal up to 120 minutes after stimulation when its association with chromatin has been almost completely lost (Fig. 6.7, red line). This pattern of exchange between the two kinases is observed on the promoter of Gba, although at a slower pace (Fig. 6.7, bottom chart). MSK1 binding becomes apparent only after 120 minutes of activation (blue line), whereas Aurora B is removed from the promoter after 60 minutes (red line). In summary, MSK1 rapidly replaces Aurora B at active promoters following activation suggesting that Aurora B plays a role in gene regulation in the quiescent state and then switches to its canonical role in regulating mitosis in activated, proliferating cells.

6.3.2 MSK1 recruitment following stimulation with LPS is independent of the presence of Aurora B

Since Aurora B cKO cells respond normally to LPS stimulation, it is possible that gene activation programmes are not compromised by the loss of Aurora B. This includes the activation of ERK/MAPK pathway and, ultimately, the binding of MSK1 to promoters. To test this idea, I performed ChIP experiments to quantify the binding of MSK1 to active genes following the knockout of Aurora B and, subsequently, a 14-hour stimulation with LPS (Fig. 6.8). MSK1 binding is not abolished by the prior knockout of Aurora B, although the levels appear to be halved in the case of b-Actin and Mapbpiip. The fact that binding of MSK1 following B cell activation is not lost in the genes analysed indicates that cKO are still able to respond to LPS stimulation via the ERK/MAPK signalling pathway. This could provide a partial explanation for why Aurora B cKO B cells activate normally despite their reduced transcription of active genes and reduced viability.
Figure 6.8: LPS-induced binding of MSK1 to promoters is not compromised in resting B cells where Aurora B as been previously knocked out. Homozygous Cre-ERT² (WT, blue bars) and homozygous Cre-ERT²/Aurkb floxed allele (Aurb KO, red bars) resting B cells were treated for 48 hours with 4-OHT to knock out Aurora B and then stimulated for 14 hours with LPS. Binding of MSK1 was measured in both cell types as a measure of the responsiveness of the cells to T-independent activation. The binding of MSK1 is expressed as enrichment relative to input DNA using the same amount of DNA in the PCR (see section 2.4.3). A dashed line indicates the threshold value of enrichment above which a protein is considered to be bound. Background levels (DNA immunoprecipitated with an anti-IgG antibody) are shown as white bars where detected. Error bars represent the standard deviation of the mean (n=2 biological replicates).

6.4 Summary

The conditional KO of Aurora B is associated with a stronger reduction of the H3S28ph histone mark at active rather than silent promoters in resting B cells. This modification could be involved in enhancing the levels of S5p Pol II at active promoters. In Aurora B conditional knockout cells, the loss of H3S28ph is not accompanied by PRC2-mediated trimethylation of H3K27. However, upon loss of Aurora B, PRC1-mediated monoubiquitination of histone H2A is observed on target genes and USP16 binding is substantially reduced. The increase in the
levels of H2Aubq does not depend on an increased binding of Ring1B or Bmi1.

No change in Cbx7 binding is observed in Aurora B knockout cells, suggesting that the composition of the PRC1 complex on active promoters is unaffected.

MSK1 replaces Aurora B on active promoters in G1-elutriated, LPS-activated B cells. The rapid kinetics of the substitution following LPS stimulation indicates that it precedes cell cycle re-entry and Aurora B commitment towards its role as a mitotic regulator. Aurora B does not seem to regulate B cell activation, as its knockout does not impair the binding of MSK1 to promoters following stimulation.
Chapter 7 - Discussion

7.1 Aurora B and Ring1B as new players in the quiescence programme

Cellular quiescence requires a specific transcriptional programme that allows the cells to reduce its size and metabolic activity and, at the same time, to rapidly resume proliferation upon stimulation. In the mammalian immune system, quiescence ensures that an adequate response will be triggered against virtually any pathogen, without compromising the energy balance of the organism. Quiescent mature B and T cells can remain in this condition throughout their lifespan, unless they encounter the specific antigen that can trigger their activation. This makes the study of how these cells regulate the expression of their genes of particular interest for understanding the correct functioning of the immune system and the pathogenesis of leukaemias and autoimmune disease.

Several transcription factors have been shown to regulate the expression of genes that mediate the acquisition and maintenance of a quiescent state in mature B and T cells. This study has uncovered previously unknown roles for Aurora B and Ring1B in this context. These proteins bind to a high proportion of active genes, particularly the highly transcribed ones. The knockout of either protein results in a dramatic reduction of RNA Pol II bound to most of the active genes. This in turn leads to downregulation of a large proportion of active genes and a reduced viability. Some of this effect could be due to downregulation of expression of RNA Polymerase subunits, but the widespread binding of Aurora B and Ring1B suggest that a large part of it is direct.

Despite the strong effects on RNA Pol II binding and transcription, our initial experiments indicate that B cell activation is not blocked in either of the two knockouts. These results suggest that Aurora B and Ring1B are recruited to
promoters in order to maintain expression of proteins that are necessary for the viability of mature lymphocytes in the resting state. The implication is that Aurora B and Ring1B are necessary for the maintenance of active transcription within the quiescent state but not for the early response to activation stimuli and exit from quiescence.

The association of Aurora B with regulatory elements in quiescent cells is lost following B cell activation and cell cycle re-entry. This suggests that Aurora B has different functions depending on the context, with the kinase acting as a transcriptional co-activator in quiescent lymphocytes and as a cell cycle regulator in activated cells. Ring1B, on the other hand, remains bound to active promoters in G1 activated B cells. Despite this difference, Aurora B and Ring1B show a remarkable functional interdependence in quiescent lymphocytes. On the one hand Ring1B promotes expression of Aurora B in resting B cells and, on the other, Aurora B regulates the activity of Ring1B as an E3 ligase by blocking the activity of the associated E2 ligase. These results suggest a model where Aurora B converts Ring1B from a repressor (histone H2A monoubiquitinating enzyme) into an activator (promoting the recruitment of RNA Pol II) (Fig. 7.1).
Based on the results presented in Chapters 3 – 6, the model proposes that Aurora B operates downstream of signalling pathways that ensure the viability and responsiveness of resting lymphocytes. Aurora B promotes the phosphorylation of serine 28 on histone H3 and of USP16, which, in this form, can bind to chromatin and exert its deubiquitinating function. Ring1B, Cbx7 and Bmi1 would be recruited by transcription factors (TF1 and TF2) and promote the formation of the pre-initiation complex at promoters. The H3S28ph mark would also act as a recognition signal that promotes recruitment of the PIC.

An important question is whether Aurora B and Ring1B play a role in enhancing the transcription of key genes in other quiescent cell types. Preliminary experiments did not detect Aurora B or Ring1B on active promoters in serum-starved fibroblasts. This could imply that their role as transcriptional activators is a unique feature of resting lymphocytes. However, serum starvation is only one type of in-vitro induced quiescence. Therefore, before drawing any conclusion on the generality of the mechanisms identified in this study, it will be necessary to test other cell types where quiescence is actively induced as part of the developmental programme, rather than an adaptive response to lack of
nutrients or mitogens (serum starvation)

To date, Aurora B has been shown to promote transcription only of thyroid hormone-inducible genes in pituitary cells via the deposition of the H3S10ph mark. Ring1B is, on the other hand, a widely studied histone modifier that promotes silencing, but this study presents evidence that it can function as a transcriptional activator in quiescent lymphocytes. Whether this behaviour occurs in other cell types is a question left unanswered by this study. Bmi1 and Suz12 have been shown to respectively promote and restrict HSC/progenitor functions (Majewski et al., 2010), raising the interesting possibility that PcG proteins have different functions and regulatory roles in different cell types and that the haematopoietic system in particular might represent an exception to the canonical role of PRC1 as a repressor.

7.2 Two new functions for Aurora B in transcriptional regulation outside mitosis

7.2.1 Aurora B phosphorylates serine 28 on histone H3 in quiescent B cells

The results presented in this thesis provide evidence that Aurora B promotes transcription via two mechanisms in resting B cells: through phosphorylation of serine 28 on histone H3 and regulation of H2A ubiquitination (Fig. 7.1). The H3S28ph mark is being increasingly linked to transcriptional activation because of its antagonistic effect on PRC2-mediated silencing, its binding affinity for 14-3-3 proteins and its physical colocalization with S5p Pol II. Several kinases activate transcription via this mechanism and, for example, MSK1 promotes the transcription of target genes by depositing the H3S28ph mark upon stimulation with mitogens, such as LPS. My results show that MSK1 substitutes for Aurora B following LPS-driven stimulation of resting B cells, where Aurora B no longer binds to the active promoters that I have analysed. It is possible that, following cell cycle re-entry, Aurora B no longer functions as a
transcriptional regulator and that other kinases take over this role. Similarly, upon cell cycle exit and engagement of the quiescent programme, Aurora B phosphorylates histone H3, a function known to be carried out by this protein at the onset of mitosis. In this respect, an interesting observation from the genome-wide ChIP analysis is that Aurora B specifically binds to promoters and enhancers in resting B cells, whereas at the onset of mitosis, it spreads all over the chromosome arms. An important question is what mediates the contrasting distribution and function of histone phosphorylation in interphase and mitosis. In the case of Aurora B, the answer might be in the different protein interactors in the two stages, as this is also what determines the different localizations and targets of Aurora B during mitosis. In this respect, my SILAC-based screening of Aurora B might prove useful, with different transcription factors (such as YY1, the Forkhead proteins and members of the CCR4-not complex) being identified as potential G1-partners. Additional work in the group has validated the CCR4-Not complex member Cnot3 as a genuine interactor with Aurora B, both in vitro and in resting and activated B cells (M. Martufi and M. Roman-Trufero, personal communication).

7.2.2 Aurora B negatively regulates the levels of monoubiquitination of histone H2A in quiescent B cells

A second function for Aurora B uncovered by this study is the indirect control of the levels of the repressive H2Aubq mark at promoters. The ablation of Aurora B by conditional knockout coincides with an increase in the levels of H2A ubiquitination, although the levels of PRC1 binding do not change. This, in turn, points to a role for Aurora B in regulating the ubiquitinating activity rather than in the recruitment of PRC1 to promoters (Fig. 7.1). The binding of Aurora B to mitotic chromosomes requires H2A deubiquitination by USP16 and ChIP-seq analysis has revealed a high degree of overlap of Aurora B and USP16 at target genes in resting B cells. Indeed, the two proteins exhibit a remarkable similarity in
their binding to chromatin, as the levels of USP16 at promoters decrease dramatically following the knockout or the displacement of Aurora B in LPS-activated cells.

The evidence suggests that USP16, like Aurora B, changes its functions following cell cycle exit and binds to the promoters of highly transcribed genes following cell cycle exit. The association of USP16 with chromatin and its deubiquitinase activity are dependent on USP16 phosphorylation. Cdk1 has been shown to be able to generate this modification on USP-16 in-vitro. However, the only identified phosphorylation site on USP16 in-vivo is an Aurora B consensus site (Zanivan et al., 2008) and in-vitro data generated in our group shows that Aurora B can phosphorylate USP16 and, in turn, promote its histone deubiquitinase activity (Dr. Monica Roman-Trufero, personal communication). Moreover, in-vivo data produced by Dr. Monica Roman-Trufero shows that Aurora B phosphorylates the E2 ligase responsible for Ring1B-mediated monoubiquitination of histone H2A (UbcH5c). This phosphorylation reduces the E3 activity of Ring1B towards nucleosomal substrates in vitro.

7.3 Interaction of Aurora B and Ring1B in quiescent and activated lymphocytes

The analysis of the Aurora B interactome in G1-elutriated, LPS-activated B cells shows that Aurora B interacts with proteins implicated in gene regulation, developmental processes and chromatin organization such as Ring1B. The interaction with Ring1B has been validated in this cell type and in transfected 293T cells by direct Co-IP and western blot. However, the ChIP data indicates that Aurora B and Ring1B physically co-occupy the same promoters in resting but not G1-elutriated, LPS-activated B cells. The Aurora B-Ring1B interaction detected in the co-IP experiments might occur in the soluble portion of the nucleus or cytoplasm or, alternatively, at an untested subset of genes where
Aurora B and Ring1B bind in interphase. Unfortunately, the low amount of DNA recovered in ChIP experiments of Aurora B in G1-elutriated cells has meant that it was not possible to test this hypothesis. Nevertheless, the possibility cannot be formally ruled out that Aurora B is still affecting the E3 ligase activity of Ring1B at some target genes in activated B cells. It is possible that Aurora B no longer binds directly to chromatin in G1-elutriated, LPS-activated cells as it no longer phosphorylates histone H3 and that, as a result, it cannot be immunoprecipitated by ChIP because its interaction with Ring1B is too dynamic.

A further consideration is the fact that even in activated cells, the presence of Ring1B on promoters does not correlate with acquisition of the H2Aubq mark on promoters or on repression. Ring1B-bound genes exhibit a transcriptional increase following activation and, therefore, it is possible that Aurora B is still preventing Ring1B from ubiquitinating histone H2A via phosphorylation of the E2 ligase or the recruitment of a histone deubiquitinase. The genome-wide distribution of USP16 in resting B cells closely resemble that of Aurora B and several USP proteins were detected in my SILAC analysis of the Aurora B G1-interactome: USP 7, 34, 47 and 48. USP7, in particular, represents an interesting candidate as its knockout results in the derepression of Polycomb regulated genes (Maertens et al., 2010) and it deubiquitinates Ring1B, effectively stabilizing but also depriving it of its E3 ligase function (de Bie et al., 2010). The histone H2A deubiquitinase MYSM1, in addition, has been shown to play a key role in B cell development by preventing the ubiquitination and repression of key target genes such as *Ebf1* and *Pax5* (Jiang et al., 2011). This makes MYSM1 a potential candidate for a role as a transcriptional regulator in activated B cells through deubiquitination of histone H2A.

**7.4 Ring1B promotes transcription in quiescent mature lymphocytes**

The observation that conditional knockout of Ring1B results in a global
reduction of the levels of unphosphorylated RNA Pol II recruited at active promoters and strongly reduces transcription is not easily reconciled with the known role of Ring1B as a transcriptional repressor. Independently of the composition of the complex it belongs to, Ring1B has generally been considered to act as a silencer (Wang et al., 2004) and knockout of the gene in ES cells leads to derepression of its target genes (Endoh et al., 2008). Recently, however, the paradigm of PRC1 and PRC2 being exclusively repressive co-factors is starting to be undermined. For instance, ChIP experiments have shown that PRC1 and PRC2 bind to the promoters of active genes in T helper cells and that their knockdown leads to transcriptional repression (Jacob et al., 2011). Moreover, in megakaryocytes and thymocytes, Ring1B is recruited to target genes by the transcriptional factors Runx1 and CBF and its knockdown results in the upregulation of 152 and downregulation of 345 genes (Yu et al., 2012). It is becoming increasingly accepted that Polycomb-repression represents a middle-ground between complete repression and the transcriptionally active state. In ES cells, although Polycomb-repressed genes show very low but detectable transcriptional activity, they have the S5p form of RNA Pol II bound at their promoters, where it is thought to be poised to initiate productive transcription upon stimulation of the appropriate developmental pathway (Brookes et al., 2012). The promoters of such genes are different from other silent genes, such as those marked by H3K9me3, which are usually found at particularly compacted regions.

Studies in *Drosophila* have also shown that Polycomb blocks transcriptional initiation but does not impair recruitment of RNA Pol II to Polycomb-repressed genes by the pre-initiation complex (Dellino et al., 2004). In light of these observations, it is possible to speculate that Ring1B might be able promote recruitment of RNA Pol II to the genes to which it binds, independently of the transcriptional status (active or poised/silenced) of the gene.
These findings lead us to propose a model where Ring1B would exert its RNA Pol II recruitment function independently of its E3-ligase activity in quiescent lymphocytes, because Aurora B prevents the deposition of H2Aubq on active promoters in these cells (Fig. 7.1). In Aurora B cKO cells, Ring1B operates as a transcriptional repressor by monoubiquitinating histone H2A, in the absence of Aurora B and USP16 (Fig. 7.1). This would, in turn, poise the gene but, at the same time, keep it silenced. It is possible that Ring1B is involved in recruiting RNA Pol II in both quiescent and activated lymphocytes, which would explain why it is retained on promoters following cell cycle re-entry. Testing of this hypothesis and identification of molecular mechanisms and partners that mediate such a function will require further investigation.

7.5 PRC1 and PRC2 display opposite localizations in quiescent lymphocytes

Several papers have now presented experimental evidence for a PRC2-independent recruitment of PRC1 to promoters. The analysis of the genome-wide distribution of Ring1B and H3K27me3 presented in this thesis provides another example of this phenomenon. One possible mechanism for the recruitment of PRC1 in the absence of PRC2 is through the binding of transcription factors. In this respect, YY1 and RYBP would represent good candidates. RYBP has been shown to permit the recruitment of PRC1 to Polycomb repressed genes in the Eed KO ES cells (Tavares et al., 2012). However, attempts at co-immunoprecipitating YY1 with RYBP-containing PRC1 complexes have so far proved unsuccessful (Gao et al., 2012) and (Tavares et al., 2012). Interestingly, the transcription factors Runx1 and CBF have been shown to associate with Ring1B and Bmi1 at active promoters. However, because of the large number of promoters bound by Ring1B in quiescent B cells, it is difficult to imagine a single transcription factor promoting the binding of a protein to so many different genes.
Experiments in the Aurora B cKO mouse show no involvement of this kinase in the recruitment of PRC1 to promoters, as the level of Ring1B binding (together with those of Cbx7 and Bmi1) are largely unaffected by the knockout. Cbx7 has been shown to interact with the ANRIL ncRNA and participates in repression of the INK4b/ARF/INK4a locus and its binding is reduced when transcription is reduced by treatment with -amanitin (Yap et al., 2010). It is possible to speculate that Cbx7 recognizes short ncRNAs originating around the TSS of active genes and, in turn, recruits PRC1 to their promoter, in a fashion similar to what has been reported for Suz12 and PRC2 recruitment (Kanhere et al., 2010). The determination of whether RNAs play a role in the recruitment of PRC1 to active promoters or whether PRC1 binding is a pre-requisite for transcription will require further study.
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Appendix A

Genome-wide distribution of Aurora B, Ring1B and other components of the transcriptional network operating in naïve B cells
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