

Cell Cycle Regulation in Mammalian Oocytes

By

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Abstract

An unusual feature of mammalian female germ cells is that they are arrested in meiotic prophase, equivalent to mitotic G2-phase, for an extended period of time. In this thesis I have investigated two aspects of this arrest. First, I examined whether cohesin replenishment is required for the maintenance of chromosome cohesion during protracted meiotic prophase arrest. Nipbl, an evolutionarily conserved protein, is a component of protein complex called kollerin, whose activity in loading cohesin onto chromosomes is necessary for accurate chromosome segregation during mitosis. However, until now its function in mammalian meiosis was unknown. I have showed that Nipbl is present on meiotic chromosomes throughout meiotic prophase in mouse spermatocytes and oocytes and it accumulates at chromosomal axes where it co-localises with cohesin. I employed conditional knockout strategy to inactivate *Nipbl* gene in mouse oocytes arrested in meiotic prophase. Although functional *Nipbl* transcripts were efficiently depleted, these oocytes underwent meiotic maturation with unaffected chiasmata and cohesion. Surprisingly, *Nipbl*-deleted eggs were fertile and the loading of mitotic cohesin containing Rad21 was unaffected in fertilized eggs. Additionally, these eggs could develop into blastocysts upon parthenogenetic activation, however harbouring a high proportion of cells with misaligned chromosomes. These results suggest that Nipbl is very stable in the oocyte.

In the second project we conceived that the maintenance of the cell cycle arrest in primordial oocytes is an important aspect of follicular survival. Previously proposed involvement of the anaphase promoting complex/cyclosome (APC/C), a cell cycle ubiquitin ligase complex in down-regulating the cyclin-dependent kinase activity in fully-grown oocyte led me to inactivate APC/C in dormant oocytes using conditional knockout system. I found that upon APC/C inactivation, primordial follicles were completely depleted before adulthood, within 5 weeks of birth, suggesting that the APC/C activity is required for the survival of primordial oocytes. These results propose the presence of previously unknown mechanism involving APC/C, essential for primordial follicle survival.

Statement of Originality

All experiments included in this thesis were performed by the author unless otherwise stated below or in the text.

Notably in Chapter 4, Dr Xiangwei Fu carried out immunohistochemistry on mouse testes presented in Figure 3.2. In the same chapter Dr Shoma Nakagawa performed parthenogenetic activation, parthenotes fixation and immunofluorescence staining presented in Figure 4.8. In Chapter 5, a Master degree student Panayiota Ploutarchou contributed to ovarian follicle counting of *Apc2^{fl/fl};Gdf-9* mice, which accounts for approximately 20% of total work presented in Figure 5.4.

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Abbreviations

AE	Axial element
APC/C	anaphase promoting complex/cyclosome
Bp, kb	base pair, kilobase
BSA	bovine serum albumin
<i>C.elegans</i>	<i>Caenorhabditis elegans</i>
cDNA	complementary DNA
CKIs	Cdk inhibitors
CREST	calcinosis, Raynaud's phenomenon, esophageal dysmobility, sclerodactyly, telangiectasia
<i>C.cereneus</i>	<i>Coprinus cereneus</i>
<i>D. melanogaster</i>	<i>Drosophila melanogaster</i>
DAB	3,3'-Diaminobenzidine
DAPI	4',6'-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dbcAMP	dibutyryl cyclic AMP
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
Emi1	early mitotic inhibitor
FACS	Fluorescence-activated cell sorted
FBS	Fetal bovine serum
g, mg, µg, ng	gram, milligram, microgram, nanogram
GCs	Granulosa cells
GFP	Green florescence protein
GV	germinal vesicle
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP1	Heterochromatin protein 1
HRP	Horseradish peroxidase
kDa	kiloDalton
L, ml, µl	litre, millilitre, microlitre

LE	Lateral element
M, mM, μ M, nM	molar, millimolar, micromolar, nanomolar
MI, MII	Meiosis I, meiosis II
MEF	mouse embryonic fibroblast
min	minutes
MPF	maturation promoting factor
mRNA	messenger RNA
NGS	Normal goat serum
NP-40	Nonidet P 40
PB, PB1, PB2	polar body, 1 st polar body, 2 nd polar body
PBE	polar body extrusion
PBS	phosphate-buffered saline
PGCs	Primordial germ cells
PCR	polymerase chain reaction
PFA	paraformaldehyde
hCG	human chorionic gonadotrophin
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
Plk1	Polo-like kinase
PMSF	phenylmethylsulphonyl fluoride
PMSG	pregnant mare's serum gonadotrophin
PN	pronuclear stage
POF	Premature ovarian failure
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
rpm	revolutions per minute
s, min, h	second, minute, hour
<i>S.cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SAC	spindle assembly checkpoint
Scc1-4	sister chromatid cohesion 1-4
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Smc1-6	structural maintenance
TBS-T	Tris-Buffered Saline with 0.5% Tween 20
TGF- β	Transforming growth factor beta
U	Units

UTR	untranslated region
v/v	volume/volume
w/v	mass/volume
<i>X. laevis</i>	<i>Xenopus laevis</i>

Chapter 1

Introduction

1. Scope of the thesis

Mammalian oocytes have extraordinary nature in respect of cell cycle. Shortly after birth, oocytes arrest at the late stage of meiotic prophase, a phase equivalent to G2-phase in mitosis. However, in high contrast to mitotic G2-phase in highly proliferative cells, the meiotic prophase arrest of mammalian oocytes is maintained for extended period of time, the shortest until puberty and the longest until menopause. In this thesis I have investigated two aspects associated with this arrest. Firstly, I examined how genome stability is maintained over this extended period of time. Secondly, I studied the involvement of a cell cycle ubiquitin ligase complex, APC/C in the maintenance of the meiotic prophase arrest. Both aspects have important clinical implications, since abnormalities in chromosome segregation lead to chromosomally imbalanced gametes and aneuploidy, while the precocious exit from meiotic prophase arrest leads to loss of oocytes, clinically recognised as premature ovarian failure (POF), which is one of major causes for female infertility.

2. The eukaryotic cell cycle

The ability of cells to proliferate is central to the life and development of complex organisms. The primary task of the cell division cycle is to achieve cellular duplication while simultaneously preserving the integrity of genomic information. The cell-division cycle consists of a series of sequential events, which can be grouped in two basic phases: interphase and mitosis (M phase). Interphase is further subdivided into three phases: Gap 1 (G1 phase), synthesis (S phase) and Gap 2 (G2 phase) and takes up to 95% of the time of a eukaryotic cell cycle. During G1 phase, cells grow and synthesize the proteins required for subsequent DNA replication in S phase. Whereas during G2, instead, cells synthesize proteins required for M phase, especially microtubules that will form the mitotic spindle (Alberts et al., 2002) (Fig. 1.1).

The correct phase progression is tightly regulated by a sequence of biochemical events such as reversible, post-translational phosphorylation or irreversible degradation of proteins (Murray and Kirschner, 1989). The phosphorylation of target proteins is mediated by the family of cyclin-dependent kinases (Cdks), whose activity is crucial for cell cycle progression. The activity of the Cdk family members, as their name implies, depends on binding to their activating partners known as cyclins. The cell-cycle stage-dependent synthesis and availability of cyclins

promotes oscillations of Cdk activities throughout the cell cycle. Consequently, this allows for the cyclical phosphorylation of cell cycle components. The activity of Cdk can be suppressed by binding of specific Cdk inhibitors proteins (CKIs). The oscillation in cyclin levels during cell cycle is not only executed by the induction and repression of their synthesis, but also by the quick degradation of cyclin proteins at transition stages. Levels of cyclins in the cell cycle are regulated by two ubiquitin ligases: Skp/Cullin/F-box containing complex (SCF complex) and anaphase-promoting complex/cyclosome (APC/C). APC/C is itself activated by Cdk-mediated phosphorylation at G2/M transition (Morgan, 2007).

Different families of cyclins and Cdks are associated with specific stages of the cell cycle. In mammalian cells the exit from G1 is controlled via phosphorylation of retinoblastoma tumour suppressor protein (Rb). Rb regulates genes involved in progression through the cell cycle, in particular through G1/S phase. In its un-phosphorylated form, Rb binds to a member of E2F family of transcription factors, which controls expression of a number of genes involved in cell cycle progression, such as cyclin E. Once in a complex, Rb acts as a repressor, preventing expression of E2F-regulated genes, and this inhibits cells from progressing through G1. Cyclin D, the first cyclin to be synthesized during cell cycle, produces an active complex by binding to Cdk4 and -6 and inhibits Rb by partial phosphorylation. This reduces Rb ability to bind to E2F and allows E2F-mediated activation of the transcription of the cyclin E gene and the cell progresses towards S-phase. Next, cyclin E fully phosphorylates Rb and completes its inactivation. This is essential for S phase progression (Resnitzky and Reed, 1995).

Mitosis, a process during which a cell that previously replicated its genetic material separates its content into two daughter cells, can be subdivided into six substages. During prophase chromosomes condense, after which the nuclear envelope breaks down and spindle microtubules attach to kinetochores in prometaphase. During metaphase, chromosomes are aligned and organised between the mitotic spindles and become separated and pulled apart to the spindle poles in anaphase. Finally, during telophase chromatids reach the spindle poles and the nuclear envelope reforms in each daughter cell. The last step is known as cytokinesis, where the cytoplasm is equally divided into daughter cells. Once a cycle is completed, cells can re-enter the G1 phase or enter a state of quiescence called G0 phase, during which cells temporarily or terminally stop proliferating (Alberts et al., 2002) (Fig. 1.1).

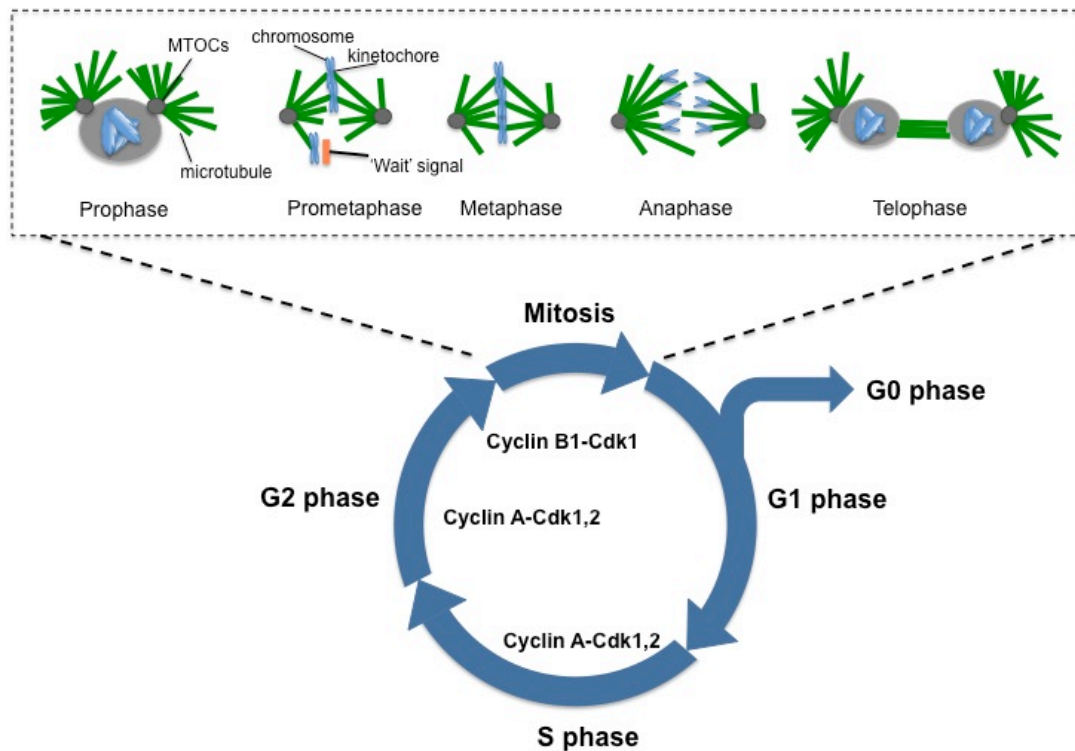


Figure 1.1 The cell cycle of a mammalian somatic cell.

The cell cycle is an ordered set of events controlled by cyclical changes in Cdk activity. Cell cycle consists of interphase and mitosis (M). Interphase is divided into three substages: G1, S phase, G2. When a cell enters mitosis chromosome condense (early prophase), nuclear envelope breaks down and spindle microtubules attach to kinetochores (prophase). This is followed by chromosome alignment at the metaphase plate (metaphase) and subsequently chromosomes are separated and pulled towards the opposite spindle poles (anaphase). Once chromosomes arrive at the spindle poles, nuclear envelope reforms for each daughter cell (telophase) and the two daughter cells separate from each other (cytokinesis) (Adopted from Pines, 2011).

3. Cyclin-dependent kinase (Cdk1)

Phosphorylation is a post-translational modification of protein, catalysed by kinases, in which a serine, a threonine or a tyrosine residue of the target protein is phosphorylated by the addition of a covalently bound phosphate molecule. It is an important regulatory mechanism that can change the features of proteins, such as their biological activity, localisation or stability (Stryer et al., 2002).

Cdk1, a serine/threonine (Ser/Thr) protein kinase, is considered to be the master regulator of mitosis (Nurse, 1990). Mitotic Cdk1 associates with two obligatory allosteric activators: cyclin B (forming an active heterodimer called MPF, maturation/mitosis promoting factor) or cyclin A (Dorée and Hunt, 2002). The entry into mitosis is driven by the upregulated activity of MPF, which is maintained within the nucleus from prophase until metaphase. Transition into anaphase is instead associated with the rapid cessation of MPF activity (Nigg, 2001). Due to its essential role, the activity of Cdk1 is controlled via several mechanisms. The majority of these mechanisms regulate the availability of cyclin B1 in the cell, which is achieved by controlling cyclin B1 transcription level, the subcellular distribution of cyclin B1 protein or the rate at which cyclin B1 is degraded. In addition, Cdk1 can be subjected to regulatory phosphorylation (Castedo et al., 2002).

From the end of S phase the cyclin B1 gene is transcribed and its mRNA stabilized. This results in slow accumulation of cyclin B1 protein in the cell and binding with Cdk1 during late S phase and G2-phase. During G2 phase, Cdk1/cyclin B1 is phosphorylated at Thr161 residue by Cdk activating kinase CAK (a heterodimer of cyclin H and Cdk7), but at the same time is negatively regulated by the phosphorylation on Thr14 and Tyr15 residues, which is catalysed by two distinct kinases, Wee1 and Myt1, thus Cdk1 remains inactive. During the transition from G2 to M phase, the inhibitory phosphorylation is counteracted by the phosphatase activity of Cdc25 that mediates dephosphorylation of Cdk1 (Nilsson and Hoffmann, 2000). Thus, Cdc25 acts as an activator for Cdk1. Activated Cdk1 complexes translocate into nucleus during prophase, where they trigger several positive feedback-loops. For example, Cdk1 activates indirectly its own activator, phosphatase Cdc25 or inhibits Wee1 and Myt1 kinases. This allows Cdk1 to sustain its activity through prophase, prometaphase and metaphase (Alberts et al., 2002).

MPF induces mitosis by phosphorylating and activating enzymes involved in chromatin condensation, nuclear envelope breakdown, mitosis-specific microtubule reorganization and the actin cytoskeleton (Nurse, 1990). Towards mitotic exit Cdk1 activity is downregulated by the anaphase promoting complex/cyclosome (APC/C) that destroys cyclin B1 (Glotzer et al., 1991). Subsequently, this enables dephosphorylation of nuclear lamins, DNA decondensation and disassembly of the mitotic spindle.

4. The Anaphase Promoting Complex/Cyclosome (APC/C)

Proteolysis involving the ubiquitin-proteasome system (UPS) controls mitotic progression at multiple stages. It is especially important for metaphase-to-anaphase transition, where sister-chromatid separation and mitotic exit are caused by the irreversible degradation of mitotic cyclins and proteins involved in control of sister chromatid cohesion. The ubiquitination process is mediated by three enzymes, an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2) and an ubiquitin ligase (E3) and is composed of three consecutive steps: activation, conjugation and ligation. The process of targeted degradation of proteins through UPS pathway involves covalent attachment of the multiple ubiquitin chains to a target protein (ubiquitination) and the subsequent recognition and degradation of the protein by the 26S proteasome complex (Harper et al., 2002; Peters, 2002).

APC/C displays E3 ubiquitin ligase activity and its main role is to trigger the transition from metaphase to anaphase by tagging specific proteins for proteasomal degradation. Depending on the organism, APC/C comprises of 15 to 18 subunits (Pines, 2011). Apc1 is the largest of them and together with Apc4 and Apc5 provides a structural scaffold that connects with two subcomplexes. The so-called 'catalytic subcomplex' contains the APC/C's catalytic core proteins: a conserved RING-finger subunit (Apc11) and cullin-domain subunit (Apc2) that are essential for catalytic activity and Doc1/Apc10, which is involved in substrate recognition. The other subcomplex is composed of subunits containing tetratricopeptide repeats (TRP): Cdc27/Apc3, Cdc16/Apc6, Cdc23/Apc8 and Apc7 (in vertebrates) (Pál et al., 2007; Yu et al., 1996). TRPs are predicted to mediate protein-protein interactions and can act as receptors for C-terminal peptide motifs (Das et al., 1998; Gatto et al., 2000). APC/C's TRP-containing subunits mediate binding to co-activators, which in mammals are Cdc20 and Cdh1 (Cdc20 homologue 1; also known as Fizzy-related 1 or Fzr1) (Thornton et al., 2006). Co-activators associate with APC/C transiently, in a

tightly regulated manner, and mediate binding of substrates to promote their poly-ubiquitination by APC/C. Most of the APC/C's targets bear either D-box or KEN-box motifs, although there are also rarer motifs including O-, G-, A- and CRY boxes (Pines, 2011). Both Cdc20 and Cdh1 recognise the D-box containing substrates, whilst Cdh1 also recognises the KEN box motif (Pfleger and Kirschner, 2000) (Fig. 1.2).

APC/C-mediated proteolysis of key cell cycle regulators drives cells from G2 through mitosis into G1 phase. The interaction of APC/C with its two co-activators, Cdh1 and Cdc20, is a key for controlling the timing of APC/C activation. Although Cdc20 is expressed during S phase, G2 phase and mitosis, its binding to APC/C is dependent on the phosphorylation of several subunits of the complex by mitotic kinases such as Polo-like kinase 1 (Plk1) and Cdk1 (Shteinberg et al., 1999; Peters, 2006). For additional control in G2 and early mitosis, APC/C activity is suppressed by binding of the early mitotic inhibitor (Emi1), which prevents APC/C-Cdc20 associations. Although, Emi1 association with Cdc20 enables stabilization of various S-phase and G2 phase cyclins, its inactivation is required for the progression of mitosis. This is executed by phosphorylation of Emi1 by Plk 1 during late prophase. Consequently, Emi1 is destroyed in prometaphase by SCF, another E3 ubiquitin ligase (Hansen et al., 2004). Active APC/C^{Cdc20} mediates the degradation of cyclin A and Nek2A in prometaphase and of securin and cyclin B1 at the metaphase-to-anaphase transition (Peters, 2006). APC/C^{Cdc20} drives mitotic exit by initiating cyclin degradation, which results in Cdk activity downregulation (Kramer et al., 2000; Peters, 2006). The regulation of APC/C association with Cdc20 is quite different from the regulation of the other APC/C's co-activator, Cdh1. Phosphorylation of Cdh1 by Cdks during S phase, G2 and mitosis prevents it from binding to APC/C. The drop in Cdk1 activity associated with cyclin B1 degradation mediated by APC/C^{Cdc20}, together with the activation of mitotic exit phosphatases, allow Cdh1 dephosphorylation and binding to APC/C during anaphase and telophase. APC/C^{Cdh1} stays active throughout G1 phase and mediates the degradation of securin, cyclins, Plk1 and Cdc20 (therefore inactivating APC/C^{Cdc20}). Eventually, APC/C^{Cdh1}-mediated degradation of the E2 enzyme leads to self-inactivation, allowing S phase entry by accumulation of S phase cyclins (Pfleger and Kirschner, 2000; Peters, 2006).

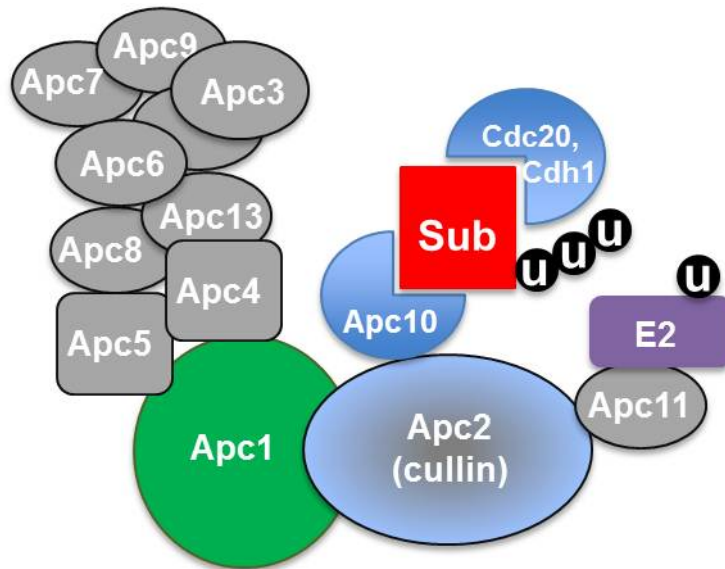


Figure 1.2 The composition of the anaphase promoting complex/cyclosome (APC/C) subunits in vertebrates.

The “catalytic subcomplex” contains the cullin subunit Apc2, the RING finger subunit Apc11 and Doc1/Apc10, which is involved in substrate recognition. The ubiquitin conjugating enzyme (E2) is associated with Apc11. The “TRP-subcomplex” consists of Cdc27/Apc3, Cdc16/Apc6, Apc7 and Apc8. The substrate protein is first binded by APC/C co-activator (Cdh1 or Cdc20) through its recognition element (co-activator specific) and then recruited to APC/C with the help of the processivity factor subunit Doc1/Apc10. Apc2 provides a structural scaffold to bring the E2 in proximity to the substrate receptor site and Doc1/Apc10. Consequently, APC/C catalyses addition of a long polyubiquitin chain to the substrate protein, which is recognised by the 26S proteasome (Adapted from Peters, 2006; Thornton et al., 2006).

In mammals, Cdc20 plays a key role in the initiation of the degradation of mitotic cyclins, the subsequent downregulation of Cdk1 and the activation of mitotic phosphatases (Li et al., 2007; Manchado et al., 2010). Unlike Cdc20, Cdh1 is dispensable for the cell cycle (Sigl et al., 2009; Li et al., 2008; Garcia-Hiugera et al., 2008), but it appears to play important function in maintaining quiescence, suppressing replication as well as regulating differentiation in a cell-type specific manner (Eguren et al., 2011).

5. Cell-type specific functions of the APC/C

APC/C complex is also active in terminally differentiated cells. While the expression of Cdc20 appears to be limited to neural cells, APC/C^{Cdh1} is involved in differentiation and function of tissues such as placenta or muscles and the hematopoietic or nervous systems (Gieffers et al., 1999; Li et al., 2007; Egure et al., 2011). APC/C^{Cdh1} has also been involved in the regulation of lens differentiation by promoting SnoN degradation (Wu et al., 2007). It was proposed that TGF- β -activated Smad2/3 mediate the recruitment of SnoN to APC/C^{Cdh1} and SnoN is targeted for degradation in the APC/C-dependent manner (Bonni et al., 2001; Nagano et al., 2007; Stroschein et al., 2001; Wan et al., 2001). SnoN represses Cdk inhibitors, while its APC/C-mediated degradation results in upregulation of these inhibitors, a process necessary to synchronize lens differentiation in response to TGF- β signalling (Wu et al., 2007).

APC/C also plays an important role in post-mitotic neurons at different levels of their development. For example, in order to prevent an unscheduled entry into the cell cycle, APC/C^{Cdh1} maintains low levels of cyclin B1 in terminally differentiated neuron (Almeida et al., 2010). In addition, APC/C^{Cdh1} directs axonal growth and differentiation by mediating degradation of two nuclear proteins SnoN and Id2 (Konishi et al., 2004; Lasorella et al., 2006; Stegmüller et al., 2006). The basal activation of TGF- β signalling in these neurons results in APC/C^{Cdh1}-dependent degradation of SnoN, which results in inhibition of axonal growth. Thus, inhibition of TGF- β stimulates axonal growth in a Cdh1-dependent manner. This implies the presence of an inherent TGF- β -APC/C pathway that is involved in modulation of transcriptional activity of SnoN and its effect on axonal growth (Stegmüller et al., 2008).

Besides antagonizing TGF- β signalling, SnoN can also transduce signals downstream from p53 signalling pathway. The elevated SnoN expression is associated with stress signalling and through direct interaction with p53 facilitates stabilising of phosphorylation and activation of p53. In epithelial and fibroblast cells SnoN uses this mechanism to induce cell senescence (Pan et al., 2012). Few studies have been conducted investigating SnoN localisation or function in the ovary. It has been reported that SnoN is expressed in somatic cell surrounding follicles in mouse neonatal ovary (Xu et al., 2009). Taken together, these studies demonstrate that APC/C functions at multiple levels in a multicellular organisms and is involved in many other roles outside metaphase-to-anaphase transition. In these processes APC/C targets cell-specific proteins for degradation.

6. Sister chromatid cohesion

The chromosome cycle begins with precise DNA duplication and the rebuilding of an equal mass of chromatin around the duplicated DNA molecules (Carson and Christman, 2001). These duplicated chromosomes are called sister chromatids and they are held together from the time of their replication until they are separated at anaphase. This phenomenon is known as sister chromatid cohesion (SCC). Cohesion must be established, maintained, and then dissolved in every cell cycle and this is essential for the maintenance of viability and for preventing genomic instability. The sister chromatid cohesion is mediated by the cohesin complex (Uhlmann et al., 1999).

6.1 The cohesin complex

The cohesin complex consists of the following four evolutionarily conserved core subunits: two members of the structural maintenance of chromosomes (Smc) protein family (Smc1, Smc3), a member of the α -kleisin family (Scc1/Rad21), and a stromalin domain protein (Scc3/SA/Stag) with two subtypes in somatic vertebrate cells SA1 and SA2 (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 2000; Losada et al., 1998; Sumara et al., 2000; Tóth et al., 1999). In mammals, mitotic cells possess two types of cohesin complexes that differ in SA subunits: Smc1/Smc3/Rad21/SA1 and Smc1/Smc3/Rad21/SA2 (Losada et al. 2000). By using genetic screening in budding yeast *Saccharomyces cerevisiae*, Michaelis and co-workers identified three genetic loci, *SMC1*, *SMC3* and *SCC1*. The temperature

sensitive mutations in these loci cause premature loss of SCC in cells arrested in metaphase by conditional inactivation of APC/C (Michaelis et al., 1997). The gene encoding the fourth cohesin subunit - SCC3 was isolated later using the same screening strategy (Tóth et al., 1999).

Globular domains of Smc1 and Smc3 proteins are connected to each other by alpha-helical structure (Melby et al., 1998), in the middle of which lies a globular hinge domain. The globular hinge domain allows Smc's polypeptide chain to fold back on itself and form an anti-parallel coiled-coil structure with two globular domains at N- and C-termini forming a head domain. Smc1 and Smc3 have ATPase activity and bind with each other at both head and hinge domain and form V-shaped Smc1/3 hetero-dimer (Haering et al., 2002; Hirano et al., 2002; Arumugam et al., 2003). The association of Scc1 with the Smc1/3 complex stabilizes their interaction and mediates the recruitment of a fourth cohesin subunit, Scc3. Scc1 forms a bridge between Smc1 and Smc3 and together they form a proteinaceous ring with an internal diameter of 40 nm, large enough to tether two chromatids in so a called embrace model (Nasmyth and Haering, 2009; Haering et al., 2002; Gruber et al., 2003; Ivanov and Nasmyth, 2005) (Fig 1.3).

The structure and function of cohesin has been extensively studied in budding yeast, while a series of studies in other eukaryotic model organisms revealed high functional conservation of cohesin. In *Xenopus* egg cell-free extracts, immunodepletion of XSmc1 or XSmc3 during interphase results in defects in SCC in mitosis (Losada et al., 1998). dsRNA-mediated Drad21 knock down in *Drosophila* S2 cells as well as embryos led to premature segregation of sister chromatids in metaphase and resulted in micronuclear formation (Vass et al., 2003). A similar phenotype was observed in chicken DT40 cells deprived of Rad21 (Sonoda et al., 2001). Therefore, the function of cohesin in mediating sister chromatid cohesion and its requirement for proper chromosome segregation are evolutionarily conserved. Moreover, cohesin also plays an important role in the repair of DNA damage (Ström et al., 2007; Unal et al., 2007) and in the control of gene expression (Donze et al., 1999). Therefore, disturbances in the function of cohesin can have broad harmful effects such as chromosome missegregation, failure to repair DNA damage and altered gene expression patterns.

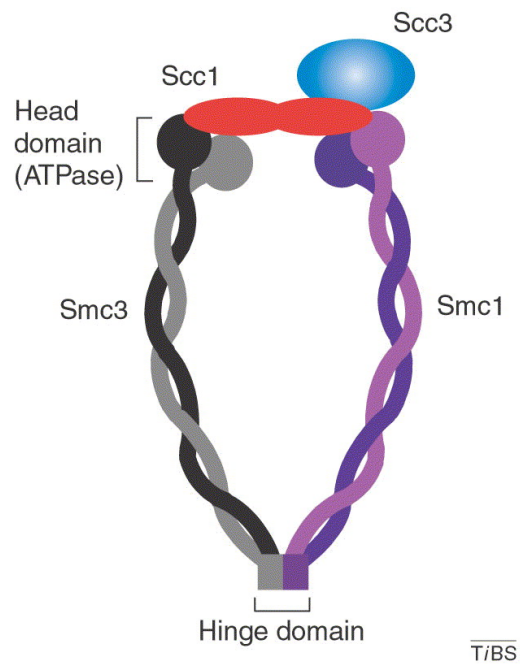


Figure 1.3 The cohesin complex.

The cohesin complex comprises of four subunits: Scc1, Scc3, Smc1 (purple) and Smc3 (black/grey). Smc1 and Smc3 are members of the Structural Maintenance of Chromosome (SMC) proteins family. Two features characterize SMC proteins: an ATP-binding head domain with ATP-ase activity, which is formed by the interaction between N- and C- termini and a hinge domain that enables dimerization of SMCs. The head domain is connected with a hinge domain via long anti-parallel coiled-coils. (Adapted from Campbell and Cohen-Fix, 2002).

6.2 Cohesin loading, maintenance and removal in mitosis

Cohesin complex is loaded onto chromosomes before DNA replication, at the end of G1 phase in budding yeast (Guacci et al., 1997; Michaelis et al., 1997) and at the end of telophase in higher organisms (Losada et al., 1998; Gerlich et al., 2006; Sumara et al., 2000). In *Xenopus* egg extracts, cohesin loading also depends on the assembly of pre-RCs on chromatin (Gillespie and Hirano 2004; Takahashi et al., 2004). In all species studied so far, loading of cohesin onto chromatin requires the loading complex comprised of Scc2 and Scc4 (Guacci et al., 1997; Michaelis et al., 1997). The loading process requires the transient opening of cohesin at the hinge domain and depends on the ability of Smc1 and Smc3 to bind and hydrolyse ATP (Arumugam et al., 2003; Hu et al., 2011; Weitzer et al., 2003). Studies in yeast, *Drosophila* and human mitotic cells suggest that this mechanism is evolutionarily conserved (Buheitel and Stemmann, 2013; Gruber et al., 2006). Cohesin binding sites identified by chromatin immunoprecipitation (ChIP) studies in yeast demonstrated that the interaction between cohesin and chromatin is enriched at specific loci known as cohesin attachment regions (CARs). They are present on chromosome arms and in larger domains at the pericentromeric region (Laloraya et al., 2000).

Once cohesin is loaded onto chromatin it requires additional factors and post-translational modifications in order to establish stable binding between two strands of DNA. The N-acetyltransferase establishment of cohesion 1 homolog 1 (Eco1/Ctf7, Esco1/Esco2 in humans) acetylates two adjacent lysine residues at the nucleotide binding domains (NBD) of Smc3 to promote cohesion establishment (Ivanov et al., 2002; Tóth et al., 1999; Unal et al., 2007). Once this is accomplished Eco1/Ctf7 becomes dispensable, which suggest a single specific function of this enzyme during DNA replication (Unal et al., 2008, 2007). Vertebrate cell culture studies revealed two other factors participating in cohesion establishment, sororin and Eco2 acetyltransferase. Sororin is essential for stable cohesin - DNA interaction and its recruitment to DNA is dependent on Eco2 (Lafont et al., 2010; Rankin et al., 2005) (Fig. 1.4).

During the prophase, immediately after nuclear envelope breakdown, the majority of cohesin bound to chromosome arms dissociates in a so called 'prophase pathway' (Losada et al., 1998; Sumara et al., 2000). This dissociation is facilitated by wings apart-like protein (Wapl) and the activity of two mitosis-specific protein kinases Plk1

and Aurora B (Gandhi et al., 2006; Kueng et al., 2006; Losada et al., 2002; Sumara et al., 2002). Wapl activity is antagonized by sororin and together they control the removal of cohesin from chromosome arms (Nishiyama et al., 2010). The remaining cohesin persists at the centromere region until metaphase. Centromeric cohesin is protected from the Plk1-mediated phosphorylation by centromeric Shugoshin (Sgo1) that acts together with protein phosphatase 2A (PP2A) (Rivera and Losada, 2006). During metaphase, centromeric cohesin is removed by cleavage of the α -kleisin subunits of cohesin Scc1 by separase, a site-specific protease (Uhlmann et al., 1999; Waizenegger et al., 2000; Yanagida, 2000). Separase activity is sequestered in a dual way: by an inhibitory chaperone securin (Pds1) and by the phosphorylation mediated by MPF (Stemmann et al., 2001) (Fig. 1.4).

6.3 DNA damage and double strand break repair

In unperturbed cells, cohesion establishment is restricted to S phase. A likely mechanism behind this time restriction is based on Cdk1-dependent negative regulation of Eco1 activity, which takes place from late S phase until mitosis. After S phase Eco1 is phosphorylated and targeted for degradation by SCF (Lyons and Morgan, 2011). However, in the response to DNA damage, Eco1 is stabilized and able to acetylate Scc1, which counteracts the anti-establishment action of Wapl (Heidinger-Pauli et al., 2009).

Induction of double strand break (DSB), where both chromatids are severed is potentially detrimental to cell survival. Upon DSB detection, cell activates checkpoints and induces cell cycle arrest in order to allow the damage to be repaired (Sjögren and Ström, 2010). Repair can be executed through re-ligation of broken ends of DNA (non-homologous end joining, NHEJ) or by homologous recombination (HR), which employs the sister chromatid or the homologous chromosome that serves as a template for replication (Wilson et al., 1997; Szostak et al., 1983). The type of DNA repair process depends on cell type and cell cycle stage.

Cohesin plays an important role in DNA damage repair. Mutations in Rad21 and other cohesin subunits result in radiation hypersensitivity in both yeast and vertebrate cells (Sjögren and Nasmyth, 2001; Sonoda et al., 2001; Schar et al., 2004). Importantly, it is the establishment of cohesion and not cohesin itself that is required for DNA damage repair, as concluded from Eco1 mutant phenotype.

Similarly to cohesin mutants, mutants of Eco1 exhibit irradiation hypersensitivity (Sjögren and Nasmyth, 2010). In addition, cohesin provides a physical topological link that keeps sister chromatids in close proximity and promotes their homologous recombination that is employed for DNA damage repair (Covo et al., 2010).

As demonstrated by immunoprecipitation experiments, in a consequence of DNA damage induction, cohesin becomes enriched in a close proximity of the break site forming a 50-100kb-long cohesin-rich domain. This process is essential for DSB repair. The re-loading of cohesin is dependent on Scc2/Scc4 complex and DNA damage response factors such as Mre11, two damage response kinases Mec1 and Tel1 as well as on phosphorylation of histone H2AX (Unal et al., 2004). Thus, yeast cohesin can be loaded *de novo* after S phase, as late as G2/M phase, at the sites of double-strand breaks and sister chromatid cohesion can be established (Ström et al., 2004; Ström and Sjögren, 2005; Unal et al., 2004). Accumulation of cohesin at the sites of double-strand breaks in mitotically dividing mammalian cells has also been observed (Kim et al., 2002). This novel form of cohesin loading was named damage-induced cohesin (DI-cohesin).

More recently, two pools of cohesin with distinct modes of binding to chromatin during the cell cycle have been identified. As observed by Gerlich and colleagues (2006) a third of nuclear cohesin is stably bound to chromosomes after replication and it persists upon until just before segregation. This pool is mainly recognized for its canonical role in sister chromatid cohesion. A second pool of cohesin is dynamically exchanged on and off chromatin every 60-120 seconds throughout the entire interphase, moving to nucleus through diffusion. This pool is absent in metaphase (Gerlich et al., 2006; McNairn and Gerton, 2009).

As shown by Ström and colleagues a single DSB is sufficient to trigger the DI-response. It is suggested that DNA damage promotes the mechanism, which inhibits cohesin formation through Wpl1. Cohesin's kleisin subunit Scc1 is post-translationally modified, which facilitates the transition from dynamic to cohesive form. This is believed to be dependent on the activity of sororin in vertebrate cells (Schmitz et al., 2007) and Eco1 acetylation (Unal et al., 2007; Heidinger-Pauli et al., 2008, 2009). These studies established a previously unknown formation of cohesion outside of DNA replication, a process crucial for the post-replicative repair of DNA damage.

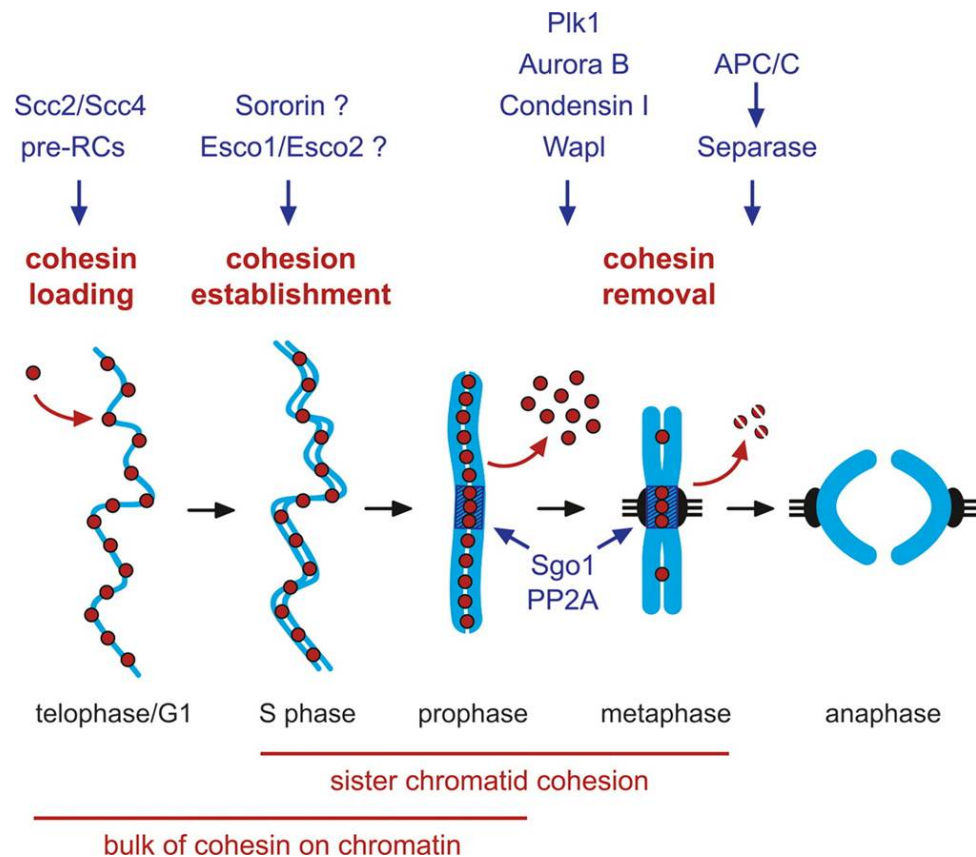


Figure 1.4 Cohesin regulation during the chromosome cycle.

*In higher eukaryotes loading of cohesin onto chromatin (represented here by red disks) occurs as early as telophase and G1 of mitosis. In *Xenopus* egg extracts, cohesin loading is associated with the assembly of pre-replicative complexes (pre-RCs). The cohesin loading depends on a binary protein complex composed of Scc2 and Scc4. Cohesion is established during S phase and additional factors such as sororin and Esco1/2 are required. Resolution of cohesion begins during prophase when the bulk of cohesin dissociates from chromosome arms in a process regulated by Plk1, Aurora B kinase and Wapl. At this time, centromeric cohesin is protected by the Sgo1-PP2A complex. Resolution of cohesion is completed at the metaphase-to-anaphase transition, when APC/C activates separase that cleaves centromeric cohesin (Adapted from Peters et al., 2008).*

6.4 Kinetochore bi-orientation and chromosome separation

Upon mitotic entry, microtubules emanating from opposite poles of the cell connect to kinetochore, the proteinaceous structure built on the centromere of each of a sister chromatids. Two kinetochores of a pair of sister chromatids, called sister kinetochores, are pulled in opposite directions by microtubules, which is counteracted by the cohesion between sister chromatids. This generates tension between them, which enables the pair of sister chromatids to be aligned at the equator of the bi-polar spindle. The connection between sister chromatids is essential for the alignment of chromosomes (Miyazaki and Orr-Weaver, 1994; Nicklas, 1997). Once all sister chromatid pairs are bi-oriented at the mitotic spindle, cohesion is destroyed at once in all chromosomes (anaphase) and chromatids are segregated to opposite sides of the cell, into the future daughter cells.

The timing of anaphase onset is controlled by a mechanism provided by the spindle assembly checkpoint (SAC) proteins that monitor kinetochore-microtubule attachment and tension generated by correct bipolar attachment (McIntosh et al., 2002). The SAC, which is conserved across eukaryotes, relies upon the activity of the following serine-threonine kinases: monopolar spindle protein 1 (Mps1) and budding uninhibited by benomyl 1 (Bub1), as well as other non-kinase components such as mitotic arrest deficient 1 (Mad1), Mad2, Bub3 and the putative pseudo-kinase Bub1-related 1 (BubR1; the human orthologue of yeast Mad3) (Hoyt et al., 1991; Li and Murray, 1991; Weiss and Winey, 1996). Mad and Bub proteins bind to the unattached kinetochore and shuttle between such kinetochores and the cytoplasm, providing the detection mechanism of missing attachment and tension. Following recruitment to unattached kinetochores, the SAC catalyses the formation of Cdc20 inhibitory complex, called mitotic checkpoint complex (MCC). MCC, a heterotetramer composed of Cdc20, Mad2, BubR1/Mad3 and Bub3, inhibits APC/C activity that leads to delayed anaphase (Chen et al., 1999). APC/C is phosphorylated by M-Cdks, which promotes its binding to Cdc20 only when all kinetochores are attached to microtubules and SAC is satisfied. This is followed by APC/C^{Cdc20}-mediated cyclin B degradation and downregulation of Cdk1 activity. Upon anaphase onset, APC/C targets for degradation the other substrate, securin, for degradation. This is essential for sudden loss of SCC (Skoufias et al., 2001). In addition, inactivation of Cdk1 results in dephosphorylation of Cdk1 substrates by protein phosphatases and enables exit from mitosis (Irniger et al., 1995; King et al.,

1995; Sudakin et al., 1995). In vertebrates, Cdk1 inactivation also contributes to separase activation (Peters, 2006).

6.5 The cohesin loading complex

Cohesin loading onto chromosomes requires the conserved binary protein complex called kollerin, consisting of a large, highly conserved protein called Nipbl (also known as Scc2, delangin or adherin, the functional homolog of budding yeast Scc2) and a less well-conserved smaller protein called Mau2 or Scc4. Mau2 and Nipbl interact with each other through their N-termini (Seitan et al., 2006; Takahashi et al., 2008). In humans, the *Nipbl* gene product has two major isoforms Nipbl A and Nipbl B, which differ at C-termini (Tonkin et al., 2004). The sequence of *Nipbl* predicts the presence of a nuclear localisation signal, nuclear export signal, seven HEAT repeats and a DNA binding domain, which implies that the Nipbl protein might be involved in numerous physical interactions. A large, highly conserved C-terminal domain of Nipbl contains five HEAT repeats, motifs implicated in protein-protein interactions that are also found in a variety of chromosome-associated proteins (Andrade et al., 2001; Neuwald and Hirano, 2000). Missense mutations in any of HEAT motifs result in cohesinopathy, Cornelia de Lange Syndrome (CdLS) (Gillespie and Hirano, 2004).

6.6 The role of the NIPBL homologs in mitosis

Inactivation of Scc2 or its homologs in yeast or immunodepletion of XScc2 from egg extract impairs the association of cohesin with chromatin (Ciosk et al., 2000; Gillespie and Hirano, 2004; Tomonaga et al., 2000). Although the precise mechanism of how the Nipbl/Scc4 catalyses cohesin loading is not yet clear, the cohesin loading complex has been proposed to promote ATPase activity of the SMC subunits (Hu et al., 2011). Knockdown of Nipbl or Scc4 reduces chromatin-bound cohesin and causes loss of cohesion in different animals, suggesting strong functional conservation (Seitan et al., 2006; Watrin et al., 2006). It has been suggested that the post-replicative cohesin recruitment induced by DSB also requires Scc2 (Strom et al., 2004; Unal et al., 2004). In addition to canonical involvement in cohesin loading, studies in yeast mitotic cells suggest that Scc2 also promotes loading of two other SMC complexes, condensin (D'Ambrosio et al., 2008) and Smc5/6 (Lindroos et al., 2006). However, it is unknown whether the condensin

or Smc5/6-related functions of cohesin loading complex are also present in mammals.

6.7 The role of Nipbl and cohesin in gene expression and its clinical relevance

As demonstrated in many organisms, mutations in the Scc2/Scc4 complex or cohesin that result in reduced levels of their expression do not lead to noticeable defects in sister chromatid cohesion but affect developmental genes expression. For example, the loss-of-function of one *Nipped-B* allele, the *Drosophila* homologue of *Nipbl*, results in reduced expression of *cut* and *Ultrabithorax (Ubx)* homeobox genes without causing defects in chromosome segregation in mitosis or meiosis (Rollins et al., 2004; Rollins et al., 1999). In zebrafish, the reduction of Rad21 levels reduces the expression of *Runx*, which is critical for embryogenesis, leading to developmental arrest (Bose and Gerton, 2010). In humans, mutations in a single copy of Smc1 β , Smc3 or Rad21, cohesin deacetylase HDAC8 or most commonly cohesin loading factor *NIPBL* is associated with Cornelia de Lange syndrome (CdLS), a multisystem developmental disorder (Deardorff et al., 2007; Krantz et al., 2004; Musio et al., 2006; Tonkin et al., 2004). Likewise in mice, heterozygous reduction of *Nipbl* mRNA level results in multiple organ system defects and transcriptional dysregulation resembling CdLS (Kawauchi et al., 2009).

7. Meiosis

7.1 The principles of meiosis

Meiosis is a specialised cell division, characterized by two consecutive rounds of chromosome segregation (meiosis I and II) without an intervening round of DNA replication. Pre-meiotic S phase generates two sister chromatids per homolog, which are linked tightly by cohesins along their entire lengths. The first meiotic division is reductional and separates homologous chromosomes, whilst the second division is equational akin to the way mitosis segregates sister chromatids. Meiosis is essential in all sexually reproducing organisms because it leads to exchange of genetic material between parental chromosomes and leads to the generation of gametes containing a single homolog of each chromosome (Petronczki et al., 2003).

Despite the fact that meiosis resembles mitosis in many respects, it is distinct by three important features. Firstly, homologous chromosomes pair and become linked to establish tension between them. This is achieved by the formation of synaptonemal complex as well as by double strand breaks (DSBs) formation. DSBs repair leads to recombination between homologous non-sister chromatids (Dobson et al., 1994). Crossovers, physical linkages between homologues, are resolved to form chiasmata that link maternal and paternal chromosomes and produce bivalent chromosomes (Page and Hawley, 2003). Secondly, unlike in mitosis, sister chromatids are required to go to the same spindle pole at the first meiotic division. This is achieved by the mono-polar attachment, where the kinetochores of sister chromatids become attached to microtubules extending from the same spindle pole (Hauf and Watanabe, 2004). Thirdly, sister chromatids have to remain connected to facilitate their bi-orientation in meiosis II. This is enabled by the two-step cohesion loss (Fig. 1.5).

Meiosis I is divided into four stages: prophase, metaphase, anaphase, and telophase. Meiotic prophase features most of the characteristic events that differentiate meiosis from mitosis and is further divided into five substages: leptotene, zygotene, pachytene, diplotene and diakinesis. The different stages of meiotic prophase are defined by the formation of a meiosis-specific structure, the synaptonemal complex.

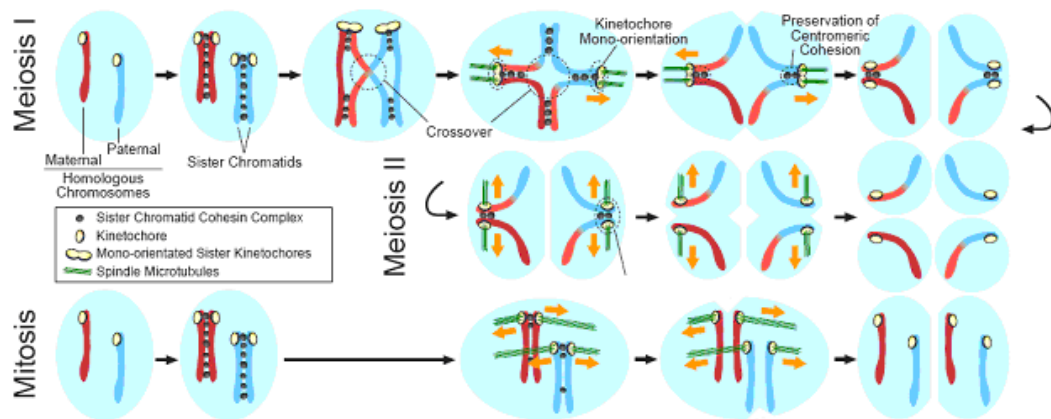


Figure 1.5 Chromosome attachment and segregation in mitosis and meiosis.

Sister chromatid cohesion is established during DNA replication and maintained until chromosome segregation into two daughter cells. It is mediated by a conserved protein complex, called cohesin (depicted by black disks). In meiosis, DNA replication is followed by recombination of sister chromatid strands between homologous paternal and maternal chromosomes, which results in crossovers. Crossovers link homologous chromosomes in addition to sister chromatid cohesion distal to crossover sites. This process is essential for the traction of maternal and paternal kinetochores toward opposite poles of the meiosis I spindles (mono-orientation) and for homologous chromosomes segregation to opposite poles during the first meiotic division. The cohesion is lost during the first meiotic division from chromosome arms, which leads to segregation of homologs. Loss of cohesion between sister centromeres allows segregation of sister chromatids in meiosis II.

7.2 Synaptonemal complex

Synaptonemal complex (SC) is a ladder-like structure that assembles at the border of paired and aligned homologous chromosomes. It is a cytological landmark, present in most sexually reproducing organisms that underlies the classification of nuclei in meiotic prophase (Page and Hawley, 2004). The synaptonemal complex functions by stabilizing the pre-synaptic alignment of the axes of the homologous chromosomes and in promoting maturation of crossovers (Handel and Schimenti, 2010). Although minor differences in the order and localisation of SC components are observed between species, a general outline of synaptonemal complex assembly is common for all of them. During pre-meiotic S phase, before homologous chromosomes pairing takes place, cohesin complexes associate broadly to the chromatin (Khetani and Bickel, 2007). The assembly of synaptonemal complex is initiated in early meiotic prophase with the formation of axial elements (AEs) along each pair of sister chromatids. In leptotene, AEs appear first thin and patchy, but progressively as chromatin condenses and chromosomes become organized in compact domains in zygotene, AEs thicken and become more prominent. The transition from leptotene to zygotene is also characterized by the appearance of pairwise associations between the AEs of homologues as they become connected by the central element (CE) and consequently AEs are called lateral elements (LEs) (Zickler and Kleckner, 1998). The central element comprises a highly ordered lattice of transverse filaments (TFs) that flank the central region. Homologue pairs become fully synapsed in the pachytene stage, during which synaptonemal complex plays an important role in the completion of crossovers. The synaptonemal complex starts to disassemble in diplotene (Kleckner, 1996). At this stage homologous chromosomes are held together by crossovers and sister chromatid cohesion.

Three classes of proteins constitute SC components. The first class is composed of proteins involved in sister chromatid cohesion and in the mouse include meiosis-specific cohesin complexes containing subunits such as Rec8, Smc1 β or Stag3 (Bannister et al., 2004; Prieto et al., 2001; Revenkova et al., 2004). The second class belongs to the meiosis-enriched HORMA domain family with a number of paralogues in different organisms. In mouse oocytes HORMAD1 and HORMAD2 are associated with unsynapsed axial elements before synapsis is achieved and after desynapsis, but are absent at synapsed regions of chromosomes when the central region associates between AEs (Wojtasz et al., 2009). The third class of SC

components is characterized by low homology to each other and in the mouse is represented by Sycp2 and Sycp3 (two LE components) Sycp1 (transverse filament protein) and Syce1, Syce2, Tex12 (components of central element) (Bolcun-Filas et al., 2007; de Vries et al., 2005; Hamer et al., 2006; Yang et al., 2006; Yuan et al., 2000).

7.3 Meiosis-specific cohesin

In meiosis cohesion between sister chromatids is established during pre-meiotic DNA replication. Meiotic cohesins are different from their mitotic counterparts. In both fission and budding yeast, the Scc1/Rad21 α -kleisin subunit is replaced by a meiotic equivalent, Rec8 (Nasmyth and Haering, 2005; Watanabe, 2004). The presence of this meiosis-specific counterpart is essential for the successful completion of meiosis, as Rec8 mutations lead to defective sister chromatid cohesion specific to meiosis (Klein et al., 1999). Similarly, Rec8 homologue inactivation in *C.elegans* results in defective chromosome synapsis and failure in meiotic double-stranded DNA breaks repair (Pasierbek et al., 2001).

Meiosis-specific subunits have been also found in mammalian cells. Mammals have two Smc1 paralogues, Smc1 α and Smc1 β (Revenkova et al., 2004). While Smc1 α is a component of the cohesin complex in both mitosis and meiosis, the presence of Smc1 β is specific to the mouse germline. Smc1 β localizes to meiotic chromosomes along the AE/LE and to the sites of future chiasmata. Smc1 α disappears in diplotene, whereas Smc1 β is lost from chromosome arms in metaphase I but stays in the centromeric region until the metaphase-to-anaphase transition of meiosis II (Revenkova et al., 2001). Smc1 β is essential for both female and male fertility (Hodges et al., 2005; Revenkova et al., 2004). Stag3 is a third mammalian homologue of Scc3 (next to Sa1/Stag1 and Sa2/Stag2) and localises to the bivalents arms in mammalian germ cells but from anaphase I onwards remains only at the centromeric region (Prieto et al., 2001; Gracia-Cruz et al., 2010). Immunoprecipitation studies demonstrated that in spermatocytes Stag3 interacts with Smc1 β and Smc3 (Revenkova et al., 2001). Mammalian meiosis-specific α -kleisin subunit, also called Rec8, is functionally essential in meiosis since Rec8 homozygous mutation results in sterility in both sexes (Xu et al., 2005). Rec8 is the first cohesin to localise along the forming structures of axial elements (Eijpe et al., 2003; Revenkova et al., 2001). During meiotic prophase, Rec8 is evenly distributed

along the chromosome axes and persists on chromosome arms into metaphase I (Parra et al., 2004). The fact that Scc1 is also detected on spermatocyte chromosomes raised a question whether Rec8 is the only meiotic α -kleisin component in mammals (Xu et al., 2004). However, recent studies demonstrated that Rec8 is solely responsible for chromosome arm and centromere cohesion at metaphase I in oocytes (Tachibana-Konwalski et al., 2010). Interestingly, expression of mutated version of Rec8, in which three separate cleavage sites are modified, results in sterility in males but not in females (Kudo et al., 2009). Recently, three groups characterized Rad21L (Rad21-like) protein, which is yet another meiosis-specific cohesin subunit (Ishiguro et al., 2011; Lee and Hirano, 2011; Gutierrez-Caballero et al., 2011). Rad21L appears to co-localise with AEs upon meiosis initiation and resides on chromosomes from the establishment of sister chromatid cohesion, through to pairing of homologs and the initiation of synapsis. It persists along AE/LE until pachytene, taking part in the formation of SC and homologous synapsis. Rad21L dissociates from chromosome axes at mid-pachytene when homologous chromosomes are synapsed and the SC is still assembled (Lee and Hirano, 2011). However, there is some disagreement on the exact time of Rad21L dissociation from chromosomes (Ishiguro et al., 2011). Rad21L is directly implicated in AEs formation, homologous synapsis and DSBs processing (Herrán et al., 2011).

7.4 Homologous recombination and chiasmata formation

Homologous recombination (HR) is a conserved aspect of DNA metabolism and plays two key functions. Firstly, HR is critical for repairing DNA double-strand breaks (DSBs), a result of DNA damage caused by e.g. replication-fork breakage in mitosis. Secondly, HR is essential for chromosomal pairing and DNA exchange during meiosis. A recombination event between two loci can result in crossovers (COs) or non-crossover (NCOs) (Cromie and Smith, 2007). In meiosis, recombination of sister chromatid strands between homologous paternal and maternal chromosomes creates crossovers, providing the basis of chiasmata that link homologues together and produce bivalents. Crossing over together with sister chromatid cohesion along chromosome arms distal to crossover is important for the alignment of chromosomes on the metaphase plate and consequent accurate segregation. Failure of a chromosome pair to undergo at least one crossover can result in both homologues segregating to the same spindle pole during meiosis I, leading to chromosome mis-segregation (Cromie and Smith, 2007). In addition to this mechanical role, the exchange of genetic information between chromosomes of

different parental origin during generation of crossover allows for increased genetic variability in offspring.

The recombination process is initiated by the generation of a number of DNA double-strand breaks (DSBs) along each of the four chromatids (paired homologs and their respective sister) mediated by Spo11 endonuclease (Keeney et al., 1997). The breaks are repaired through a series of steps involving resection, synthesis and ligation using the homologous non-sister chromatid as a template for recombinational repair.

In mammals, the generation of DSBs result in the rapid phosphorylation of histones H2AX and H2B conducted by ataxia telangiectasia mutated (ATM), yielding in a specific modified form called γ -H2AX that accumulates at the sites of DSBs (Mahadevaiah et al., 2001). After removal of Spo11, the 5' ends are resected to yield single-strand 3' protruding overhangs on either side of the break. One 3' protruding overhang invades a homologous non-sister chromatid, which is crucial for generating exchanges instead of repairing a break using sister sequences, as observed in mitotic cells. The 3' end of the invading strand from the paternal chromatid is paired with a complementary strand from a maternal chromatid, creating a template for repair synthesis (Petronczki et al., 2003). This process is mediated by a cascade of repair proteins loaded onto the damage site in order to restore DNA correctly. The first loaded recombinases, Rad51 and Dmc1 localise to early recombination nodules (ENs) along the developing AEs (Schwacha and Kleckner, 1997; Hunter and Kleckner, 2001; Keeney, 2001). The 3' overhang at the other side of the break subsequently follows the same fate. Eventually 5' end left over from resection of the original DSB invades and is ligated to the newly synthesized DNA, creating a joint molecule. This exchange generates a structure called double Holliday junction (DHJ) (Petronczki et al., 2003).

In mid-pachytene the final step of recombination takes place, which is the resolution of DHJs. This process is essential for disjunction of the maternal from the paternal chromosome and generation of exchanges that will link them until the anaphase I. This coincides with disappearance of phosphorylated H2AX (γ -H2AX) and Rad51 (Fernandez-Capetillo et al., 2004; Lee et al., 2011). Resolution of DHJs requires horizontal or vertical cleavages of a pair of strands at each end of the junction and their reciprocal ligation. The CO is generated when one junction is cleaved horizontally and the other vertically (Petronczki et al., 2003). During pachytene, COs

are marked by the mismatch repair proteins Mlh1 and Mlh3, which also localise to late recombination nodules (LNs) (Anderson et al., 1999). These signals replace Rad51 and γ -H2AX that dissociate from chromosomal axis in late pachytene (Lee et al., 2011) (Fig. 1.6).

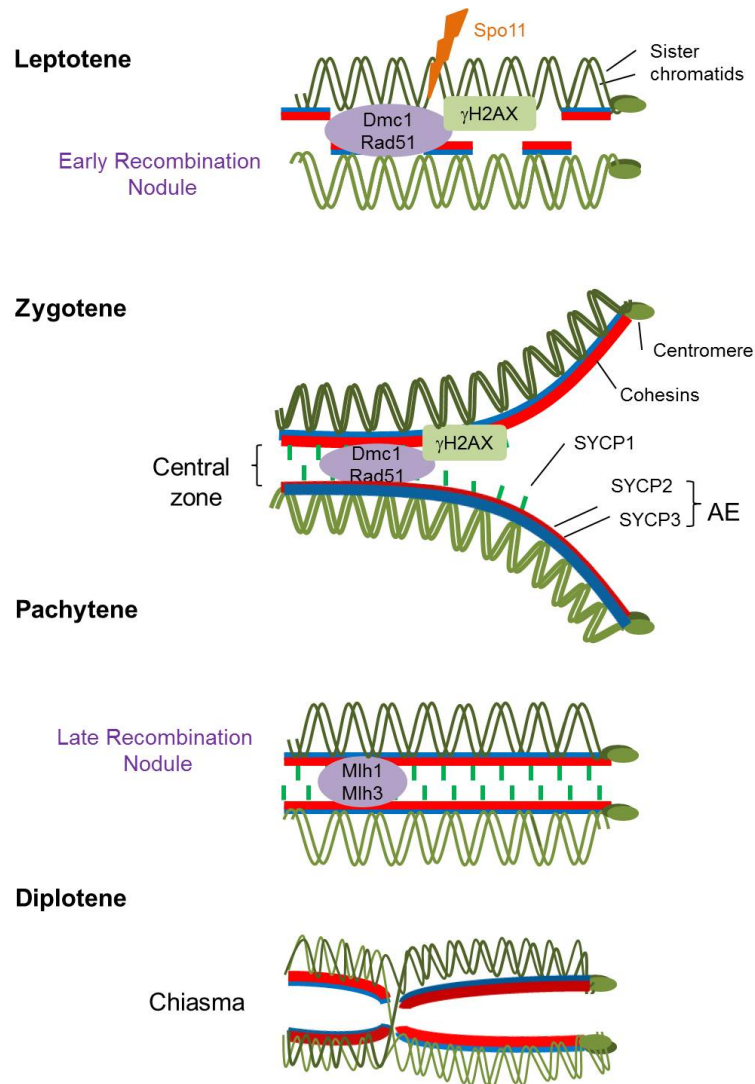


Figure 1.6 Formation of synaptonemal complex and chromosome dynamics during meiotic prophase of mouse meiosis.

During **leptotene**, homologous chromosomes begin to align and chromosomal scaffold begins to form through the assembly of axial elements (AEs) from cohesins and synaptonemal complex-specific proteins (Sycp3 and Sycp2). Spo11 introduces DSBs along each of four chromatids, which provide the substrate for recombination (the two chromatids of the upper homologue are dark green lines, and the two chromatids of the lower homologue are light green lines). DNA breaks are recognised by homologous recombination repair machinery leading to phosphorylation of histone H2AX to a special form called γ -H2AX and are resected, which promotes binding by the Dmc1 and Rad51 proteins (related to recombinase A (RECA)) and other proteins. They all co-localise to early recombination nodules along the developing AEs. In **zygotene**, pairing extends and synapsis is initiated, generating the synaptonemal complex. Axial elements (AEs) become connected by central element (containing Sycp1) in pairwise associations, leading to the generation of lateral elements (LEs). In **pachytene** synapsis is completed. During pachytene, a subset of meiotic recombination sites matures into crossovers marked by the mismatch repair proteins MutL protein homologue 1 (Mlh1) and Mlh3 that co-localises to late recombination nodules. When recombination is completed, chromosomes desynapse during **diplotene**. At this stage, homologues are held together by the recombination sites, crossovers. CE; central element, LE; lateral element. (Adapted from Handel and Schimenti, 2010).

7.5 The role of the NIPBL homologs in meiosis

Depletion of the NIPBL ortholog (*Scs2*) in yeast results in defective sister chromatid cohesion, nuclear division and transcriptional dysregulation of multiple genes, including meiotic cohesin subunits (Lin et al., 2011). Nipbl homologs have been shown to localize to meiotic chromosomal axes in fruit flies (*Nipped-B*), nematodes (*Scs-2*), plants (*AtScs2*) and fungi *Coprinus cinereus* (*Rad9*) (Cummings et al., 2002; Gause et al., 2008; Lightfoot et al., 2011; Sebastian et al., 2009). Downregulation of meiotic *Rad9*, Nipbl homolog in fungi *Coprinus cinereus* leads to DNA repair defects (Valentine et al., 1995) and failure to assemble synaptonemal complex (SC) (Cummings et al., 2002; Seitz, Tang et al., 1996). Similar phenotype has been observed in *Arabidopsis thaliana* when *AtScs2* is inactivated (Sebastian et al., 2009). In *Caenorhabditis elegans*, mutation in *Scs2* prevents it from loading cohesin that leads to multiple cytological defects and failure to repair Spo-11 induced breaks (Lightfoot et al., 2011). Thus, Nipbl is the candidate protein responsible for cohesin loading onto DNA in mammalian meiosis.

7.6 Stepwise cleavage of cohesin during meiosis

Unlike mitosis, where chromosome segregation is triggered by a complete loss of sister chromatid cohesion at anaphase onset during meiosis there are two distinct phases of chromosomal cohesion dissociation. During the first meiotic division, at anaphase I, cohesion is lost along chromosome arms together with the resolution of chiasmata. In contrast to mitosis, separase activity is required for proteolytic cleavage of meiotic cohesin at chromosome arms, which leads to separation of homologous chromosomes (Buonomo et al., 2000; Kudo et al., 2006). However, prior to its removal the cohesion along chromosome arms distal to crossover site with respect to the centromere provides physical linkage is essential to hold bivalent structure together (Petronczki et al., 2003). Centromeric cohesion is protected from the action of separase during the first meiotic division, similarly to mitosis and its preservation until second meiotic division is required for accurate segregation of parental and recombined sister chromatids during anaphase II. The protection depends on a class of proteins called shugoshins (*Sgo1*) associated with PP2A (Watanabe, 2005; Kitajima et al., 2006). The centromeric cohesion is resolved during meiosis II and results in sister chromatids separation (Miyazaki and Orr-Weaver, 1994; Petronczki et al., 2003).

8. Folliculogenesis

Mammalian oocytes develop from primordial germ cells (PGCs) that migrate and colonize the gonadal ridge to become oogonia during embryonic life. PGCs form oogonial nests in which they undergo multiple divisions and stay clustered together (Pepling and Spradling, 1998, 2001). The process of folliculogenesis is initiated when flattened epithelial pre-granulosa cells are recruited to the oocyte in the foetal (mid-gestation in humans) or neonatal (2-4 days postnatal in rodents) ovary and surround germ cell resulting in primordial follicle formation (Sforza and Forabosco, 1998; Hirshfield, 2000). Primordial follicles remain dormant, arrested in meiotic prophase (Baker and Franchi, 1967) for up to 50 years in humans, until they are recruited into growth in a gonadotropin independent process called initial recruitment (McGee and Hsueh, 2000; Greenwald, 1972). The primordial pool of follicles is progressively depleted as follicles are recruited into further development or lost through atresia (Greenwald, 1972). The first signs of primordial follicle recruitment are the cuboidalization of the squamous pre-granulosa cells into cuboidal granulosa cells (GCs) and the initiation of transcription (Da Silva-Buttkus et al., 2008). Oocyte growth involves also development of large nuclei referred to as germinal vesicle (GV). Initially primordial follicle develops into transitional stage, followed by transformation into a primary follicle. In primary follicle granulosa cells start to proliferate and once they grow beyond a single layer, follicle is called a secondary follicle. The growth phase is characterized by extensive cytoplasm reorganisation and simultaneous production of proteins, lipids, mRNAs and a protein coat called zona pellucida (ZP). Further follicle growth is dependent on gonadotrophic hormones. Rapid multiplications of GCs form multiple layers and they become surrounded by a layer of theca cells. The following inward divisions of the outermost layer of GCs generate a fluid-filled cavity (antrum) that divides a population of granulosa cells into cumulus cells, surrounding the oocyte, and mural granulosa cells, located next to the basal lamina. The follicle growth takes 3 weeks in mice, after which these oocytes give rise to fully-grown competent oocytes included within the antral follicle. Hormonal cues that lead to ovulation induce oocytes to resume and complete meiosis I. After ovulation, the remaining of the follicle develops into a temporary endocrine gland, corpus luteum (CL) that provides hormones to support the following pregnancy (Kidder and Vanderhyden, 2011; Fair, 2003).

The pituitary gland is a source of gonadotropins that regulates the processes of ovarian folliculogenesis, oogenesis and ovulation. The biological activity of gonadotropins on the ovary is exerted by multiple paracrine factors that serve as intra-ovarian signals as well as by bi-directional cell to cell communication that is mediated by gap junction channels. These bi-directional signals operate within the oocyte-granulosa cell complex and they regulate oocyte and follicle growth and differentiation and are important for ensuring oocyte developmental competence (Kidder and Mhawi, 2002; Kidder and Vanderhyden, 2011).

9. Meiotic development of mammalian germ cells

Before birth, the entire population of mammalian oocytes undergoes pre-meiotic DNA replication and establishes cohesion between sister chromatids. Immediately after replication, during early stages of meiotic prophase, homologous chromosomes create crossovers, which is followed by a cell cycle arrest at a late stage of meiotic prophase (a period also known as dictyate; equivalent to G2-phase). Soon after, each of these dormant oocytes becomes enclosed by somatic cells forming a primordial follicle. Some of the primordial oocytes are recruited into the growing pool, which leads to follicle growth in accordance with follicular expansion. Following oocyte growth, these cells develop meiotic competence and are ovulated upon LH surge. This triggers oocytes to resume and complete meiosis I. Meiotic resumption is a result of the upregulation of MPF, which induces the breakdown of the membrane of oocyte's large nucleus. This is referred to as GV breakdown (GVBD). GVBD is the first visible manifestation of meiotic resumption followed by condensation of the interphase chromatin and spindle formation (Jones, 2004). Bi-orientation of bivalent chromosomes where four chromatids are connected by a chiasma (crossover) and sister chromatid cohesion mediated by cohesin is established at metaphase I (Petronczki et al., 2003). Chiasma resolution is facilitated by dissociation of cohesion at chromosome arms in anaphase I, which creates univalent chromosomes containing two chromatids connected by cohesion at centromeres. Completion of first meiotic division is marked by cytokinesis, which is highly asymmetrical and results in the extrusion of a small cell called the first polar body (PB1). Notably, the arrest between the creation of crossovers (before birth) and homologs bi-orientation and segregation resulting in first polar body extrusion (PBE) (in adulthood) is extremely protracted and lasts up to 50 years in humans and more than a year in mouse (Hassold and Hunt, 2001) (Fig. 1.7). Following first PBE, oocytes progress to metaphase of the second meiotic division, after which they

become arrested for the second time under the influence of the cytostatic factor (CSF). The following fertilization results in the release from the second meiotic arrest and triggers chromosome segregation. One set of chromosomes is retained in oocyte and the other segregated into second polar body (PB2) (Kubiak et al., 1993; Ciemerych and Kubiak, 1999; Angell, 1997; Masui, 2001; Holt and Jones, 2009; Jones, 2004).

The protracted nature of female meiosis contrasts with meiotic progression in males. Diploid germ cells that give rise to sperm do not enter meiosis in the embryo, but instead they arrest at an early stage of mitotic cell cycle in the embryonic testis. After birth these cells resume mitotic proliferation, while after sexual maturation spermatogonia are transformed into primary spermatocytes, the largest germ cells in the seminiferous tubules. Primary spermatocytes subsequently undergo meiosis, each forming four haploid spermatids. The spermatids are gradually transformed into four mature sperms in a process of spermiogenesis. The entire process of spermatogenesis, which includes spermiogenesis, takes approximately 2 months. When spermiogenesis is complete, the sperms enter the seminiferous tubules (Wistuba et al., 2007).

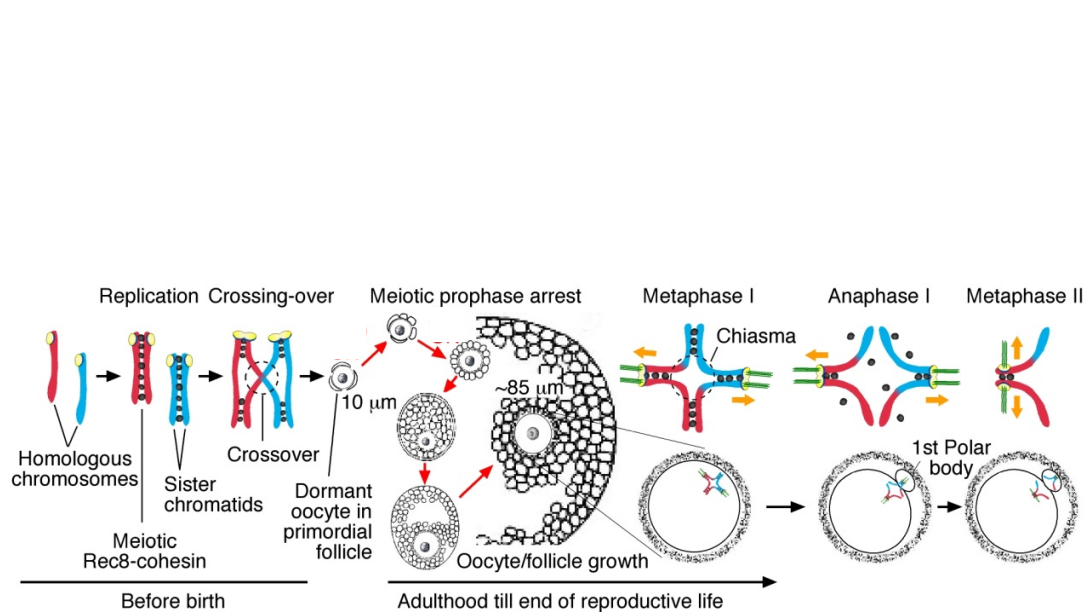


Figure 1.7 Meiotic development of mammalian oocytes.

After mitotic proliferation, the entire population of mammalian female germ cells enter meiosis by replicating DNA and establishing cohesion, which in mice occurs within the embryonic ovaries 13-14 days after conception. After replication, during early stages of meiotic prophase, homologous recombination creates crossovers by the time of partum (E14-E19) and primordial oocytes enter a cell cycle arrest in a late stage of meiotic prophase arrest (all these events are before birth). Throughout female life, after sexual maturation, some of oocytes are recruited into a growing pool. Around 3 weeks later oocytes give rise to fully-grown meiotically competent oocytes included within the antral follicles. Upon meiotic resumption (entry into meiosis I) upregulation of cyclin-dependent kinase 1 (Cdk1) induces germinal vesicle breakdown (GVBD). Bi-orientation of bivalent chromosomes where four chromatids are connected by a chiasma (crossover) and sister chromatid cohesion mediated by cohesion is established at metaphase I. Chiasma resolution is facilitated by dissociation of cohesion at chromosome arms in anaphase I. This creates dyads containing two chromatids connected by cohesion at centromeres. Extrusion of the first polar body (PB1) represents completion of meiosis I.

9.1 The SAC in mammalian meiosis

Although the existence of the SAC in mouse oocytes has been questioned in the past, its functionality has been confirmed when the disruption of any one of a SAC components in mouse oocytes accelerated the first meiotic division progression as a result of premature securin and cyclin B1 destruction, leading to aneuploidy (Hached et al., 2011; Homer et al., 2005; McGuinness et al., 2009; Niaux et al., 2007; Tsurumi et al., 2004).

APC/C is the only known target of SAC. SAC inhibits the activity of APC/C^{Cdc20} and delays the onset of anaphase if any chromosome is not correctly attached to bi-polar spindle (Niaux et al., 2007). Key components of SAC such as Mad1 and Mad2, BubR1 and Bub3 localise to the kinetochore until stable interaction between chromosome and microtubule is established (Homer et al., 2005; Leland et al., 2009; McGuinness et al., 2009; Niaux et al., 2007). Overexpression of Mad2 results in cell cycle arrest during meiosis I, while the expression of dominant negative version of Mad2 interferes with the correct spindle checkpoint arrest (Niaux et al., 2007; Wassmann et al., 2003). It has been shown that Mad2 interacts directly with Cdc20 and inhibits APC/C activation until the bi-orientation is achieved (Wassmann et al., 2003). Depletion of Bub1, another SAC component, results in precocious resolution of chiasmata, defective bi-orientation of bivalents, massive chromosome missegregation at meiosis I and precocious loss of centromeric cohesion (McGuinness et al., 2009), which is consistent with the abrogation of SAC function.

On the other hand, oocytes containing even several univalents can still progress to metaphase II stage, regardless of SAC activation in response to meiotic spindle defects during meiosis I, which may account for high incidence of aneuploidy (LeMaire-Adkins et al., 1997; Kouznetsova et al., 2007). Since stable attachment of only part of chromosomes is sufficient to satisfy SAC requirements in oocytes, this implies that SAC satisfaction might be less stringent in oocytes than in spermatocytes (Gui and Homer, 2012; Nagaoka et al., 2011; Kolano et al., 2012; Lane et al., 2012).

9.2 Aneuploidy in oocytes

Errors in chromosome segregation during gametogenesis produce aneuploid gametes that contain either too many or too few chromosomes. Chromosome mis-segregation during meiosis may directly contribute to infertility, genetic abnormalities in embryos, miscarriages and congenital defects including Trisomy 21 (Down Syndrome). Although chromosome mis-segregation can arise during both meiotic divisions, the vast majority of aneuploidy is the consequence of errors occurring specifically during the first meiotic division in oocytes with relatively minor contributions from second meiotic division or from spermatogenic errors. Notably, the chances of these female meiosis I segregation errors increase with the advancing maternal age, which currently is considered the strongest risk factor for embryonic aneuploidy in humans. Whereas the risk of embryonic trisomy is about 2-3% in women in their twenties, it increases to more than 30% at the age of forty (Hassold and Hunt, 2001; Hunt and Hassold, 2002).

Given the prominence of the SAC in prevention of aneuploidy during the first meiotic division in oocytes, it was proposed that age-related decline in SAC integrity in oocytes might be an important factor contributing to the rise in aneuploidy associated with maternal age. In support of this hypothesis, it was suggested that the components of SAC are less abundant in oocytes and ovaries from older mice (Baker et al., 2004; Pan et al., 2008) and older women (Battaglia et al., 1996; Steuerwald et al., 2001). However, other studies suggested that SAC functions equally efficient irrespective of oocyte age (Duncan et al., 2009; Lister et al., 2010). Thus, the involvement of dysfunctional SAC to maternal age related aneuploidy is still unknown (Kubiak, 2012).

More recently, several reports have focused their attention on the cohesin complex. The prevailing hypothesis is that segregation errors in oocytes from older mice arise in large part because of unusually protracted nature of meiotic prophase (Warburton, 2005; Hassold and Hunt, 2001). The integrity of bivalent-associated cohesion, which is established during pre-meiotic S phase in foetal life, must be maintained for the protracted time, a staggering 40-50 years in humans. Therefore, the hypothesis of cohesin degradation during protracted meiotic arrest was proposed to lead to the non-disjunction or pre-division of homologous chromosomes, thereby leading to age-dependent aneuploidy in mammalian females, including humans (Eichenlaub-Ritter et al., 2004; Hodges et al., 2005;

Wolstenholme and Angell, 2000). Several recent studies provide compelling evidence supporting this hypothesis. The analysis of *Smc1 β* -null oocytes revealed synaptic defects in form of shortened synaptonemal complex. In a direct consequence *Smc1 β* deficiency is associated with reduction in the number of crossovers as assessed by the number of Mlh1 foci on pachytene SC, despite their unaltered distribution. (Hodges et al., 2005; Revenkova et al., 2004). This at later stages gave rise to distally localised chiasmata in 85% of chromosomes, where cohesion could not be established distally to crossover sites. These chromosomes were not able to sustain tension exerted by microtubules attachment during prometaphase I, resulting in precocious segregation of bivalents followed by the loss of sister chromatid cohesion in metaphase II. Hodges and co-workers observed that the frequency of these defects were significantly higher in older mice (Hodges et al., 2005). This observation was the first evidence proposing the link between gradual loss of cohesins and age-related aneuploidy in mouse. This discovery has been followed by a series of studies on non-transgenic mice. In ageing-accelerated wild-type mice the advancing maternal age was associated with the decline of the amount of cohesins bound to chromosomes in oocytes consequently followed by the increase in segregation errors (Liu and Keefe, 2008; Chiang et al., 2010; Lister et al., 2010). In addition to age-related cohesin loss during meiotic prophase arrest, also Sgo2 (shugoshin) that serves to protect cohesin, is progressively lost (Lister et al., 2010).

Over the course of my PhD it was observed that remarkably, deleting the gene encoding meiosis-specific cohesin subunit *Smc1 β* in mouse knockout model shortly after birth does not affect the structure of bivalents or chromosome segregation during first meiotic division (Revenkova et al., 2010a), which implies that *de novo* synthesis of *Smc1 β* during meiotic prophase is dispensable. Another work further demonstrated stable nature of cohesin in arrested oocytes. Expression of TEV (Tobacco Etch Virus)-protease cleavable mutant of Rec8 before the pre-meiotic S phase creates oocyte bivalents that are connected by TEV-sensitive cohesion. However, the expression of wild-type Rec8 (TEV-resistant) from a BAC transgene during the oocyte's growth phase does not make cohesion resistant to TEV, suggesting that Rec8 synthesized during oocyte growth does not serve for functional cohesion (Tachibana-Konwalski et al., 2010).

10. The APC/C in mammalian oocytes

An accumulating body of evidence suggests that APC/C is involved in practically every stage of mammalian oocyte development, namely G2/prophase arrest, the G2-M transition, prometaphase I, the anaphase-to-metaphase transition, the transition between the first and second meiotic division, CSF-mediated meiosis II arrest and calcium-related events at fertilization. Consistent with this, the depletion of APC/C activity results in the major fertility defects. (Homer et al., 2009; Marangos et al., 2007; Reis et al., 2006; Holt et al., 2011; McGuinness et al., 2009; Holt et al., 2010; Marangos et al., 2008; Schindler et al., 2009a and 2009b; Reis et al., 2007; Shoji et al., 2006; Chang et al., 2004; Malureanu et al., 2009; Herbert et al., 2003). As mentioned before, during mitosis APC/C is activated by binding first to Cdc20 (prometaphase to anaphase), which is then followed by APC/C activation by binding with Cdh1 during mitotic exit. In mammalian oocytes, the sequence in which APC/C becomes activated by Cdc20 and Cdh1 is reversed. APC/C^{Cdh1} is active at an earlier stage than in mitosis, during meiotic prophase arrest and prometaphase I. During late prometaphase the activity of APC/C^{Cdc20} rises and triggers segregation of homologous chromosomes (Homer, 2013).

11. Meiotic prophase arrest of oocytes

The meiotic prophase arrest in the follicle-enclosed oocyte is associated with low MPF activity, while the meiotic resumption (entry into M-phase) is mediated by MPF upregulation in the nucleus (Sagata, 1996). The nucleus is the main site for the Cdk1 function to facilitate GVBD followed by condensation of the interphase chromatin, spindle formation and completion of meiosis I. Meiotic resumption can be triggered by the preovulatory, luteinizing hormone (LH) surge (*in vivo*) (Jones, 2004; Eppig et al., 2004) or by the mechanical release of the oocyte from the follicle and subsequent *in vitro* culture (Pincus and Enzmann, 1935). Cdk1 is the sole Cdk that is essential and sufficient to drive meiotic resumption and its loss cannot be compensated by any other Cdks (Adhikari et al., 2012). Multiple signaling pathways are involved in the regulation of MPF activity during the meiotic prophase arrest and meiotic resumption.

11.1 Regulation of Cdk1 by cAMP

The inhibitory phosphorylation of Cdk1 is induced by high levels of second messenger molecule cyclic adenosine 3',5'-monophosphate (cAMP) (Mehlmann, 2005). cAMP activates protein kinase A (PKA), while PKA in turn simultaneously activates inhibitory kinase Wee1B (Conti et al., 2002; Han, Chen et al., 2005; Kovo et al., 2006) and inactivates phosphatase Cdc25B. Whilst Wee1B localises to the oocyte nucleus and inhibits Cdk1 there, another inhibitory kinase Myt1 inhibits Cdk1 activity in the cytoplasm (Oh et al., 2010). Cdc25B dephosphorylating activity is essential for Cdk1 activity and meiotic resumption in mouse oocytes (Han et al., 2005; Lincoln et al., 2002; Pirino et al., 2009). Therefore, cAMP is at the top of a cascade of events leading to the inactivation of MPF (Von Stetina and Orr-Weaver, 2011).

A high level of cAMP required to maintain meiotic arrest is presumably produced by the oocyte itself, however several lines of evidence have also indicated that cAMP is generated by somatic follicular cells surrounding the oocytes and transported into the oocyte through gap junctions (Dekel, 1988). Activation of the G-proteins Gs mediated by the G protein-coupled receptor (GPCR) localised on the oocyte plasma membrane stimulates adenylyl cyclase (AC) enzyme involved in cAMP synthesis, together known as GPCR/Gs/AC pathway (DiLuigi et al., 2008; Freudzon et al., 2005; Hinckley et al., 2005; Horner et al., 2003; Kalinowski et al., 2004; Mehlmann, 2005; Mehlmann et al., 2004; Mehlmann et al., 2002). This is enhanced by guanosine 3',5'-cyclic monophosphate (cGMP) that diffuses through gap junctions from the surrounding follicular cells into the oocyte, where it inhibits cAMP hydrolysis mediated by oocyte-specific phosphodiesterase 3A (PDE3A). These events promote the maintenance of high levels of cAMP (Norris et al., 2009; Sela-Abramovich et al., 2008; Vaccari et al., 2009; Zhang et al., 2010). In addition, cGMP production can also be promoted by mural granulosa cells that secrete natriuretic peptide precursor type C (Nppc) that consequently activates the guanylyl cyclase, natriuretic peptide receptor 2 (Npr2), expressed in cumulus granulosa cells (Zhang et al., 2010) (Fig. 1.8 A).

11.2 Regulation of cyclin B1 levels - APC/C^{Cdh1} activity control in mammalian oocytes

Another key contributor suppressing MPF activity is to prevent cyclin B1 accumulation in the nucleus. Indeed, the overexpression of cyclin B1 in GV stage oocytes promotes GVBD even under conditions in which cAMP levels are chemically sustained using phosphodiesterase inhibitors (Ledan et al., 2001; Reis et al., 2006). Recently it was proposed that the reduction of cyclin B1 levels in GV oocytes is mediated by APC/C and its co-activator Cdh1 (Holt et al., 2011; Reis et al., 2006) (Fig 1.7 A). The importance of APC/C^{Cdh1} in restraining the nuclear accumulation of cyclin B1 was demonstrated by the *in vitro* depletion of Cdh1 in fully grown oocytes, which resulted in their increased susceptibility to undergo GVBD even in the presence of drugs that inhibit GVBD (Reis et al., 2006). Further, this mechanism has been shown to be involved in sustaining the pool of oocytes arrested in meiotic prophase in the ovary. *In vivo* inactivation of *Fzr1*, gene encoding Cdh1, from growing and mature oocytes using Cre-LoxP recombination leads to premature spontaneous resumption of meiosis even within inhibitory cAMP-rich environment in the ovary, which was related to increased cyclin B1 levels (Holt et al., 2011). Thus, inhibiting MPF activity associated with the maintenance of meiotic prophase arrest requires continuous degradation of cyclin B1.

Unexpectedly, the accompanying mechanism to support APC/C^{Cdh1} activity is associated with the SAC protein BubR1 (Homer et al., 2009), whose knockdown results in Cdh1 instability. APC/C^{Cdh1} is also regulated by Cdc14B phosphatase (Schindler and Schultz, 2009) that counteracts Cdk1-mediated inhibition of APC/C^{Cdh1}. Together, BubR1 and Cdc14B are important factors for supporting APC/C^{Cdh1} activity and maintaining meiotic prophase arrest. This is counterbalanced by mechanisms employed to limit cyclin B proteolysis. Early mitosis inhibitor 1 (Emi1) inhibits APC/C^{Cdh1}-dependent cyclin B1 destruction and enables oocytes to enter meiosis I (Marangos et al., 2007). The activity of Emi1 is restricted to the time when fully-grown oocytes are due to respond to hormonal surge. Low MPF activity also favours hypophosphorylation and activation of PP1, protein phosphatases (PP) that localise to the nucleus. This in turn keeps meiotic phosphoproteins, like nuclear lamins that maintain structural integrity of the nuclear envelope, in hypophosphorylated state (Adhikari et al., 2012; Wang et al., 2004; Smith et al., 1998; Swain et al., 2003). (Fig. 1.8 A).

11.3 Meiotic resumption

Meiotic resumption triggered by the LH surge or release from the follicle leads to the end of a signal transduction cascade mediated by the surrounding follicular cells that eventually results in the activation of nuclear MPF. Closure of gap junctions throughout the follicle stops the supply of cGMP to the oocyte (Dekel et al., 1981; Dekel et al., 1996; Kidder and Mhawi, 2002, Granot and Dekel, 1994). This in turn increases cAMP hydrolysis by PDE3A. Reduced cAMP and PKA activity leads to increase in Cdc25B activity and at the same time reduces Wee/Myt1 kinase activity leading to Cdk1 dephosphorylation and catalytic activation (Lincoln et al., 2002; Duckworth et al., 2002; Zhang et al., 2008; Pirino et al., 2009; Han et al., 2005). Cyclin B, which is primarily cytoplasmic in GV oocytes translocates to the nucleus (Marangos and Carroll, 2004). Active MPF phosphorylates and inactivates PP1 that promotes maintenance of the phosphorylation status of other Cdk1 substrates. Active MPF phosphorylates nuclear lamin proteins, which causes their depolymerisation and GVBD (Peter et al., 1990) (Fig. 1.8 B).

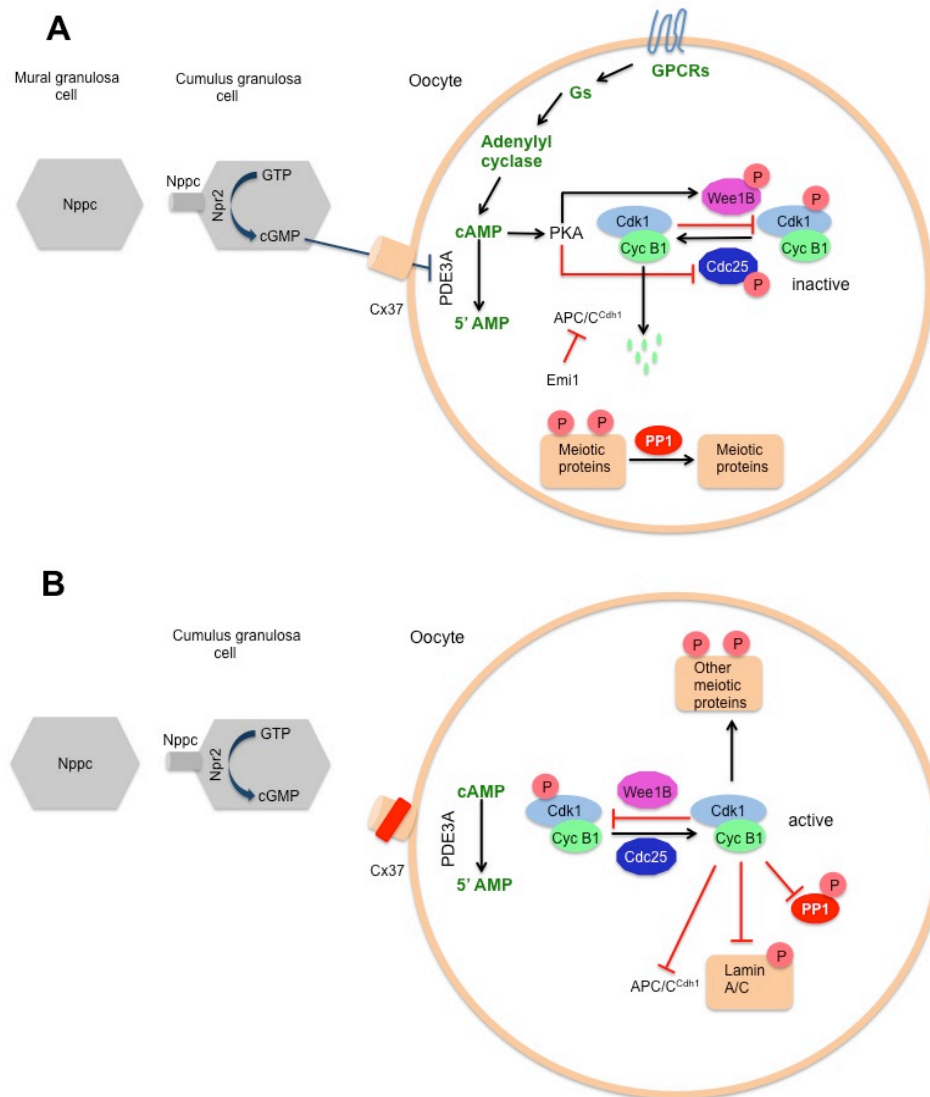


Figure 1.8 Cell signaling regulation of MPF during meiotic prophase arrest and after a preovulatory surge.

(A) Meiotic prophase arrest. High levels of cAMP within the mammalian oocyte are essential to keep MPF in an inactive state during meiotic prophase arrest. cAMP is produced by the oocyte itself via the activation of the GPCR/Gs/adenylyl cyclase pathway. Mural granulosa cells induce the generation of cGMP by secreting Nppc, a ligand of Npr2 (a guanylyl cyclase) present in cumulus granulosa cells. cGMP diffuses into oocytes through Cx37 gap junctions and inhibits PDE3A from hydrolysing cAMP in the oocyte. cAMP activates PKA that in turn activates the Wee1B kinase and inhibits the Cdc25B phosphatase leading to the inactivation of Cdk1. Constant degradation of cyclin B1 (cycB1) by APC^{Cdh1} prevents MPF activation in oocytes arrested in meiotic prophase. Emi1 serves to limit APC^{Cdh1}–mediated proteolysis of cyclin B1 and inhibits APC^{Cdh1} thereby allowing cyclin B1 accumulation required for GVBD. Low MPF activity promotes the activation of PP1 that constantly dephosphorylates meiotic proteins. **(B)** Meiotic resumption. As a result of preovulatory LH surge, gap junctions are shut throughout the follicle, which results in the reduction of cGMP in the oocyte. This in turn results in the increase in cAMP hydrolysis by PDE3A. Low levels of cAMP and PKA are not sufficient to activate Wee1B and inactivate Cdc25B. Consequently Cdk1 becomes dephosphorylated and catalytically active. MPF activity inactivates PP1, and this in turn promotes the maintenance of the phosphorylation of other Cdk1 substrates e.g phosphorylation of lamin A/C causes the GVBD. P-phosphorylation (Adapted from Adhikari and Liu, 2013).

12. Regulators of primordial follicles activation

Recruitment of follicles to the primary follicle stage or the maintenance of primordial follicles in a dormant state requires close bi-directional communication between the oocyte and the surrounding somatic cells (McLaughlin and McIver, 2008). Members of the transforming growth factor- β (TGF- β) superfamily are markedly key players in follicle recruitment. In the primordial follicle, the pre-granulosa cells express kit ligand (KL) and leukaemia inhibitory factor (LIF), a multifunctional cytokine, while oocytes and theca cells express KL receptor, c-kit. The expression of KL and LIF as well as KITL and KIT interaction have been shown to be essential for primordial follicles activation and growth (Parrott and Skinner, 1999; Yoshida et al., 1997). c-kit-KL interaction activates multiple pathways, such as RAS, RAF, mitogen activated protein kinase (MAPK), Protein Kinase B (AKT) or phosphatidylinositol -3- kinase (PI3K) (Kinoshita et al., 1997). Bone morphogenetic proteins (BMPs) BMP-4 and BMP-7, members of TGF- β superfamily, are positive regulators of primordial follicle activation (Lee et al., 2001). GDF-9, BMP-15 and BMP-6, three other TGF- β superfamily members that are selectively expressed in oocytes from early folliculogenesis are also essential for primordial follicle progression (Jaatinen et al., 1999; McGrath et al., 1995). In contrast, anti-Mullerian hormone (AMH) has an inhibitory role on the initiation of primordial follicle growth (Durlinger et al., 1999; Durlinger et al., 2002).

Recently, the PI3K signalling pathway has been implicated in maintaining dormancy and survival of primordial follicles (Liu et al., 2006; Reddy et al., 2008, 2009). It is proposed that PI3K regulation is stage specific and various levels of PI3K signalling may regulate many states of primordial oocytes through other regulatory proteins (Epifano et al., 1995; Gallardo et al., 2008; Reddy et al., 2008). The basal PI3K activity that is negatively regulated by phosphatase and tensin homolog (PTEN) has been proposed to maintain follicular survival of the primordial follicle pool. Inactivation of negative regulator PTEN in primordial follicles leads to enhanced PI3K signalling and as a result the entire pool of primordial follicles becomes prematurely activated and further undergo atresia during early adulthood leading to premature ovarian failure (POF) (Reddy et al., 2008). These results indicate that PTEN is an indispensable molecule in maintenance of primordial follicle dormancy. PTEN, Foxo3a, Pdk1 and other factors such as negative regulators of mTOR, mammalian target of rapamycin, have been shown to be the part of a signalling

cascade PI3K/PTEN-Akt-mTORC1-S6K-rp63 that controls the initiation of oocyte growth during primordial follicle activation (Reddy et al. 2008; Reddy et al., 2009; Castrillon et al., 2003). However, factors outside of PI3K and TGF- β signalling were also reported to control follicular activation. p27^{Kip1} (p27), a Cdk1 inhibitor, known for its ability to suppress the cell cycle progression, is expressed in the nucleus of primordial and primary oocytes in mice. Mice deficient in p27 have their dormant follicles activated at once in the time around puberty that leads to POF (Rajareddy et al., 2007). Taken together, these studies and many other not mentioned here demonstrate that follicle activation process is controlled by many positive and negative factors. No single factor seems to have an overpowering control, instead they balance each other for the controlled follicle recruitment.

13. Apoptosis in the ovary

Extensive germ cell death is a crucial process for the establishment of the ovarian reserve of high-quality oocytes that will support female fertility throughout the reproductive life (Morita and Tilly, 1999; Perez et al., 2000). It is estimated that up to 70% of oocytes are lost and only 1% out of the surviving ones will be ovulated (Pepling and Spradling, 2001). The oocyte death takes place both prenatally and postnatally. At 13.5 dpc and 15.5-17.5 dpc, a population of mitotic oogonia and early meiotic oocytes undergo programmed cell death, known as attrition (Coucovanis et al., 1993). The second round of extensive apoptosis takes place by the third week of life, during follicle assembly and is known as atresia. Interestingly, the process of follicular death does not affect all follicular stages at the same rate, with the highest being among dormant primordial follicles arrested in meiotic prophase (Bakken and McClanahan, 1978). The unbalanced loss of ovarian follicles can negatively impact the ovarian dynamics during adulthood and result in POF. Despite the consequences, little is known about reasons for programmed cell death, although several hypotheses have been proposed. For example, loss of c-kit and KL survival factors (De Felici et al., 2005), failures in entering meiosis (Wartenberg et al., 2001) or arresting at meiotic prophase (Yang and Fortune, 2008), errors in homologs synapsis and recombination (Mittwoch and Mahadevalah, 1992) or failure in follicle assembly (Guigon and Magre, 2006) can all potentially lead to oocyte degeneration and cease further development. The cellular mechanism of the primordial follicles death is poorly characterized as they lack classical morphological features of apoptotic follicles, such as active caspase-3 (Glamoclija et al., 2005) or DNA fragmentation (Hurst et al., 2006; Vaskivuo et al., 2001).

The p53 family of proteins consists of p53, p63 and p73, DNA sequence-specific transcription factors, which are mostly known to be involved in the prevention of cancer (Wade et al., 2010) as well as in many aspects of development (Candi et al., 2007; Mills et al., 1999; Yang et al., 2000). Members of p53 family exist in truncated or full length isoforms that exhibit reciprocal biological functions; truncated Δ isoforms support proliferation, while TA promote cell cycle arrest, cellular senescence and apoptosis (Murray-Zmijewski et al., 2006). Recently, the p53 family of proteins has also been implicated in the regulation of female reproduction through the maintenance of genomic integrity of female germ cells. TAp63, expressed during the late meiotic prophase arrest of germ cells, has been shown to protect female germ line fidelity through elimination of damaged oocytes (Kurita et al., 2005; Suh et al., 2006). The loss of another p53 family member, p73, results in reduced ovarian follicle pool size, decreased ovulatory abilities and poor quality of oocytes, leading to reduced ovulation rate and infertility (Tomasini et al., 2008).

p53, a positive regulator of apoptosis, plays a crucial role in maintaining genomic stability in somatic cells (Levine et al., 2006). In response to stress or DNA damage, upregulated gene transcription and stabilization of p53 results in the increase in total concentration of p53. Consequently, this results in p53-mediated cell cycle arrest that can serve for DNA repair (Moallem and Hales, 1998) or if cells are lethally damaged, it leads to apoptosis (Smith and Fornace, 1996). p53 is expressed during mouse embryogenesis (Moallem and Hales, 1998) and the peak of its expression is observed at 9-11 days of gestation (Tanaka et al., 2000). p53 expression is activated by DSBs generated during meiotic recombination and is followed by expression levels oscillation throughout meiotic prophase (Lu et al., 2010). It was suggested that p53 is a positive regulator of apoptosis in oocytes arrested in meiotic prophase and it slows down the execution of leptotene, zygotene and pachytene stages possibly to allow checking the genomic integrity (Ghafari et al., 2009).

Aims and Objectives

The majority of human aneuploidies are derived from mis-segregation of homologous chromosomes during meiosis I of oocytes (Hassold and Hunt, 2001). To prevent this, sister chromatid cohesion must stably connect recombinant homologous chromosomes (manifested as bivalent chromosomes with chiasmata) at distal part of chromosome arms (Petronczki et al., 2003). As previously reported mouse oocytes lacking the meiotic cohesin subunit Smc1 β exhibit progressive loss of chiasmata and an increase in aneuploidy (Hodges et al., 2005). The discovery of maternal age-associated reduction of chromosome-bound cohesin further suggested that cohesin degeneration might promote age-related increase in aneuploidy (Liu and Keefe, 2008; Lister et al., 2010; Chiang et al., 2010). One possibility is that degeneration of cohesin during ageing might be due to lack of cohesin turnover (Revenkova et al., 2010a; Tachibana-Konwalski et al., 2010). Thus, dysfunction of cohesin loading mechanism may also promote degeneration of cohesion. However, the cohesin loading factor Nipbl has not been studied so far in mammalian meiotic cells. Nipbl homologues have been shown to localise to meiotic chromosomal axes in yeast, fruit flies, nematodes, *Arabidopsis* and *Coprinus* and Nipbl mutations in some of these organisms are shown to cause meiotic cohesion defects and infertility, suggesting that Nipbl is responsible for cohesin loading also in meiotic cells. To gain insights into the function of Nipbl during mammalian meiosis, I investigated the localisation of Nipbl in mouse spermatocytes and oocytes and generated oocytes whose *Nipbl* allele has been conditionally eliminated and examined its effect on chromosome cohesion and cohesin binding. These results are presented in Chapter 3 and 4.

At birth, female germ cells are enclosed in primordial follicles. Subsequently follicles are progressively lost (atresia) but surviving primordial follicle pool must be reserved for as long as reproductive lifespan to supply fertile ova. In adulthood a subset of primordial follicles is periodically recruited for oocyte growth and granulosa cell expansion (follicular activation). Upon hormonal stimuli fully-grown oocytes finally resume meiosis and are ovulated. Precise regulatory mechanisms for controlling the fates of oocytes in primordial follicles, namely survival, death and differentiation, are still largely unknown. An unusual feature of female germ cells is that they are arrested in G2-phase for an extremely extended period of time, thus I hypothesized that the maintenance of the cell cycle arrest of oocytes is an important aspect of

follicular survival. Recent studies proposed the involvement of the anaphase-promoting complex/cyclosome (APC/C), a cell cycle ubiquitin ligase complex, in down-regulating the cyclin-dependent kinase activity in fully-grown oocytes. To study the role of APC/C in the maintenance of cell cycle arrest I inactivated the APC/C activity in dormant oocytes by a conditional *Apc2* allele (encoding an essential subunit of APC/C). The results are presented in Chapter 5.

Chapter 2

Methods and Materials

2.1 Mouse handling and dissection

2.1.1 Transgenic mouse lines

Targeted locus mouse lines

- Nipbl*^{fl/fl}** Transgenic mouse strain with targeted *Nipbl* allele (*Nipbl*^{fl/fl}) whose exon 2, containing the initiation codon, is flanked by two *loxP* sequences. The targeting strategy can be found in Figure 4.1. Elimination of floxed exon 2 by the expression of Cre recombinase creates a translational frameshift and results in a deletion allele lacking exon 2 (Δ allele). This strain has been generated in laboratory of Prof. Tom Strachan at University of Newcastle, UK. Mice in which *Nipbl* was inactivated in oocytes of primordial follicles are referred to as *Nipbl*^{fl/fl}; *Gdf-9-iCre*. Mice in which *Nipbl* was inactivated in growing oocytes are referred to as *Nipbl*^{fl/fl}; *Zp3-cre*.
- Apc2*^{fl/fl}** Transgenic mouse strain in which exons 2 to 4 (*Apc2* locus), encoding APC/C's catalytic core cullin, are flanked by *loxP* sites. Elimination of floxed region by the expression of Cre recombinase leads to a translational frameshift with stop codons in all three open reading frames and creates a deletion allele lacking exons 2-4 (Δ allele). This strain was generated by Prof. Nasmyth's group at the Research Institute of Molecular Pathology, Vienna, Austria and is described in Wirth et al. (2004). Targeting strategy is presented in Figure 5.1. Mice in which *Apc2* was deleted in oocytes of primordial follicles are referred to as *Apc2*^{fl/fl}; *Gdf-9-iCre* mice. Mice in which *Apc2* was deleted in growing oocytes are referred to as *Apc2*^{fl/fl}; *Zp3-cre*.
- Rosa26*^{Stop-YFP/Stop-YFP}** Transgenic mouse strain, which has a *loxP*-flanked STOP sequence followed by the Enhanced Yellow Fluorescent Protein gene (EYFP) inserted into the *Gt(ROSA)26Sor* locus. When *R26-YFP* mice are bred to mice expressing Cre recombinase, the STOP sequence is deleted and EYFP expression is observed in the *cre*-expressing tissue(s) of the double mutant offspring.
- SnoN*^{LacZ/LacZ}** Transgenic mouse strain with targeted *SnoN* allele. Exon 1 containing ATG codon has been replaced by *lacZ* gene with a nuclear localization signal. This resulted in the inactivation of biological activities of SnoN and created *SnoN*^{LacZ/LacZ} allele (Pearson-White and McDuffie, 2003). This mouse strain is a gift of Prof. Vasso Episkopou.

p53^{neo/neo} Transgenic mouse strain with targeted p53 allele. The neo cassette is inserted into intron 4, which results in premature termination of p53 RNA transcription after exon 3 and creates Δ allele. This mouse strain is a gift of Dr Tristan Rodriguez.

Oocyte-specific Cre recombinase mouse lines

Zp3-Cre In *Zp3-cre* mouse transgenic line, *Cre* expression is controlled by the regulatory sequences from the mouse *zona pellucida 3* (*Zp3*) gene. Zona pellucida 3 protein is a component of mammalian egg coat. *Zp3* promoter is active exclusively during oocyte growth and drives the expression of *Cre* recombinase in oocytes from the primary follicle stage, soon after entry into the growth phase at late stage of meiotic prophase arrest but before the completion of the first meiotic division (Lewandoski et al., 1997).

Gdf-9-iCre *Gdf-9-iCre* line contains sequence encoding improved *Cre* recombinase (iCre) under the control of the mouse growth differentiation factor 9 (*Gdf9*) promoter. iCre expression driven by *Gdf-9* promoter eliminates floxed region in oocytes within primordial follicles from postnatal day 3 (Lan et al., 2004). Figure 5.4 presents a monitoring study of *Gdf-9-iCre* mediated deletion.

Double knockouts (dKOs) have been generated in a result of multiple breeding steps. Double mutants that have been used in this thesis are:

Apc2^{fl/fl} SnoN^{LacZ/LacZ}; Gdf-9 iCre

Apc2^{fl/fl} p53^{neo/neo}; Gdf-9-iCre

2.1.2 Ethics

All mice were housed in accordance with the Animals (Scientific Procedures) Act of 1986 and associated Codes of Practice and all experiments were performed under approved project licences issued by Home Office, UK.

2.1.3 Breeding

If not stated otherwise all experiment were performed using 4-8 week old females. For testicular chromosome spreads 3-week-old males were used. The mouse (*Mus*

musculus) strains used in this study have mixed genetic background of C57BL/6J, 129/SV, CBA/J and FVB/N.

2.1.4 Fertility analysis

Reproductive capacity of *Nipbl^{fl/fl};Zp3-cre* or *Nipbl^{fl/fl};Gdf-9-iCre* and *Apc2^{fl/fl};Gdf-9-iCre* or *Apc2^{fl/fl};Zp3-cre* females was assayed by cohabitation with wild-type, fertile males for the minimum period of 3 months. During this time, the number of litters and pups were recorded. For the long term fertility studies, *Nipbl^{fl/fl};Gdf-9-iCre* females were housed with wild-type fertile males for a period of 6 months and the number of litters and pups were recorded.

2.1.5 Hormonal priming

Hormonal priming was employed to increase the yield of MI oocytes, MII eggs and zygotes and reduce the number of animals required for experiments. Mice were injected with 5 IU of pregnant mare's serum gonadotrophin (PMSG) 48 hours before use. For zygotes collection, 3-5-week old females were injected with PMSG, which was followed 48 h later by 5IU human chorionic gonadotrophin (hCG). For parthenogenetic activation experiments, eggs were isolated from ampulla of uterine tube 14-17 h after hCG injection. For experiments requiring *in vivo* fertilization, hormonally primed females were mated with fertile males.

2.1.6 Dissection

All mice were culled by cervical dislocation. The abdominal skin was sanitized with 75% ethanol and cut open with scissors. The ovaries were dissected out and depending on the aim of experiment collected into pre-warmed M2 medium or directly fixed in 10% neutral buffered formalin. The ovaries from mouse pups of postnatal day 1, 3 and 5 were dissected out under a stereo-microscope with heated stage.

2.1.7 Genotyping

PCR reaction was used for routine genotyping of mouse progeny. Genomic DNA was extracted from mouse ear notches by 1 h long incubation in 102 μ L of Tail Buffer with 20 mg/ml of Proteinase K at 55°C. Incubation mixtures were pelleted and

supernatant re-suspended in dH₂O and further used in PCR reaction. Each reaction contained 1X Reaction Buffer, 0.2 mM dNTP mix, 0.2 µM each primer, 0.5 U Taq Polymerase and 1-50 ng template DNA. Following denaturation at 95°C for 2 min, 35 cycles of PCR were carried out (20 s at 95°C, 60 s at 65°C, 30 s at 72°C). Specific PCR primers used for genotyping of each mouse strain can be found in Table 1.1. To confirm the correct size of amplicons, each reaction was subjected to separation via 2% (w/v) agarose gel electrophoresis containing 3 µL of 0.2 g/mL Ethidium Bromide per 100 mL gel. Electrophoresis was carried out in 1X TBE buffer and run at 100V.

Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Mutant band (bp)	WT band (bp)
Nipbl flox	GCTCAGTGATGGAAGC TTTCTGGA	ACAGCAAATGCTCTTTC AGCTAAGCTAT	480	450
NipblΔ	GCTCAGTGATGGAAGC TTTCTGGA	GTGATGACATATCTCAT ACCTGTT	360	2000
Apc2 flox	GTTCTGCAGCACTTCCA CGAACCA	GGCGACAATTATTGCCT CCGATGA	570	472
Cre	CATCGCCATCTTCCAGC AG	GATCGCTGCCAGGATAT ACG	600	no
iCre	TCTGATGAAGTCAGGAA GAACC	GAGATGTCCTTCACTCT GATTC	500	no
Rosa26-YFP	GTTATCAGTAAGGGAGC TGCAGTG	GTGGGAAGTCTTGTCC CTCCAATT	no	320
SnoN	CTCAACCATTAAACACCG AACTTTGGA	AAATGTGAGCGAGTAAC AACCCG	550	no
LacZ	AAGCTGACCCTGAAGTT CATCTGC	GATGTTGCCGTCCTCCT TGAAGTC	290	no
EGFP	CCAACCAAACCTCAACCA TTACACC	ATCATCCTCCATGCCGT TCAGCTT	no	412
SnoN	GTGTGTTGGCCATCTCT GTGAGTT	AGCCGAGTAACGATCA GGTGTCAA	no	500
p53	ACAACAGACAATCGGCT GCTCTGA	CAAGGTGAGATGACAG GAGATCCT	290	no
Neo				

Table 1.1 Primers for genotyping

2.2 Oocyte and zygote techniques

2.2.1 Oocyte collection and culture

Oocyte collection was performed on a stereomicroscope (Leica) with a heated stage to help maintain a 37°C environment during collection. Fully-grown mouse GV oocytes were retrieved by physical disaggregation of ovaries and collected into M2 medium supplemented with 200 mM IBMX or 100 µg/mL of dbcAMP with the use of a glass-handling pipette of a diameter greater than the one of an oocyte. Commercially available glass, firepolished pipettes (BioMedical Instruments) with 108 µm-114 µm of diameter were used. The pipette with the smaller diameter was used to denude oocytes from cumulous cells. M2 media was used for oocyte collection and short-term incubation and for some imaging experiments where providing a 5% CO₂ atmosphere was not possible. M16 media is HCO₃ buffered, therefore it requires pre-incubation for several hours in a humidified incubator with 5% CO₂ in order to attain physiological pH. Oocytes were cultured in micro-drop culture (50 µL each drop) covered with mineral oil to maintain stable culture condition. Mineral oil was also pre-incubated in 37°C, 5% CO₂. Oocytes that had undergone GVBD within 90 min following release from IBMX or dbcAMP were selected and cultured further in M16 for experiments.

2.2.2 Zygotes collection, staging and culture

Zygotes of desired pronuclear stages were released from ampulla, cumulus cells were removed by short digestion with 30 mg/mL Pronase and washed in M2 medium. Pronuclear stages (PN) were classified according to the distance between pronuclei (Adenot et al., 1997) and corresponded approximately to the following times after hCG injection in our strain background: PN0-PN1, 17 h; PN2, 19 h; PN3-PN4, 23 h; PN5, 28 h. PN1 and PN5 zygotes used in my experiments were collected 17 and 28 h after hCG injection respectively.

2.2.3 *In vitro* parthenogenesis

For generation of parthenogenetic embryos, MII-stage oocytes were collected from 3-5-week old superovulated females (as described in 2.1.2.2). Ovulated eggs were first treated with 0.1% hyaluronidase in M2 medium to denude them of the

surrounding cumulus cells. They were then transferred to activation medium (7% ethanol in M2) for 5 min at RT. Subsequently they were cultured for 6 h in microdrop cultures of KSOM media equilibrated at 37°C in 5% CO₂ and supplemented with Cytochalasin B (5 mg/mL) to prevent second polar body extrusion. Note that commonly used strontium chloride (0.125 to 10 mM) did not activate specifically eggs from the *Nipbl^{fl}* strain. Activated eggs, with clearly visible pronuclei were washed in Cytochalasin B-free KSOM media and further cultured. Embryonic development was observed every 24 h after activation until 120 h and cleavage rates and blastocyst numbers were recorded (Protocol was conducted by Dr Shoma Nakagawa).

2.2.4 Kinetics of GVBD and PBE

Fully-grown oocytes were collected into M2 media supplemented with 200 mM of IBMX or 100 µg/mL of dbcAMP at 37°C as described above. If the aim of experiment was to examine kinetics of GVBD, oocytes were released into 50 µl droplets of M2 media supplemented with appropriate inhibitor concentration covered with mineral oil and monitored every 30 min for 2 hours. If the aim of experiment was to examine kinetics of PBE, oocytes that have undergone GVBD within 90 min after the release to inhibitor-free medium were selected (at time 0 after GVBD) and used for time-lapse microscopy. Polar body extrusion (PBE) curve was obtained by analysing bright field images acquired at 20 min intervals for up to 16 h.

2.3 Quantitative analysis of oocyte RNA

2.3.1 RNA extraction

RNA was isolated from three independent oocyte collections. Approximately one hundred oocytes were freed from granulosa cells and zona pellucida by treatment in 30 mg/mL Pronase and collected in 1 mL of Trizol per experiment. Total RNA was isolated following the manufacturer's instructions. Briefly, each sample was supplemented with 1 µL (20 µg) of glycogene as a carrier and total RNA was eluted in 20 µL DEPC-treated water. RNA concentration and purity were quantified using a Nanodrop ND-1000 Spectrophotometer (Biolab Ltd.).

2.3.2 Complementary DNA synthesis and quantitative PCR

Synthesis of first-strand cDNA was performed on 1 µg of extracted RNA with the use of QuantiTect Reverse Transcription Kit following the manufacturer's instructions. Reaction mixture of the final volume of 25 µL containing cDNA synthesized from 50 ng RNA, SYBR Green premix and 0.2 µM forward and reverse primers was subjected to a thermal cycle program (initial denaturation at 94°C for 2 min; 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s and extension at 72°C for 30 s) on a Chromo4 DNA Engine driven by Optikon Monitor 3 software (BioRad). Primer sequences for specific genes as well as housekeeping gene used as a control are listed in Table 1.2. RT-PCR conditions for each primer set were optimized using mouse testis cDNA, prior to quantification experiments. A melting program (melting curve from 67°C to 91°C, read every 0.3°C) was performed after the amplification and melting curves were monitored for the production of a single peak to confirm PCR product identity and exclude non-specific signals. Additionally, PCR products were analysed by agarose gel electrophoresis to confirm product sizes. Each sample within an experiment was performed in duplicate and analysed using $\Delta\Delta C(T)$ method (Pfaffl, 2001). Experiments were repeated 3 times.

Name	Forward Primer sequence (5'-3')	Reverse Primer sequence (5'-3')	Amplicon size
Nipbl A	GAGGATAGAGGAGGA GGCACTT	GGTCGGTCTTTGTAC TTTGGAC	145 bp
			99 bp
Nipbl B	TAGAGGAGGAGGCAC TTCAG	AGACTGACACACATA AGCACA	114 bp
			136 bp
Nipbl AB	AGTCCATATGCCCCA CAGAG	ACCGGCAACAATAGG ACTTG	182 bp
Nipbl Ex2	CCATGTCCCCATAACT ACGCT	AGTTCACCTCTTCTG CTATTCGT	
GAPDH	ACGGCCGCATCTTCT TGTGCA	GCCACTGCAAATGGC AGCCCT	

Table 1.2 Primers for the amplification of cDNA from mouse transcripts by qPCR

2.4 Chromosome spreads

2.4.1 Chromosome spreads from foetal ovaries

In mammalian females meiosis initiates during fetal development. In order to analyse the first stages of meiotic prophase, fetal ovaries from E13.5 embryos were prepared. Ovaries were placed in a hypotonic solution for 10 min and then transferred to 50 μ L of 100 μ M Sucrose (pH 8.2). The ovaries were disaggregated using sharp forceps and pipetting to obtain a single cell suspension. The cell suspension was transferred onto a PolylysineTM-coated microscope slides that had been immersed in fixative. Cells were dispersed on the slide by tilting and then slowly dried.

2.4.2 Chromosome spreads from fully-grown oocytes or pronuclei spreads from zygotes

Fully-grown GV stage oocytes were isolated and cultured for 6 h (Metaphase I) or 18 h (Metaphase II) after GVBD. Zona pellucida was removed by incubation in M2 medium containing 30 mg/mL Pronase at 37°C for 5 min, followed by 3x5 min washes in M2 medium. Zona pellucida-free oocytes (PBs detached and was lost in most cases) were then placed in a dish containing hypotonic solution and incubated at 37°C for 10-30 min. Next, 2-3 oocytes were transferred into each drop of fixative on a 15-well multitest glass slide (MP Biomedicals). Slides were left to dry overnight in a humid chamber before being stored at -80°C. The same method was used to prepare pronuclei spreads from zygotes.

2.4.3 Chromosome spreads from spermatocytes

Testes from 3-week-old male mice were collected and used for the preparation of chromosome spreads using a drying-down technique described previously (Peters et al., 1997). Briefly, testes were washed several times in PBS then incubated for 10 min in hypotonic solution. The tubules were then disaggregated thoroughly with forceps in 100 mM Sucrose to isolate testicular cells. Cells were spread on PolylysineTM-coated microscope slides covered with fixative. Slides were left to dry overnight in a humid chamber before being stored at -80°C.

2.4.4 Carnoy's chromosome spreads from MEFs

For chromosome analysis in MEFs, cells collected after FACS analysis were grown in 6-well plate and harvested 9 days after infection. On the day of spreading, iMEFs were pretreated with 10 µg/ml demecolcine for 1 h prior to harvest. MEFs were harvested by trypsinization and treated with hypotonic solution containing ice-cold 0.56% KCl solution for 6 min at RT. Cells were then spun (1200g for 4 min at RT), supernatant aspirated and the pellet was resuspended in remaining 200 µL of hypotonic solution. Next, cells were carefully resuspended in 5 ml of freshly made Carnoy's solution. Resuspended MEFs were pelleted (1200g for 4 min at RT) and then resuspended again in 1ml of new fixative. For spreading, cell suspension was dropped onto PolylysineTM-coated microscope slides and dried at RT. Slides were stained with mounting medium Vectashield containing DAPI.

2.5 Oocyte and embryo fixation

Fully-grown GV stage oocytes were isolated and cultured for 6 h (Metaphase I) or 18 h (Metaphase II) after GVBD. Oocytes were fixed in PHEM buffer containing 4% paraformaldehyde and 0.5% Triton X-100 for 20 min and washed 2x5 min in 1% BSA in PBS. Oocytes were then permeabilized in 0.25% TritonX-100 in PBS for 10 min. The same protocol has been used for fixation of blastocysts.

2.6 Immunofluorescence

2.6.1 General principles

Following fixation, samples were labeled with primary antibodies detecting the protein of interest. Next, primary antibodies were targeted by fluorescently conjugated secondary antibodies. In experiments where co-localisation was performed two primary antibodies raised in two different host animals were used. This enabled simultaneous detection of two different antigens using species-specific secondary antibodies with two different fluorescent dyes. Chromatin was counterstained with DAPI (1 µg/ml).

2.6.2 Immunofluorescence staining of spindle

Following fixation and permeabilization, oocytes or blastocysts were blocked in 3% BSA in PBS at 37°C for 1 h followed by 2x10 min washes in PBS. Next, they were incubated overnight at 4°C in micro-drops of primary antibody diluted in PBS covered with mineral oil. The following day, oocytes were washed 2x10 min in PBS and transferred to micro-drops containing secondary antibody diluted in PBS for 1 h at 37°C. This was followed by 10 min wash in PBS. Chromatin was counterstained with DAPI (1 µg/ml) in Vectashield mounting medium for 25 min at RT.

2.6.3 Immunofluorescent staining of spreads from spermatocytes, fetal ovary spreads and ES cells.

PolylysineTM-coated microscope slides with chromosome spreads were rinsed in PBS and washed 2x5 min in IF Wash Buffer. Next, they were blocked in IF Block Buffer under the cover slip for 30 min in a humid chamber at RT. Primary antibodies were diluted in IF Block Buffer at indicated dilutions (materials and methods 2.9.1) and incubated on slides overnight at 4°C in a humid chamber. Then, slides were again rinsed and washed 2x5 min in IF Wash Buffer. Next, slides were blocked for 30 min in IF Block Buffer under cover slips in a humid chamber at RT. Secondary antibodies were applied on slides and incubated for 1 h in 37°C in a humid chamber. Following, slides were washed 2x5 min in IF Wash Buffer, dried at RT, mounted in Vectashield medium containing 1 µg/ml DAPI and covered with a glass coverslip.

2.7 Immunohistochemistry

2.7.1 General principles of chromogenic staining

Avidin-biotin complex (ABC) method is based on a very high affinity of avidin for biotin. This technique involves application of three layers of reagents. The first layer consists of unlabeled primary antibody, which is detected by secondary biotinylated antibody (second layer). The third layer is a complex of avidin-biotin and peroxidase. Peroxidase is then developed by the DAB or other substrate to produce different colorimetric end products. Many tissues have endogenous peroxidase activity, which can generate background staining by reacting with DAB substrate. Endogenous peroxidase activity can be eliminated by the pretreatment of the tissue section with hydrogen peroxide prior to incubation of primary antibody.

2.7.2 Chromogenic staining of paraffin-embedded sections

Slides containing ovarian sections were dewaxed 2x10 min in Histoclear and rehydrated in 70% and absolute ethanol (1 min each) with a final 2 min wash in distilled water. Next, slides were washed 2x5 min in PBS, endogenous peroxidase activity was quenched with 3% hydrogen peroxide in 70% methanol for 20 min and slides were washed again in PBS (2x5 min). Next, sections were blocked for 20 min with 20% NGS in PBS followed by incubation in primary antibody diluted in blocking solution overnight at 4°C. Following day, slides were washed for 3x10 min in PBS after which the appropriate biotinylated secondary antibody diluted in PBS was applied to sections for 1 h at RT. After washing in PBS (2x5 min), an avidin-biotin complex (ABC) was added to slides for 30 min at RT. Samples were washed again in PBS (2x5 min) before DAB staining kit was applied for 1 min to visualise signal. Washing slides in distilled water ceased the development of signal. Stained slides were then counterstained with haematoxylin for 1 min. Residual haematoxylin was gently washed away under a tap of cold water before being dipped in 1% acid alcohol for 5 s and further rinsed in cold water. Sections were dehydrated in 100% and 70% ethanol (1 min each), incubated 2x5 min in Histoclear and finally mounted with mounting medium and covered with a glass coverslip.

2.8 Western blotting

2.8.1 Sample preparation

Whole testicular protein extracts from wild-type mouse were prepared by physical disaggregation of seminiferous tubules with forceps in PBS and removal of precipitated extracellular matrices. Cells were collected by centrifugation, suspended in SDS-PAGE sample buffer and boiled. DNA was sheared by sonication and passed through a 27-gauge needle. Control protein extract was prepared from pre-B3 cell line. Briefly, confluent cells were collected by centrifugation, washed 2x10 min in PBS and suspended in SDS-PAGE sample buffer and boiled. DNA was sheared by sonication and passed through a 27-gauge needle.

Whole protein extracts were prepared from iMEFs that were harvested 5 and 10 days after infection. Cultured iMEFs were washed in PBS then lysed in 50 µL ice-cold RIPA/10cm² containing Protease Inhibitor Cocktail (1:1000) and PMSF (1:1000). Samples were incubated with rotation at 4°C for 30 min and span in a

bench top centrifuge (1200g for 30 min at 4°C) to pellet any cell debris. Supernatants containing proteins were collected and protein concentration determined by BCA Assay. Samples were stored at -80°C until further use. For analysis of protein levels in whole cell lysates, 30 µg of total protein extract was heated at 98°C in 1x Loading Buffer supplemented with 5% β-mercaptoethanol for 10 min and loaded onto SDS-PAGE gel.

2.8.2 Protein separation, immunolabelling and chemiluminescence

Samples were loaded onto 6% SDS-PAGE gel and electrophoresed in Running buffer at 100V for 1.3-5 h. Proteins were transferred onto a PVDF membrane (Millipore) in Transfer Buffer containing 5% methanol using a wet transfer system at 30V, overnight at 4°C. After transferring, membranes were blocked in 5% milk-TBST for 1 h before incubation with the relevant primary antibody at 4°C overnight. The next day, membranes were washed for 3x10 min in TBS-T then incubated with secondary antibodies conjugated with HRP for 1 h at RT. Proteins were detected using ECL or ECL Plus Western Blotting Substrate.

2.9 Antibodies

Antibodies used in this study are listed below, including the dilutions used for immunofluorescence (IF) western blotting (WB) and immunohistochemistry (IHC).

2.9.1 Primary Antibodies

Antibody	Supplier	Dilution	Host Species
α-Nipbl A	KT54, 2F1; Thermo Scientific (Seitan et al., 2006)	1:500 (IF) 1:2500 (WB)	Rat monoclonal
α-Nipbl B	KT55, 16H10; Thermo Scientific	1:500 (IF) 1:2500 (WB)	Rat monoclonal
α-Nipbl AB	Gift from J.M.Peters (Watrín et al., 2006)	1:500 (IF) 1:5000 (WB)	Rabbit polyclonal
α-SYCP3	10G11; Abcam	1:500 (IF)	Mouse monoclonal

CREST serum	a gift of A. Kromminga, Hamburg, Germany	1:1000 (IF)	human CREST autoimmune serum
α - γ H2AX	Cell Signalling Tech.	1:1000 (IF)	Rabbit polyclonal
α -Lamin B1	Ab16048 Abcam	1:5000 (WB)	Rabbit polyclonal
α -Mlh1	BD, Pharmingen	1:500 (IF)	Mouse monoclonal
α -Rec8	(Eijpe et al., 2003)	1:500 (IF)	Rabbit monoclonal
α -Rad51	14B4, Abcam	1:500 (IF)	Mouse monoclonal
α -tubulin	[DM1A+DM1B]; Abcam	1:1000 (IF) 1:5000 (WB)	Mouse monoclonal
α -GFP	Ab290; Abcam	1:1000 (IF)	Rabbit polyclonal
α -BrdU	GE Healthcare	1:500 (IF)	Mouse monoclonal

2.9.2 Secondary Antibodies

Immunofluorescence

Antibody	Supplier	Dilution
Alexa488 Goat anti-rabbit IgG	Invitrogen	1:500
Alexa488 Goat anti-rat IgG	Invitrogen	1:500
Alexa568 Goat Anti-mouse IgG	Invitrogen	1:500
Alexa568 Goat anti-rabbit IgG	Invitrogen	1:500
Alexa568 Goat anti-rat IgG	Invitrogen	1:500
Alexa568 Goat anti-human IgG	Invitrogen	1:500
Alexa568 Goat anti-human IgG	Invitrogen	1:500

Western Blotting

Antibody	Supplier	Dilution
Goat Anti-mouse-HRP	ThermoScientific	1:1000
Goat Anti-rat-HRP	ThermoScientific	1:1000
Goat Anti-rabbit-HRP	ThermoScientific	1:1000

Immunohistochemistry

Antibody	Supplier	Dilution
Goat Anti-mouse-HRP	ThermoScientific	1:1000
Goat Anti-rat-HRP	ThermoScientific	1:1000
Goat Anti-rabbit-HRP	ThermoScientific	1:1000

2.10 Histology

2.10.1 Ovarian section preparation

Mouse ovaries were fixed in 10% neutral-buffered formalin (NBF) for approximately 24 h before being washed briefly with PBS and placed in 70% ethanol for 2 h. This was followed by 3 changes of 70% ethanol at 4°C for up to 24 h. Then tissues were placed in fresh 70%, 90% and absolute ethanol (each for 1 h) before incubating for 2 h in Histoclear. Next, tissues were submerged in paraffin wax for 2 h and cooled on ice for at least 30 min. Then, 6-µm serial sections of entire ovaries were collected using a Leica RM 2135 microtome (Milton Keynes, UK) and left to dry on PolylysineTM-coated microscope slides overnight at 37°C.

2.10.2 Haematoxylin and eosin staining

Slides containing ovarian sections were dewaxed 2x10 min in Histoclear and rehydrated in 70% and absolute ethanol (1 min each) with a final wash in distilled water (2 min). Next, they were stained in hematoxylin solution for 5 min followed by

wash in running tap water for 5 min. Slides were then differentiated in 1% acid alcohol for 30 s and subsequently washed under running tap water for 1 min. Sections were counterstained by incubation in eosin solution for 1 min. Finally, sections were dehydrated in 95% alcohol (2x1 min) and absolute alcohol (1x5 min), cleared in HistoClear (2x5 min), mounted in DPX mounting medium and covered with a glass coverslip.

2.10.3 Ovarian follicles staging and quantification

Ovarian follicles at different developmental stages were classified by their granulosa cell morphology and number of complete granulosa cell layers as previously described (Da Silva-Buttkus et al., 2008). Examples of ovarian follicles including primordial (Pmd), transitional (T), primary (1), primary + (1+), secondary (2), secondary + (2+), pre-antral (pA), early antral (eA), late antral (lA) and preovulatory (pO) are presented in Figure 2.1 and the classification is based on Pedersen and Peters, (1968). Briefly, follicles were classified as primordial when a primordial oocyte is surrounded by partial or complete layer of squamous (flattened) pre-granulosa cells within a follicle (Fig. 2.1 a). A layer of both types of cells, flattened pre-granulosa and cuboidal granulosa, surround oocyte in transitional follicle (Fig. 2.1 b). Primary follicle has an oocyte surrounded by a single layer of cuboidal granulosa cells (Fig. 2.1 c), while primary+ follicle has a single layer of granulosa cells and a few cells that form the second layer (Fig. 2.1 d). Secondary follicles have two layers of granulosa cells (Fig. 2.1 e), while in secondary+ follicle two complete layers are separated by a space where other layers form (Fig. 2.1 f). Pre-antral follicles have many layers of cuboidal cells but no visible antrum (Fig. 2.1 g). Early and mid-antral follicles have gradually larger areas of antrum filled with follicular fluid (Fig. 2.1 h and 2.1 i). Pre-ovulatory follicles have large antrum and a rim of cumulus cells surrounding the oocyte (Fig. 2.1 j). To calculate total follicle numbers, every fourth section was counted throughout the entire ovary, beginning with the first section, and the final count was multiplied by 4 as a correction factor. Infrequent follicles with oocytes of unhealthy morphology were not analysed. In each section follicles containing oocytes where nucleus is clearly visible were scored, which allowed reducing the chance of missing the follicle or counting the same follicle twice. This method was also used to calculate the total number of GFP-positive follicles in ovarian sections from *Gdf9-iCre;Rosa26^{YFP/YFP}* mice.

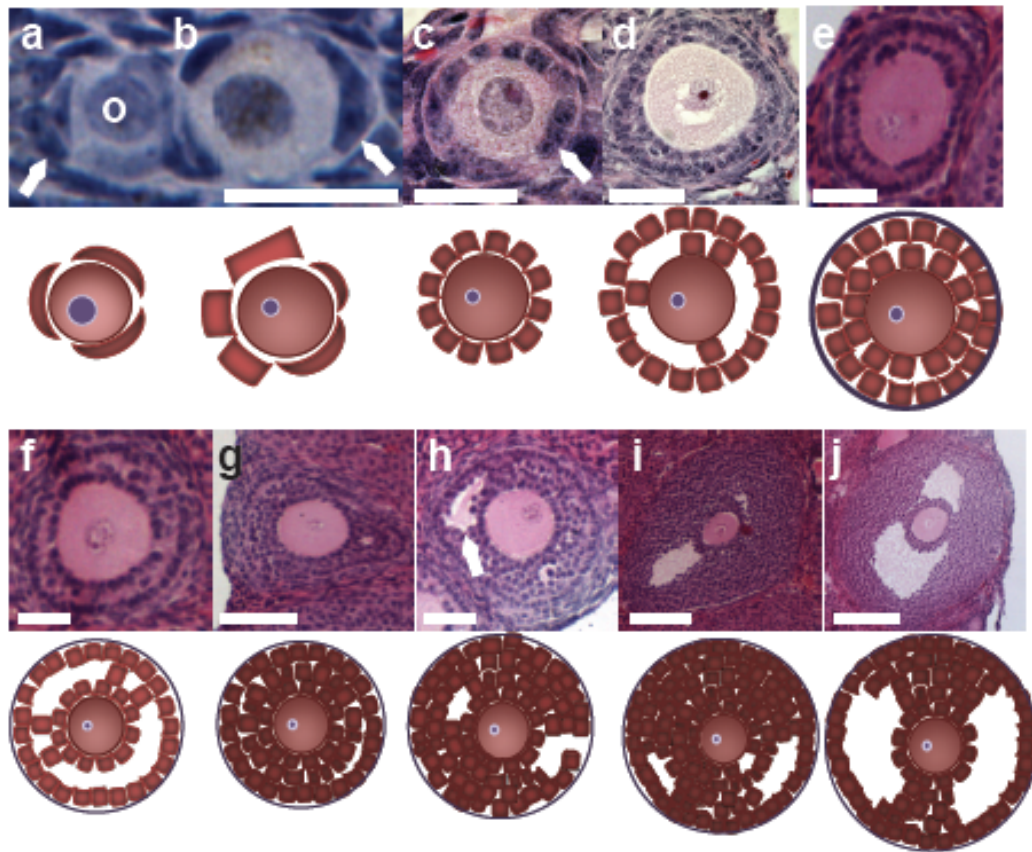


Figure 2.1 Morphological classification of mouse ovarian follicles.

The classification of follicular stages is described in 2.10.3. (a) primordial follicle, arrow indicates flattened pre-granulosa cell; o, nucleus (b) transitional follicle, arrow indicated cuboidalised granulosa cell (c) primary follicle, arrow indicates a single layer of granulosa cells (d) primary plus follicle (e) secondary follicle (f) secondary plus follicle (g) pre-antral follicle (h) early antral follicle, arrow indicates forming antrum (i) mid-antral follicle (j) pre-ovulatory follicle. Scale bar in a-d 20 μm ; e-f 50 μm ; g-i 100 μm ; j 200 μm . Note that graphics are not in scale.

2.11 Tissue Culture

2.11.1 Cell lines

HEK 293T Human embryonic kidney cell line immortalized with adenovirus type 5 DNA (Belandia et al., 2005).

MEFs Mouse embryonic fibroblasts (MEFs) derived from *Nipbl^{fl/fl}* mouse embryos.

Pre-B3 cells. Precursor to the development of B cells cell line (Brown et al., 1997)

2.11.2 Derivation of *Nipbl^{fl/fl}* mouse embryonic fibroblasts (MEFs)

Mouse embryonic fibroblasts (MEFs) were isolated from day 13.5 (E13.5) embryos (following the protocol by Nagy et al., 2003) that were generated by mating *Nipbl^{fl/fl}* female with *Nipbl^{fl/fl}* male. E13.5 embryos from which the head and internal organs were removed were cut in pieces, and stirred in 0.1% Trypsin for 30 min in order to disaggregate them. Primary cells were cultured in MEF culture medium at 37°C and 5% CO₂.

2.11.3 Maintenance of Cell Lines

All cell lines were cultured at 37°C and 5% CO₂ in their respective media as described in 2.11.1. 293T cells as well as B3 cells were passaged 1:5 to 1:10 every 4-5 days. Primary MEFs were passaged 1:3 every 2-3 days. Immortalized MEFs (iMEFs) were obtained by prolonged passaging (> 20 passages). iMEFs were passaged 1:10 every 3-4 days.

2.11.4 Production of lentivirus expressing Cre recombinase

Two types of viruses were produced: Cre recombinase-expressing lentivirus vector (LV-Cre-EGFP) and control EGFP expressing (LV-EGFP). Viral particles were packaged in 293T cells that were seeded at a density of 2x10⁶ cells per 10 cm² tissue culture dishes and cultured overnight to reach 80% confluence. The next day 293Ts were co-transfected with 5 µg of pMDG2-vsvg DNA (envelope vector), 10 µg of pCMV-d8.91 DNA (packaging vector) and 15 µg of either transfer vector LV-Cre-

EGFP or *pLKO.3G* (to generate LV-Cre-EGFP or LV-EGFP, respectively) per tissue culture dish. Vectors were mixed in 1 mL serum-free media with 75 μ L of polyethylenimine (PEI) transfection reagent and incubated for 30 min, then added to each plate of 293T dropwise. The next day, the transfection mix was exchanged for 6.5 mL/plate of viral collection media. Supernatants containing viral particles were harvested 24 h and 48 h after the first media change and stored in -80°C . To concentrate, two aliquots of harvested supernatant were thawed, pooled together and spun at 200 $\times$ g for 5 min to pellet any debris of cells. Viral supernatant were concentrated to final volume of 200 μ L using centrifugal filter units (Millipore Amicon ultra-4 10,000 NWML) according to manufacturer's instructions. Aliquots were stored in -80°C . Viral titer was calculated using serial dilutions of LV-CRE-EGFP and LV-EGFP-expressing plasmid to infect 293T and MEF cells in the presence of 8 mg/mL Polybrene. The percentage of GFP- expressing cells was analysed 72 h after infection by fluorescence activated cell sorting, FACS (BD FACSAria IIu; BD Biosciences) and the minimum volume of virus (typically 10-15 μ L) was required to give ~80% GFP-positive when 1.5×10^5 cells MEFs were infected.

2.11.5 Targeted deletion of Nipbl Exon2 in Nipbl^{fl/fl} iMEFs and FACS analysis

iMEFs were transfected with *Cre* recombinase-expressing lentivirus vector. $1.5-1.7 \times 10^5$ iMEFs were infected with either 15 μ L of LV-CRE-EGFP or 15 μ L of LV-EGFP (control infection) or left untreated followed by 3 days of incubation at 37°C and 5% CO_2 . After 72 hours cells infected with either LV-CRE-EGFP or LV-EGFP were analysed for GFP fluorescence with fluorescence activated cell sorting (FACS) (BD FACSAria IIu; BD Biosciences). GFP-positive population of MEFs infected with LV-CRE-EGFP (iMEFs^{Cre}), iMEFs infected with LV-EGFP (iMEFs^{EGFP}) and uninfected MEFs were further grown in culture and used for other experiments.

2.11.6 Genotyping of MEFs

DNA was extracted from 2.5 k of MEF cells in culture. Briefly, cells were trypsinized, washed in PBS and pelleted by centrifugation (1200g for 5 min at RT). Cells pellet was incubated in Tail Buffer with 20 mg/ml of Proteinase K and DNA was collected from supernatant. PCR amplifications were carried out as described for mouse genotyping (2.1.7).

2.11.7 BrdU incorporation and staining

iMEFs were treated with 10 μ M of BrdU in MEF culture media 16 h prior to fixation and staining. Cells were washed in PBS and fixed with 4% PFA in BSA for 15 min. Next, they were permeabilized with 0.2% TritonX-100 in PBS for 15 min and later blocked in 1% BSA solution supplemented with 0.4% fish gelatin for 30 min. Samples were incubated with anti-BrdU primary antibody in blocking solution containing 0.5U/ μ L of DNase I and 1mM MgCl₂ for 1 hour. Cells were then washed for 15 min in PBS and appropriate secondary antibody conjugated to Alexa fluor fluorophores was applied and incubated in 37°C for 1 h. Slides were mounted with Vectashield containing DAPI and visualized using an epifluorescent microscope. Labelling indices were calculated by counting BrdU-positive cells as a percentage of total number of cells within each randomly selected microscopic field. Results represent the mean $\pm$ SEM.

2.12 Microscopy and Image Analysis

2.12.1 Bright-field microscopy for ovarian sections

Bright-field microscopy was performed on an Olympus BX50 equipped with PlanApo and Plan N objectives and a Leica DFC295 CCD camera operated by the LAS software.

2.12.2 Wide-field epi-fluorescence microscopy

Wide-field epi-fluorescence microscopy was performed on a Leica DMI6000B equipped with an HCX PL APO 100x/1.40 Oil CS objective, dichroic filters (A4, L5, N3, TX2, Y5) and a Hamamatsu ORCA-ER CCD camera operated by the MetaMorph software (Molecular Devices).

2.12.3 Confocal microscopy

Confocal microscopy was performed on a Leica SP5/AOBS equipped with an HCX PL APO 63x/1.40-0.6 lambda blue objective.

2.13 Statistical data analysis

All statistical analyses in this study were performed using the software Prism 4.0 GraphPad. Results of RT-qPCR assay and fertility analysis were expressed as an average $\pm$ SD. Comparisons between the mean variables of 2 groups were made by a 2-tailed Student's t-test. P values less than 0.05 were considered to be statistically significant. Statistical examination of genotypes of offspring was analysed by chi-squared analysis against Mendelian expectations. *Gdf-9-iCre;Rosa26-YFP* monitoring was analysed by Wald's test.

2.2. Chemicals, Solvents and Reagents

Chemical	Supplier
Acrylamide/Bis Solution	Biorad
Agarose	Sigma-Aldrich
Ammonium Persulfate (APS)	Sigma-Aldrich
β -Mercaptoethanol	Sigma
Bovine Serum Albumin (BSA)	Sigma
Complete Protease Inhibitor Tablets	Roche
Deoxynucleotide Triphosphate (dNTPs) 10mM	New England Biolabs
Dimethyl Sulfoxide (DMSO)	Sigma
Ethanol	Fisher Scientific
Glycerol	BDH
Glycogene	Roche
Glycine	Sigma-Aldrich
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Gibco
Histoclear	National Diagnostics
Isopropanol	Fisher Scientific
Methanol	Fisher Scientific
Nonidet P-40 (NP-40)	Sigma-Aldrich
Normal Goat Serum	Sigma-Aldrich
Neutral-buffered formalin (10%)	Sigma-Aldrich
Oligonucleotides	Invitrogen

Paraformaldehyde	Sigma
Phenylmethanesulphonylfluoride (PMSF)	Sigma-Aldrich
Phosphate-buffered Saline (PBS)	Sigma-Aldrich
Polyethylenimine Linear (PEI) MW 25.000	Polysciences
PVDF Membrane	Millipore
DEPC-treated	Invitrogen
Skimmed Milk Powder	Fluka
Sodium Dodecyl Sulfate (SDS) Solution 10%	Fluka
SYBR Green	Sigma-Aldrich
TEMED	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Trizol	Invitrogen
Tyrode's Solution	Sigma
Tween-20	BDH
Vectashield with DAPI	Vector Laboratories

2.3 Kits

Kit Name	Supplier
Bicinchoninic acid (BCA) Protein Assay	Pierce
ECL Western Blotting Reagent	Pierce
ECL Plus Western Blotting Reagent	GE Healthcare
QIAprep Spin Miniprep Kit	QIAGEN
QuantiTect Reverse Transcription Kit	QIAGEN
Avidin-biotin complex (ABC)	Vector Laboratories

2.4 Antibiotics

Antibiotic	Supplier
Ampicillin	Sigma-Aldrich
Penicillin/Streptomycin	Sigma

2.5 Enzymes

Enzyme	Supplier
Proteinase K	Invitrogen
Polymerase	Sigma-Aldrich
SYBR Green Premix	Invitrogen

2.6 Buffers, Solutions, Gels

DNA Manipulation

TBE 10x	900 mM Tris Borate 20 mM EDTA pH 8.0
Tris-EDTA (TE) Buffer	1 mM EDTA 10 mM Tris pH 8.0
DNA Loading Buffer 10x	0.2% (w/v) Bromophenol Blue 40% (v/v) Glycerol 100 mM EDTA pH 8.0
Tail Buffer	100 mM Tris-HCl pH 8.0 5 mM EDTA 200 mM NaCl 0.1% SDS
Proteinase K	Sigma

Plasmid Transfection

Polyethylenimine Linear (PEI) 1 mg/mL	Dissolve PEI in water heated at 80°C Cool and adjust to pH 7.0 Sterile-filter (0.2 µm) (Store at -20°C)
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Protein manipulation

Radioimmunoprecipitation assay (RIPA) Buffer	50mM Tris pH 8.0 1mM EDTA 0.5 mM EGTA 1% Triton X-100 0.1% Na-deoxycholate 140 mM NaCl (Aliquot and store at -20°C)
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Polyacrylamide Gels (Resolving)	375 mM Tris HCl pH 8.8 6% (w/v) Acrylamide/Bis 0.1% (w/v) SDS 0.05% (w/v) Ammonium Persulfate 0.005% (v/v) TEMED
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Polyacrylamide Gels (Stacking)	125 mM Tris HCl pH 8.8 4% (w/v) Acrylamide/Bis 0.1% (w/v) SDS 0.05% (w/v) Ammonium Persulfate 0.01% (v/v) TEMED
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SDS Running Buffer	25 mM Tris base 190 mM Glycine 0.1% (w/v) SDS
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Transfer Buffer (1X)	3.03 g Tris base 14.4 g Glycine 0.01% SDS 10% (v/v) methanol
----------------------	---

TBS-T	130 mM NaCl 20 mM Tris pH 7.6 0.1% (v/v) Tween-20
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Protein Loading Buffer	1.0M TrisHCl (pH 8.5) 8% (w/v) lithium dodecyl sulfate 40% (v/w) glycerol 2 mM EDTA DTT Bromophenol Blue (BPB)
------------------------	---

Immunofluorescence (IF)

Buffers

IF Wash Buffer	1xPBS 0.2%BSA 0.1%Tween-20
IF Permeabilization Buffer	IF Block buffer plus 0.25% (v/v) triton X-100
IF Block Buffer	Wash Buffer with 10% (v/v) normal goat serum 2.5% (w/v) BSA
PHEM Buffer (2x)	60 mM PIPES 25 mM HEPES 10 mM EGTA 2 mM MgCl ₂ pH 6.9

Fixatives

4% Paraformaldehyde (PFA)	4% (w/v) PFA dissolved in PBS pre-heated to 40°C containing 2 mM NaOH Sterile-filter (0.2 µm) (Aliquot and store at -20°C)
4% PFA in PHEM Buffer	4% Paraformaldehyde, 0.2-0.5% Triton X-100 in PHEM buffer.
Fetal oocyte fixative	1% paraformaldehyde 0.15% Triton X-100 3 mM DTT pH 9.2 adjusted by NaOH
Carnoy's Solution	75% methanol 25% glacial acetic acid

Hypotonic Solutions

Fully-grown oocytes and zygotes chromosome spreads hypotonic solution	50% FBS diluted in dH ₂ O
Fetal ovaries and testes chromosome spreads hypotonic buffer	30 mM Tris-Cl (pH 8.2), 50 mM sucrose pH 8.2, and 17 mM Na- citrate.
Hypotonic Solution for Carnoy's Spreads	0.56% KCl

2.7 Plasmids

Plasmid	Source
<i>pCMV-8.91</i>	Anil Chandrashekan (IRDB, Imperial College London)
<i>pMDG2-vsug</i>	Anil Chandrashekan (IRDB, Imperial College London)
<i>LV-CRE-EGFP</i>	Joost Verhaagen (Netherlands Institute for Neuroscience, Amsterdam) (Ahmed et al., 2004)
<i>pLKO.3G</i>	Anil Chandrashekan (IRDB, Imperial College London)

2.8 Chemical Inhibitors

Stocks of drugs or inhibitors were prepared by suspending in DMSO, typically at a concentration thousand times of the desired final concentration. The stock was then aliquoted and stored in -20°C. When the drug-supplemented media was prepared, media was pre-heated in 37°C and the drug rapidly added and mixed to ensure that it is fully dissolved.

Cdk1 inhibitor (RO3306)	Millipore
IBMX	Sigma
dbcAMP	Sigma
Cytochalasin B	Sigma
Demecolcin	Sigma

2.9 Hormones

Both hormones were prepared under sterile conditions by dissolving the lyophilized hormone in filter sterile Phosphate Buffered Saline solution (PBS) at a final concentration 5 IU/0.1 ml. The solution was aliquoted into 1.5 ml Eppendorf tubes before freezing at -20°C until just prior to use when the hormone was thawed at RT.

PMSG	Choluron, Intervet
hCG	Choluron, Intervet

2.10 Tissue Culture Components

Reagent	Supplier
Dulbecco's MEM (DMEM), High Glucose, no Glutamine	Gibco
Iscove's Modified Dulbecco's Medium (IMDM)	Gibco
Fetal Bovine Serum, Heat-inactivated (HI FBS)	Gibco
L-Glutamine 200mM (100X)	Gibco
Penicillin/Streptomycin (100X)	Gibco
Trypsin 0.25% 1X with phenol-red	Sigma

2.11 Media

2.11.1 Mammalian Tissue Culture Media

293T Media	DMEM high-glucose supplemented with: 10% (v/v) HI-FBS 2 mM L-Glutamine 50 U/mL Penicillin 50 µg/mL Streptomycin
Mouse Embryonic Fibroblast (MEF) Media	As 293T media but 15% (v/v) HI-FBS
B3 cell Media	IMDM supplemented with: 2 mM L-Glutamine and 25 mM HEPES, 10% (v/v) HI-FBS, 50 U/mL Penicillin and 50 µg/mL Streptomycin
Viral Collection Media	As 293T media but 5% (v/v) HI- FBS

2.11.2 Oocyte and Zygote Culture

M2 medium	Sigma
M16 medium	Sigma
KSOM medium	Millipore
Mineral oil	Sigma

Chapter 3

Localisation of the cohesin loading factor
Nipbl in mammalian meiotic cells.

3.1 Introduction

The association of cohesin with chromosomes in proliferating cells requires kollerin, a binary protein complex composed of Nipbl (Scc2) and Mau-2 (Scc4) (Nasmyth, 2011). The role of kollerin in mediating cohesin loading is evolutionarily conserved in mitosis. Inactivation of Nipbl in plants, nematodes and *Coprinus* causes cohesion defects and infertility suggesting that Nipbl is also responsible for cohesin loading during meiosis (Ciosk et al., 2000; Tomonaga et al., 2000; Gillespie and Hirano, 2004; Seitan et al., 2006; Cummings et al., 2002; Gause et al., 2008; Lightfoot et al., 2011; Lin et al., 2011; Sebastian et al., 2009; Watrin et al., 2006).

Meiotic prophase is a period of dynamic chromosome behaviour, during which chromatin undergoes large-scale reorganization. This includes processes such as establishment of chromosomal structure, followed by pairing of homologs associated with dynamic chromosome movements and crossing-over. The correct execution of these events leads to the formation of stable bivalents, which is essential for accurate chromosome segregation. Careful examination of protein localisation on meiotic chromosomes at different stages gives great insight into their functions. Immunolocalisation studies in some experimental models demonstrated that Nipbl homologues reside at meiotic chromosomal axes in the proximity to cohesin (Cummings et al., 2002; Gause et al., 2008; Lightfoot et al., 2011; Lin et al., 2011; Sebastian et al., 2009). However, Nipbl has not been characterized in mammalian meiotic cells and the binding pattern on meiotic chromosomes is unknown. Therefore, I set out to characterize the localisation of Nipbl and its association with cohesin during mammalian meiosis.

Here, I show that the cohesin loading factor Nipbl, a mammalian homologue of yeast Scc2, is present on chromosomes throughout meiotic prophase in mouse spermatocytes and oocytes. Nipbl decorates the axial/lateral element of the synaptonemal complex in zygotene. Later, Nipbl translocates from chromosome axes to chromocenters concomitantly with the completion of homologous recombination during mid-pachytene in spermatocytes.

3.2 Results

3.2.1 Nipbl is expressed in mammalian spermatocytes

Nipbl expression was analysed at the protein level in mouse testes by Western blotting assays. Total protein extracts from wild-type mouse testes and proliferating somatic cells from the mouse pre-B cell line B3 (Brown et al., 1997) were run on SDS-PAGE gels. The human NIPBL has two major isoforms, Nipbl A that is 2804 amino acids long (315 kDa), and Nipbl B that translates into 2697 amino acids (304 kDa). Both isoforms are identical up to the 2683 amino acid residue, but differ at the C-termini (Tonkin et al., 2004). Three antibodies were used to detect human NIPBL: two rat monoclonal antibodies, KT54 and KT55 that were raised against C-terminal fragments specific to NIPBL isoforms A and B, respectively (Seitan et al., 2006) and a rabbit polyclonal anti-Nipbl antibody (114) that recognizes both isoforms (Watrin et al., 2006). The peptide sequences in NIPBL A and B used for raising the isoform-specific antibodies are conserved between human and mouse homologs. As shown in Figure 3.1, all three antibodies detected one major protein in both testis and somatic cell extracts that migrated at approximately 300 kDa, which is expected for mouse Nipbl A and B isoforms. These results demonstrate that Nipbl is expressed in mammalian testes.

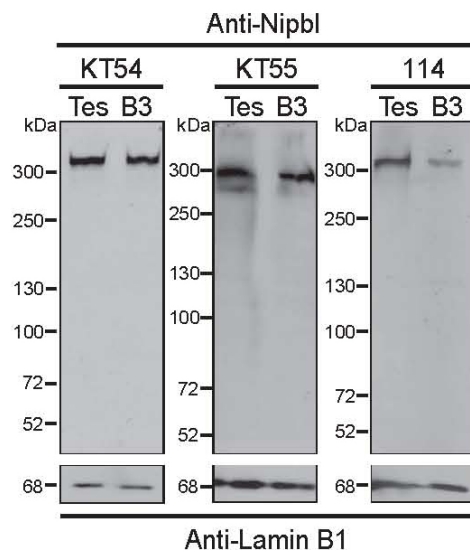


Figure 3.1 The specificity of anti-Nipbl antibodies.

Western blotting assays on total protein extracts from whole mouse testis (Tes) and mouse pre-B cell line B3 (B3) using three antibodies against Nipbl. Rat monoclonal antibodies KT54 and KT55 were raised against the C-terminal fragments of two splicing isoforms of human NIPBL: A; long form (315 kDa) and B, short form (304 kDa). The rabbit polyclonal antibody 114 recognizes both splicing variants. The peptide sequences in NIPBL A and B used for raising these isoform-specific antibodies are conserved between human and mouse homologs. Rabbit polyclonal anti-Lamin B1 antibody was used as a positive loading control for each blot.

Because testes are composed of somatic and germ cells, I further set out to determine which cell types in the testes are expressing Nipbl. Immunohistochemistry was performed on mouse testis sections using anti-Nipbl B (KT55) antibody. Within the seminiferous tubules, spermatogonia are localised on the peripheral basement membrane, and the developmentally more advanced cell types are arranged in a sequential order (spermatocytes, spermatids, spermatozoa) towards the lumen of a tubule (Russell et al., 1990). Immunostaining with the anti-Nipbl antibody revealed high expression levels of Nipbl in mitotically proliferating spermatogonia (marked as G in Fig. 3.2) and meiotic spermatocytes (marked as C in Fig. 3.2). Nipbl was also detectable at chromocenters of postmeiotic round spermatids (marked as R in Fig. 3.2), but not in spermatozoa (marked as Z in Fig. 3.2). Accumulation of Nipbl was observed in quiescent Sertoli cells (marked as S in Fig. 3.2), however at lower levels than in spermatogonia. Taken together, these results suggest that mouse Nipbl is expressed in spermatocytes, which is consistent with meiotic function. This experiment has been performed by Dr Xiangwei Fu.

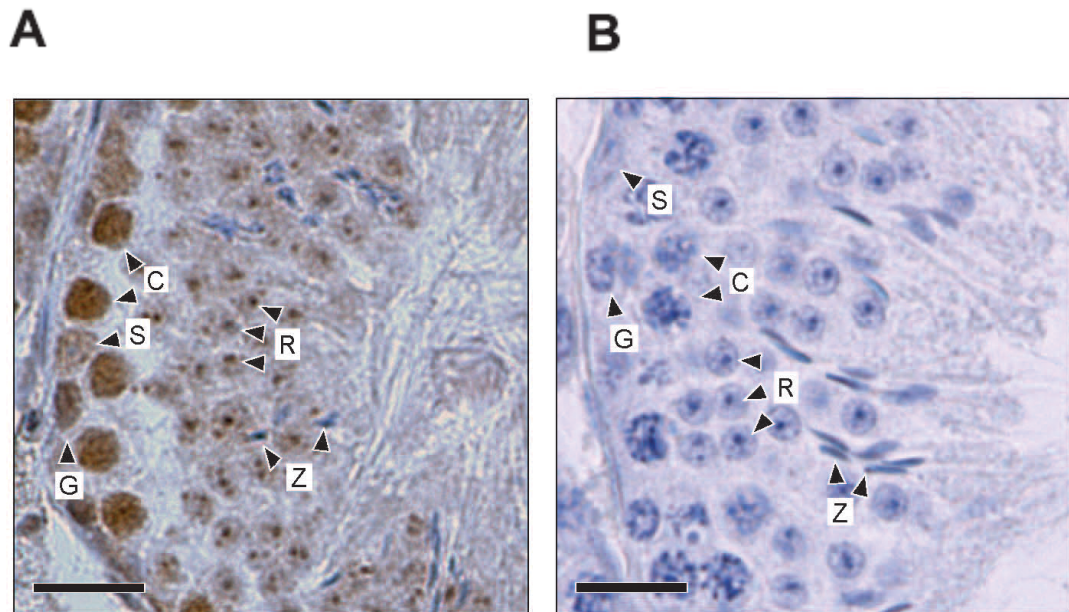


Figure 3.2 Nipbl is expressed in mouse spermatocytes.

(A) Immunohistochemistry on seminiferous tubule cross-section from wild-type adult mouse testis. Sections were stained with rat monoclonal anti-Nipbl B (KT55) antibody, a horseradish peroxidase-conjugated secondary antibody and the stain was developed with DAB. Sections were counterstained with haematoxylin. Brown staining indicates positive immunoreactivity
(B) Control immunohistochemistry staining with no primary antibody. Specific cell types in the seminiferous epithelium are marked: G, spermatogonium; C spermatocyte; R, round spermatid; Z, spermatozoa; S, Sertoli cell. Scale bar 200 μ m.

In order to determine Nipbl localisation on meiotic chromosomes during spermatogenesis and oogenesis, I prepared testicular and embryonic ovarian chromosome spreads and performed three sets of staining. Two isoform-specific anti-Nipbl antibodies and an antibody that recognizes both splicing variants of Nipbl (Watrin et al., 2006) were separately used to co-label chromosome spreads with an antibody against Sycp3. The anti-Sycp3 antibody marks a component of the synaptonemal complex (SC) and enables to recognize distinct sub-stages of meiosis. To investigate the relationship between cohesin and Nipbl, I visualised α -kleisin subunit Rec8 in spermatocytes. Very similar binding patterns were observed for all three Nipbl antibodies, thus in the description below I present a summary description of Nipbl localisation on meiotic chromosomes in spermatocytes and oocytes with representative images reported in Figure 3.3 and 3.6. The images of all co-staining patterns with isoform-specific antibodies can be found in Appendix I.

3.2.2 Nipbl localisation on meiotic chromosomes during spermatogenesis.

A) Leptotene

Upon the completion of DNA replication in S phase, meiocytes enter the first stage of meiotic prophase, called leptotene. During leptotene, Spo11 induces DSBs formation. Meiotic cohesin complexes, which contain proteins such as Rec8, are loaded onto chromosomes before replication and start to form axial elements (AEs) by associating with proteins such as Sycp3 or Sycp2 (Eijpe et al., 2000; Lee et al., 2003; Pelttari et al., 2001). AEs form along the places where homologous chromosomes synapse and can be observed as progressively elongating strands as the leptotene stage proceeds (Eijpe et al., 2003). Co-labelling with anti-Sycp3 and anti-Nipbl antibodies on testicular chromosome spreads revealed that Nipbl is present to chromatin and its staining pattern is visible as dot-like aggregations scattered all over the nucleus (Fig. 3.3, b). The Nipbl signals co-localise irregularly with those of Sycp3 (Fig. 3.3, a). To investigate the relationship between the localisation of cohesin and Nipbl, testicular chromosome spreads were co-stained with antibodies against Nipbl and Rec8. The signals of cohesin and Nipbl are partially overlapping on the forming AEs (Fig. 3.4, a). Together, these results demonstrate that Nipbl is expressed at an early stage of meiotic prophase in spermatocytes and that the binding patterns of Nipbl are very similar to those of cohesin and Sycp3, which suggests that Nipbl is also a component of AEs.

B) Zygotene

In zygotene, meiotic DSBs are repaired by homologous recombination using non-sister chromatids as template. This repair process promotes homologous chromosome pairing and the formation of the synaptonemal complex. Short segments of the chromosomal axis, which are visible in leptotene, fuse to form the full-length AEs detectable as elongated bars when stained with an anti-Sycp3 antibody (Fig. 3.3, b). Zygotene is characterized by the appearance of pairwise associations between the AEs of homologous chromosomes as they become connected by the central element (CE) and consequently AEs are called lateral elements (LEs). Zygotene nuclei co-stained with anti-Nipbl and anti-Sycp3 antibodies show that Nipbl signals start to form discrete thread-like contiguous foci along both synapsed (LEs) and unsynapsed (AEs) regions of the assembling SC (Fig. 3.3, b). Nipbl signals are not evenly spread and some regions of the chromosomal axis have weak while others strong, punctuated staining (Fig. 3.3, b). Co-labeling with antibodies against Nipbl and Rec8 revealed that Nipbl partially co-localises with cohesin on the forming synaptonemal complex (Fig. 3.4, b).

C) Pachytene

In pachytene, the process of synapsis along the entire length of homologous chromosomes is completed. DSBs are repaired in mid-pachytene, which consequently leads to crossovers and non-crossovers. Co-staining with antibodies against Nipbl and Sycp3 revealed three distinct patterns of Nipbl in early, mid and late pachytene. In early pachytene when anti-Sycp3 signals mark fully synapsed chromosomes, Nipbl localise to AE/LE (Fig. 3.3, c). In mid-pachytene, Nipbl signals continue to be visible at AE/LE but additionally they are enriched at chromocenters, which are visible as DAPI dense patches around centromere (Fig. 3.4, d). In late pachytene, the AE/LE-localised fraction of anti-Nipbl signals became weaker while the accumulation at the chromocenters persisted (Fig. 3.3, e). The co-localisation analysis of Rec8 and Nipbl showed that the signals along AE/LE partially overlap during early and mid-pachytene (Fig. 3.4, c-d). Thus, Nipbl remains a component of the AE/LE after the completion of synapsis until the mid-pachytene stage but relocates in late pachytene from AE/LE to sites adjacent to centromeres of sister chromatid pairs.

D) Diplotene

During diplotene, SCs gradually disassemble whilst AE/LE remain structurally intact, despite the re-localisation of several components such as Sycp3 or cohesin subunits to the centromeric region of sister chromatids (Eijpe et al., 2003; Lee et al., 2003). Homologous chromosomes are desynapsed except for crossovers, which are regions of homologous recombination (Page and Hawley, 2003). Nipbl does not reside any longer at AE/LE marked by Sycp3 but is enriched only at chromocenters (Fig. 3.4, f). Thus, these results indicate that Nipbl is no longer localised along the AE/LEs during the diplotene stage.

E) Metaphase I and Anaphase I

At metaphase I, the pairs of sister kinetochores are attached to microtubules of the spindle and are oriented towards opposite poles (Page and Hawley, 2003). Sycp3 is mostly lost from the chromosome arms (Fig. 3.3, g). No anti-Nipbl signals were detected on metaphase I chromosome spreads (Fig. 3.3, g) suggesting that Nipbl dissociates from chromosomes upon entry into meiosis I (Fig. 3.3, g). In anaphase I, anti-Nipbl signals appeared at the centromeres mostly co-localising with anti-Sycp3 signals (Fig. 3.3, h).

F) Interkinesis (Interphase)

Upon completion of the first meiotic division, primary spermatocytes enter interkinesis. A nucleus undergoing interkinesis is characterized by the presence of a variable number of chromocenters strongly stained with DAPI and associated with Sycp3 signals (Kudo et al., 2009). Co-staining of interkinesis nuclei with antibodies against Nipbl and Sycp3 showed weak Nipbl signals that localise to chromocenters (Fig. 3.3, i).

G) Metaphase II

At metaphase II sister chromatids are joined at the centromere. Later, during the metaphase-to-anaphase transition in meiosis II, sister chromatids eventually separate from each other in the result of centromeric cohesion dissociation. No anti-Nipbl signals are present on metaphase II chromosomes (Fig. 3.3, j).

H) Round Spermatids

Anaphase II is followed by spermatocyte differentiation that generates mature spermatozoa. The co-staining with anti-Sycp3 and anti-Nipbl demonstrated that Nipbl appears at chromocenters (Fig. 3.3, k).

I) Mitotic metaphase

As previously reported, Scc2 binds to chromatin from the end of mitosis until prophase, thus it is not present on mitotic metaphase chromosomes in HeLa cells (Watrin et al., 2006). Co-labelling of mitotic metaphase chromosome spreads with anti-Nipbl and anti-Sycp3 antibodies, demonstrated that Nipbl is not present (Fig. 3.3, l).

In summary, Nipbl is detectable on chromosomes soon after meiotic entry. In leptotene nuclei Nipbl is visible as scattered foci throughout the nucleus. These signals partially overlap with anti-Rec8 signals. Subsequently in zygotene, Nipbl accumulates to the axial/lateral element (AE/LE) of the synaptonemal complex that contains Sycp3 as well as cohesin. I have also observed overlaps of signals between anti-Nipbl and anti-Rec8 at AE/LE. The localisation of Nipbl to AE/LE is maintained in early pachytene, but by late pachytene stage the anti-Nipbl signals at AE/LE become faint, while intense anti-Nipbl signals are visible at chromocenters. In diplotene, Nipbl is enriched only at chromocenters. During metaphase I stage, no anti-Nipbl signals can be detected, suggesting that Nipbl may dissociate from chromosomes upon entry into meiosis I. However, weak signals on chromosomes undergoing anaphase I are observed. In nuclei at interkinesis, weak Nipbl signals are detected at chromocenters. Chromosomes undergoing meiosis II also exhibit weak anti-Nipbl signals. Anti-Nipbl signals are also detectable in postmeiotic spermatids with accumulation at chromocenters.

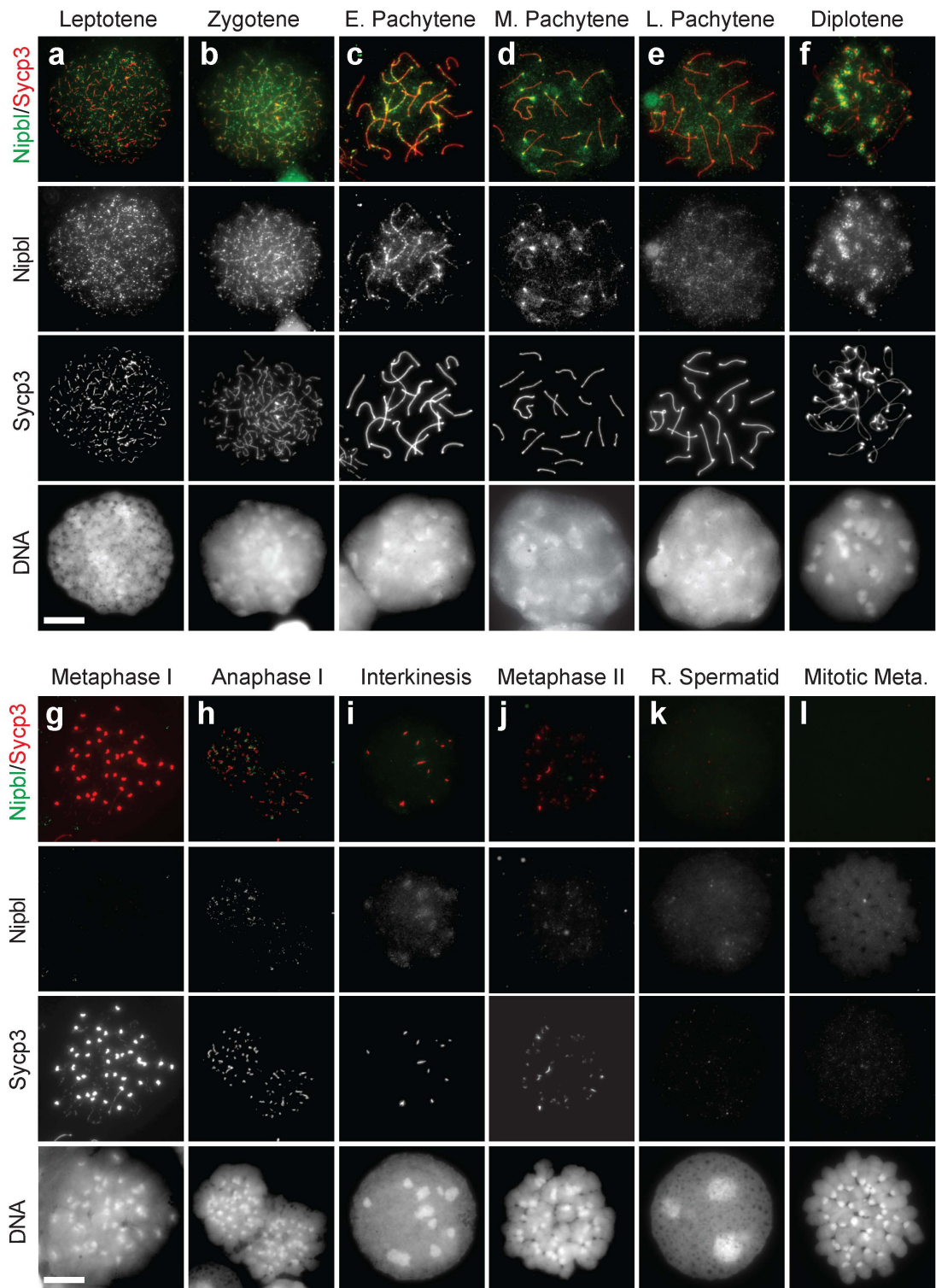


Figure 3.3 Nipbl localisation on spermatocyte chromosomes.

Chromosome spreads from testicular cells were subjected to immunofluorescent staining with rat monoclonal antibody against Nipbl and anti-Sycp3 mouse monoclonal antibody. Sycp3 marks the AE/LE of the synaptonemal complex. DNA was counterstained with DAPI and merged images are shown. a: Leptotene, b: Zygotene, c: Early Pachytene, d: Mid-Pachytene e: Late Pachytene, f: Diplotene, g: Metaphase I, h: Anaphase I, i: Interkinesis, j: Metaphase II, k: Round Spermatid, l: Mitotic Metaphase. Sycp3 is in red and Nipbl is in green. In merge images co-localisation of red and green signals appear yellow. E., early, M., mid. L., late. Scale bar 5 μ m.

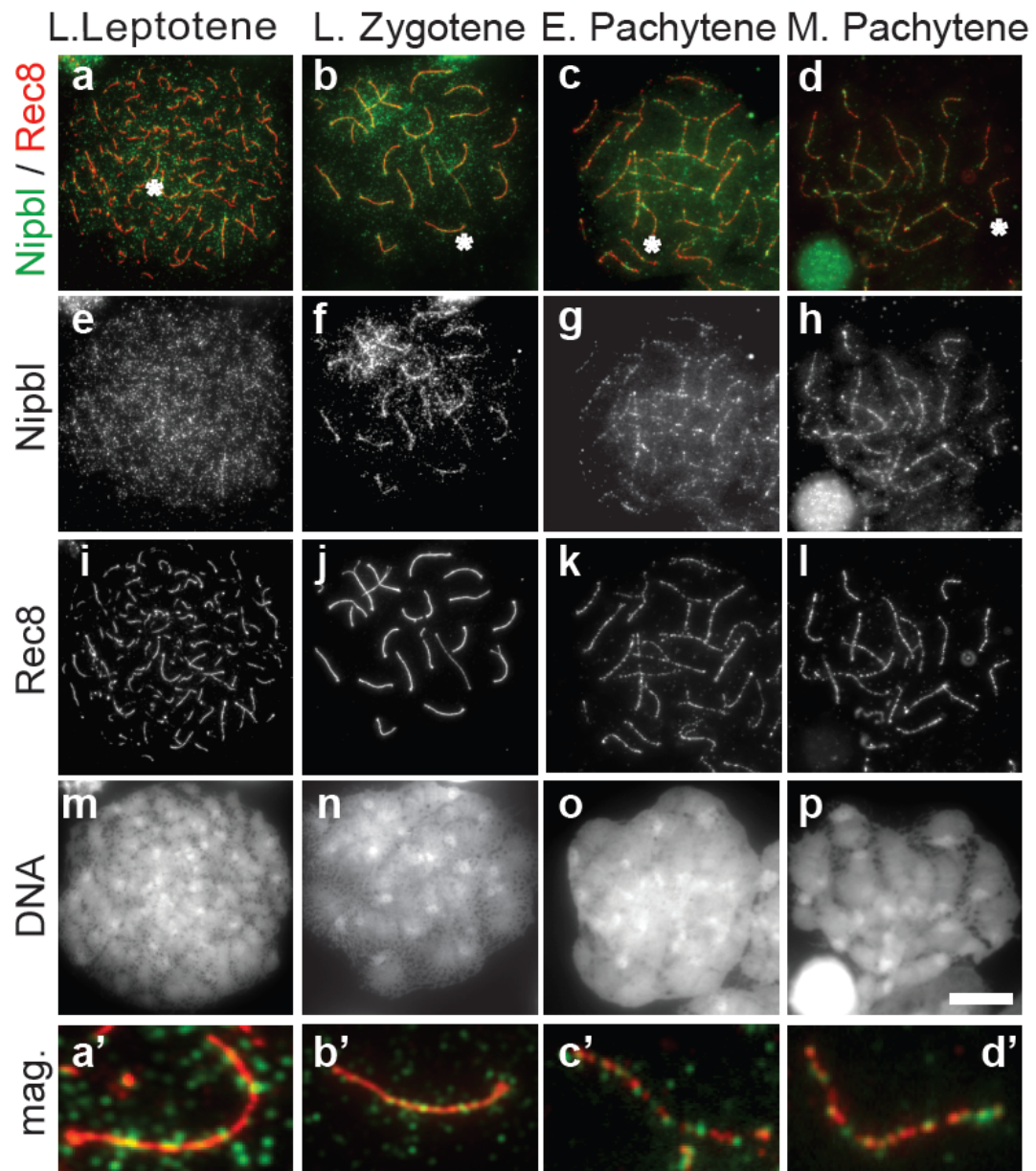


Figure 3.4 Differential distributions of Nipbl and Rec8 on chromosomal axis.

Nuclear spreads from testicular cells were subjected to immunofluorescent staining with a rat monoclonal antibody against Nipbl and a rabbit polyclonal antibody against Rec8. Rec8 localises to AE/LE during the stages shown. DNA was counterstained with DAPI and merged images are shown (a: Late Leptotene, b: Late Zygotene, c: Early Pachytene, d: Mid Pachytene). The regions indicated by asterisks in a, b, c, d are magnified in a', b', c', d'. Rec8 is shown in red and Nipbl is shown in green. In merge images co-localisation of red and green signals appear yellow. E, early; M., mid. L, late. Scale bar 5 μ m.

3.2.3 Nipbl re-localisation takes place concomitantly with completion of DSBs repair during spermatogenesis.

Mid-pachytene is the stage when DSB repair is completed by the resolution of the double Holliday junction, which coincides with the disappearance of phosphorylated H2AX (γ -H2AX) and Rad51 signals from the nuclei and the appearance of recombination nodules marked by Mlh1 (Lee et al., 2003). As concluded from Nipbl localisation studies in mouse spermatocytes (Fig. 3.3), Nipbl reside at AE/LE of chromosomal axes in early pachytene, but re-localise from AE/LE to chromocenters by late pachytene. To investigate further whether the Nipbl re-localisation from AE/LE in mid-pachytene correlates with other chromosomal events, I performed co-labelling experiments with antibodies staining Nipbl and markers of meiotic prophase events: γ -H2AX, Rad51, Mlh1.

A) γ -H2AX

One of the first cellular responses to the introduction of DSBs by Spo11 is the phosphorylation of H2AX, a highly conserved histone variant, in chromatin flanking DSBs (Mahadevaiah et al., 2001; Rogakou et al., 1998; Nagata et al., 1991). This process promotes structural chromosome changes and is spatially and temporally linked to synapsis and meiotic DSBs in male mouse spermatocytes (Mahadevaiah et al., 2001; Bassing et al., 2004; Fernandez-Capetillo et al., 2004; Rogakou et al., 1999). Testicular meiotic spreads co-stained with anti- γ -H2AX and anti-Nipbl antibodies revealed a strong and chromosome-wide signal of γ -H2AX in leptotene and zygotene with foci localising to AEs and those protruding along chromatin loops (Fig. 3.5 A, a-b), which is similar to results described by Chicheportiche and co-workers (2007). At these stages Nipbl did not co-localise with anti- γ -H2AX signals (Fig. 3.5 A, a-b). In early pachytene, anti- γ -H2AX signals persisted on chromosomal axes and localised with Nipbl in a mutually exclusive manner. The second population of γ -H2AX signals began to accumulate on the sex chromosomes, marking sex body formation (Fig. 3.5 A, c) (Blanco-Rodríguez, 2012; Chicheportiche et al., 2007). Later, in mid-pachytene, the anti- γ -H2AX signals at AE/LE became weaker, which was also observed for Nipbl signals (Fig. 3.5 A, d). In late pachytene, anti- γ -H2AX signals were absent from AE/LE whilst Nipbl localised to chromocenters (Fig. 3.5 A, e). These results suggest that DSBs repair and weakening of anti- γ -H2AX signals at the AE/LE coincide with Nipbl re-localisation.

B) Rad51

To further explore Nipbl re-localisation during pachytene, I examined the temporal relationship between Nipbl signals and Rad51 foci. Following the formation of multiple DSBs in leptotene, Rad51 foci appear in high abundance, at about 300 foci per mouse male nucleus. Rad51 catalyses the DNA strand exchange reaction between homologous chromosomes and subsequently marks early recombination intermediates. The number of Rad51 foci gradually declines by zygotene and completely disappears in diplotene (Ashley et al., 1995; Barlow et al., 1997; Moens et al., 1997). Immunofluorescent labelling of testicular chromosome spreads with antibodies against Rad51 revealed the gradual decline in the number of Rad51 foci from early zygotene to pachytene, which confirms the previous observations (Fig. 3.5 B, a-c). Co-labelling studies with antibodies against Nipbl demonstrated that Nipbl co-exists with Rad51 at chromosomal axis throughout zygotene, but the gradual loss of Rad51 signal from meiotic chromosomes precedes the re-localisation of Nipbl (Fig. 3.5 B, a-c).

D) Mlh1

Next, I investigated the temporal relationship between Nipbl and Mlh1 foci. In pachytene, Mlh1, a mismatch repair protein homolog, localises to chromosomal axes of bivalents, where it is involved in reciprocal recombination. Consequently, Mlh1 marks places where crossover occurs (Anderson et al., 1999; Marcon and Moens, 2003). Co-immunolabelling with antibodies against Mlh1 and Nipbl revealed that in early pachytene when Nipbl signals localise along the AE/LE, Mlh1 foci were not observed (Fig. 3.5 C, a). A few Mlh1 foci started to appear in mid-pachytene when the Nipbl signals were visible on AE/LE and started accumulating at chromocenters (Fig. 3.5 C, b). These signals co-existed in late pachytene when the number of Mlh1 foci increased, while Nipbl accumulated at chromocenters (Fig. 3.5 C, c).

Taken together, the co-localisation analysis of Nipbl and markers of the meiotic prophase events demonstrates that the Nipbl re-localisation from the AE/LE to chromocenters takes place concomitantly with completion of DSBs repair.

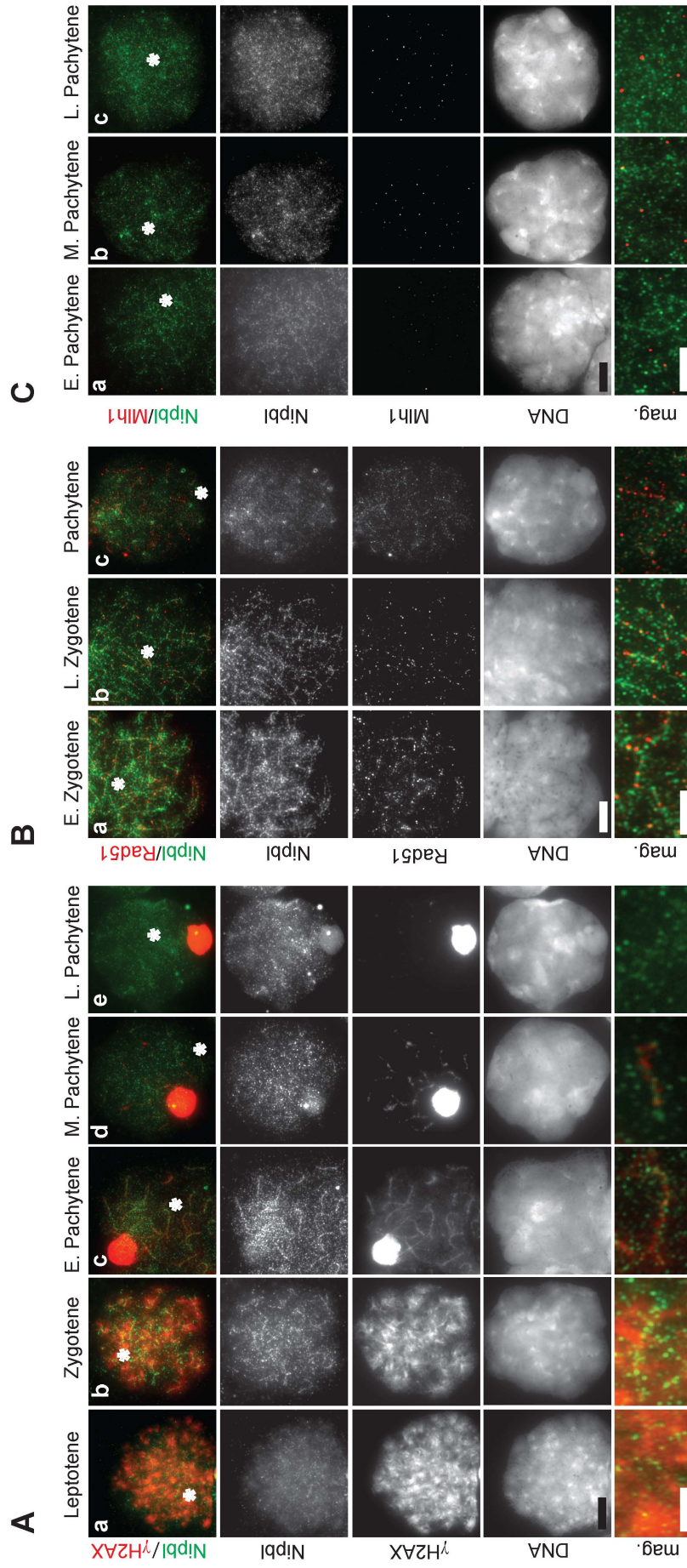


Figure 3.5 Temporal correlation between the re-localisation of Nipbl and other meiotic prophase events during spermatogenesis.

Chromosomal spreads from testicular cells were immunofluorescently labelled with a rat monoclonal antibody against Nipbl and marker antibodies: (A) rabbit polyclonal anti- γ H2AX antibody marking double strand breaks (DSBs). Note that anti- γ H2AX highly accumulates at the sex body during the stages shown. The regions indicated by asterisks in a: Leptotene, b: Zygotene, c: Early Pachytene, d: Mid Pachytene, e: Late Pachytene are magnified at the bottom. (B) mouse monoclonal anti-Rad51 antibody marking early recombination intermediates. The regions indicated by asterisks in a: Early Zygotene, b: Late Zygotene, c: Pachytene are magnified at the bottom. (C) mouse monoclonal anti-Mlh1 antibody marking the appearance of recombination nodules. The regions indicated by asterisks in a: Early Pachytene, b: Mid Pachytene, c: Late Pachytene are magnified at the bottom. DNA was counterstained with DAPI and merged images are shown (γ H2AX, Rad51 and Mlh1 are in red and Nipbl is in green). In colour merge images co-localisation of red and green signals appear yellow. E., early; M., mid; L., late. Scale bar 5 μ m.

3.2.4 Nipbl localisation on meiotic chromosomes during oogenesis.

To investigate Nipbl localisation during meiotic prophase in female mice, chromosome spreads from wild-type foetal ovaries were prepared and subjected to immunolabelling with anti-Sycp3 antibodies and three anti-Nipbl antibodies (as above) (Fig. 3.6). In leptotene nuclei, Nipbl was detected as scattered foci throughout the nucleus, which were partially co-localising with Sycp3 (Fig. 3.6, a). In zygotene, Nipbl accumulated on axial element (AE) and co-localised with Sycp3 signals (Fig. 3.6, b-c). Anti-Nipbl signals were enriched at chromosome axes throughout the pachytene and diplotene stages (Fig. 3.6, d-e). To examine Nipbl localisation in oocytes during late meiotic stages, I prepared chromosome spreads from fully-grown germinal vesicle (GV) stage oocytes and oocytes cultured *in vitro* until metaphase I and II stages. Chromosome spreads were immunostained with an anti-Nipbl antibody and CREST serum that labels kinetochores. Anti-Nipbl signals were faint over the germinal vesicle chromatin and absent from chromosomes in metaphase I and II (Fig. 3.6, f-h).

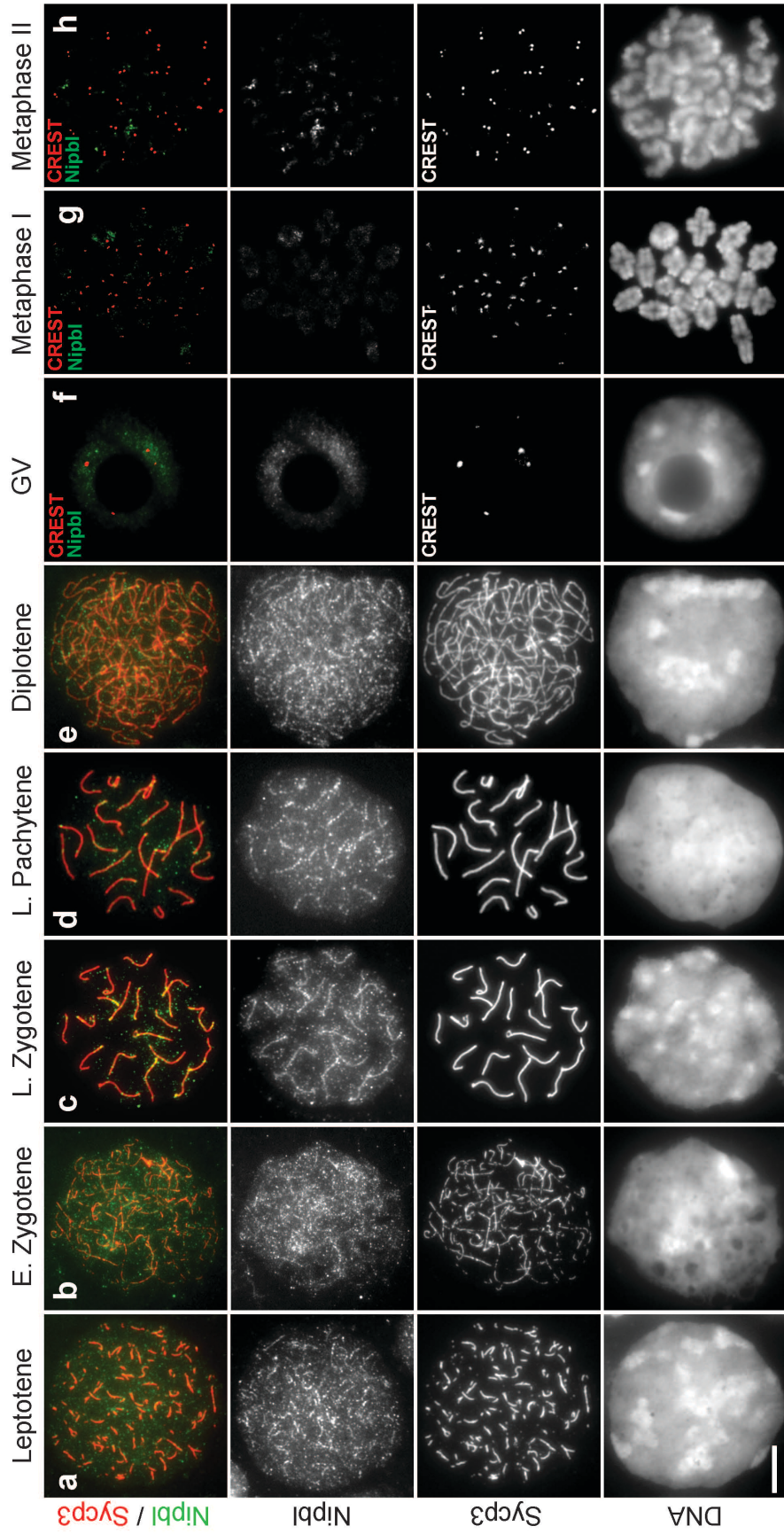


Figure 3.6 Nipbl localisation on oocyte chromosomes.

Chromosomal spread assay during meiotic prophase stages in E15.5 wild-type fetal ovaries. Spreads were immunofluorescently labelled with rat monoclonal antibody against Nipbl and an anti-Sycp3 mouse monoclonal antibody. Sycp3 marks the AE/LE of synaptonemal complex. DNA was counterstained with DAPI and merged images are shown. a: Leptotene, b: Early Zygotene, c: Late Zygotene, d: Late Pachytene, e: Diplotene, f: GV, g: Metaphase I, h: Metaphase II. Sycp3 is in red and Nipbl is in green. In colour merge images co-localisation of red and green signals appear yellow. E., early; L., late; GV: Germinal vesicle. Scale bar 5µm.

3.3 Discussion

3.3.1 Summary of observations

Nipbl and its functional homologues have been characterised in different eukaryotic model organisms, which demonstrated that Nipbl is required for chromatin association of cohesin and therefore correct chromosome segregation in mitosis. Nipbl homologues have been also shown to localise to meiotic chromosomal axes in yeast, fruit fly, nematodes, *Arabidopsis thaliana* and mushroom *Coprinus cinereus*. Inactivation of Nipbl homologues in plants, nematodes and *Coprinus* causes cohesion defects and infertility, which suggest that Nipbl is responsible for cohesin loading also in meiotic cells (Cummings et al., 2002; Gause et al., 2008; Lightfoot et al., 2011; Lin et al., 2011; Sebastian et al., 2009). However, the cohesin loading factor has not been studied in mammalian meiotic cells so far.

The goal of this study was to analyse the localisation of the cohesin loading factor Nipbl in different meiotic stages of wild-type mouse germ cells. For immunolabelling I used three different antibodies. Two of them recognise two known Nipbl isoforms (Nipbl A and B), while the third antibody detects both isoforms. I have not observed significant differences in localisation patterns at any stage of meiosis with these three antibodies, which suggest that Nipbl isoforms are indistinguishable in terms of localisation during meiotic prophase.

To this end I found that Nipbl is expressed in mitotically proliferating spermatogonia, as well as in meiotic spermatocytes and post-meiotic round spermatids. For further cytological analysis, I have employed a drying-down technique for the spreading of mammalian meiocytes, a very useful tool for studying nuclei of all meiotic stages (Peters et al, 1997). These studies revealed that Nipbl is present on meiotic chromosome axis from zygotene to mid-pachytene, after which it re-locates to chromocenters where it is detectable throughout meiotic prophase. In developing oocytes, Nipbl is present on chromosomal axes at least until diplotene stage, thus no re-localisation was observed in oocytes. In spermatocytes, I could observe significant overlap in staining between cohesin and Nipbl during zygotene, consistent with a role for Nipbl as a cohesin loader. After mid-pachytene, however there was little overlap in staining indicating that when at chromocentres, Nipbl does not load detectable levels of cohesin. Co-localisation studies between markers of crossover formation events and Nipbl suggest that homologous recombination may

require Nipbl-mediated cohesin reloading.

3.3.2 Nipbl is expressed in post-meiotic cells

Although the involvement of cohesin and its regulatory proteins in sister chromatid cohesion from S phase until metaphase is well established, cohesin can also play other functions in the cell. As shown by a number of recent studies cohesin complex regulates gene expression in both proliferating and post-mitotic cells and participates in DNA damage responses. For example, cohesin or its accessory protein Pds5B are widely expressed in mouse post-mitotic neurons that normally do not replicate their DNA again and therefore cannot establish cohesion (Wendt et al., 2008; Zhang et al., 2007). Disruption of Rad21/Scc1 function in *Drosophila* results in precocious separation of sister chromatids and massive chromosome missegregation in proliferating cells but not in post-mitotic mushroom body neurons, where Rad21 disruption results in defects in axon pruning (Pauli et al., 2008). This provides strong evidence for a function of cohesin on chromosomes that is distinct from its role in cell division. While many of cohesin's known roles in DNA damage responses are associated with its function in sister chromatid cohesion, some of cohesin's functions in gene regulation are independent from its role in cohesion. Presumably both, sister chromatid cohesion and gene regulation, involve the same molecular mechanism that is based on direct association with DNA (Peters et al., 2008).

As mentioned in Chapter 1, *Nipped-B*, the fly ortholog of *Nipbl/Scc2*, was discovered as a gene that regulates long-range activation of homeobox genes, which regulates wing and limb development (Rollins et al. 1999). In humans, mutations in cohesin loading complex *Nipbl/Scc4* that affect only one allele do not result in sister chromatid cohesion defects but instead can result in the severe developmental disorder Cornelia de Lange Syndrome (Krantz et al., 2004; Tonkin et al., 2004). This observation attributes importance to the correct expression levels of the cohesin loading complex, which plays an important role in transcriptional regulation.

The presence of cohesin has also been investigated in mammalian meiotic and post-meiotic cells. Most of cohesin is lost from centrosomes at metaphase II-to-anaphase II transition in both oocytes and spermatocytes (Eijpe et al., 2000; Eijpe et al., 2003). However, immunostaining of formalin-fixed testis sections with anti-cohesin antibodies detected Rec8 and Smc3 signals in post-meiotic terminally

differentiated round spermatids. Also, as presented in this thesis, cohesin loading factor Nipbl is expressed not only in mitotically proliferating spermatogonia and meiotic spermatocytes, but also in post-meiotic round spermatids. This observation indicates that Nipbl mediates cohesin loading during mammalian meiosis but also that Nipbl may be required for cohesin loading and turnover outside S phase to support cohesin's function in quiescent cells. The exact role of both cohesin and Nipbl in terminally differentiated meiotic cells remains to be investigated.

3.3.3 Chromosomal localisation of Nipbl in spermatocytes and oocytes during the meiotic prophase in mouse

Immunostaining with antibodies against Nipbl on both oocytes and spermatocytes, revealed that Nipbl is present on chromosomes from leptotene stage. Co-staining with antibodies against Nipbl and meiotic cohesin kleisin Rec8 demonstrated that in spermatocytes cohesin and Nipbl appear in the nucleus at the same time. Nipbl is detectable as spread foci throughout the nucleus, whilst surprisingly, cohesin organises itself in recognisable axes before its loading factor Nipbl. This observation could suggest that Nipbl does not mediate cohesin loading in mammalian meiotic cells. However, this can also be interpreted in the context of recent study proposing that Nipbl loads cohesin at distinct loading positions, from which cohesin may slide along DNA in an ATP-dependent manner (Hu et al., 2011).

In the subsequent stage, zygotene, Nipbl accumulates at the AE/LE where it reaches a maximum co-localisation level with meiosis-specific α -kleisin cohesin subunit Rec8. These results suggest that cohesin loading activity is maintained during early stages of meiotic prophase in mammals. In spermatocytes, the axial localisation is maintained in early pachytene, where homologues are paired and synapsed, but during mid-pachytene Nipbl dissociates from the AE/LE and accumulates at chromocenters. Surprisingly this re-localisation cannot be observed in oocytes and Nipbl remains stably associated with chromosomal axes in developing oocytes. In addition to chromosome bound Nipbl I have also detected a pool of Nipbl that is not associated with meiotic chromosomes. The localisation of Nipbl to chromosomal axes is consistent with previous observations of Nipbl homologues in non-vertebrate meiotic cells suggesting that Scc2 may be a conserved component of the axial element of meiotic chromosomes (Cummings et al., 2002; Gause et al., 2008; Lightfoot et al., 2011; Lin et al., 2011; Sebastian et al., 2009). In a fruit fly Scc2 orthologue Nipped-B has been shown to co-localise

extensively with the cohesin subunit Smc1 and Smc3 along the arms of meiotic chromosomes but not at centromeres, at which cohesin loading factor is not present in meiotic cells (Gause et al., 2008). Further, shortly after submission of this thesis, my immunocytological observations have been confirmed in a report by Visnes and colleagues that investigated the localisation of both factors of cohesin loading complex, Nipbl/Scs2 and Mau2/Scs4, during mammalian meiotic prophase. They showed highly matching staining pattern in both male and female germ cells (Visnes et al., 2013).

While Nipbl is found to co-localise with cohesin from late leptotene until mid-pachytene on chromosomal axis, at chromocenters where Nipbl re-localises in late pachytene, co-localisation with cohesin is not present in spermatocytes. This suggests that at chromocenters Nipbl may play a role that is unconnected to cohesin loading and cohesion. This aspect has been discussed in the next paragraph of the discussion. Interestingly, a novel member of meiosis-specific α -kleisin family called Rad21L binds to the AE/LE until mid-pachytene and then dissociates from there with similar timing to Nipbl translocation (Ishiguro et al., 2011; Lee and Hirano, 2011). Consequently, in mid-pachytene the mitotic α -kleisin Rad21 becomes detectable on the AE/LE, as if the cohesin complex containing Rad21 replaces that containing Rad21L (Ishiguro et al., 2011; Lee and Hirano, 2011). Due to this temporal concurrence I propose that Nipbl might need to stay at the AE/LE until the loading of Rad21-containing cohesin, after which it dissociates from the AE/LE. Figure 3.7 illustrates schematic summary of Nipbl localisation during mammalian meiotic prophase and sexual dimorphism observed in the localisation.

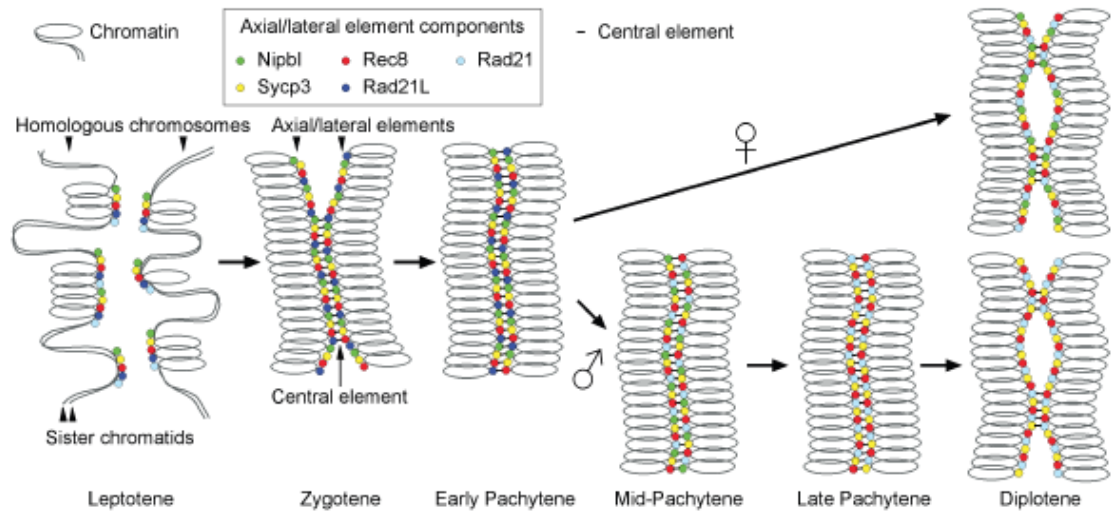


Figure 3.7 Schematic for Nipbl localisation during mammalian meiotic prophase.

Summary of the observations on Nipbl localisation are illustrated together with major components of AE/LE (Sycp3) and previously reported localisation of the cohesin complexes containing different α -kleisin subunits (Rec8, Rad21 and Rad21L) during mouse meiotic prophase in spermatocytes and oocytes. Note that this schematic does not represent co-localisation of each molecule in sub-regions of the AE/LE or interactions between the molecules.

As previously mentioned, Nipbl re-localisation from chromosomal axes to chromocenters is characteristic for mouse spermatocytes and is not observed in oocytes, where Nipbl remains on chromosomal axis until dictyate stage. The reason for this sexual dimorphism is unknown. Since the majority of cohesin required for cohesion in meiosis is assumed to be loaded during meiotic S phase, what is the role of chromatin-bound Nipbl in pachytene or dictyate? Also, as shown by a study by Ravenkova and co-workers cohesin subunits are expressed during late pachytene in oocytes (Ravenkova et al., 2010). One possibility is that Nipbl is needed on chromosomal axis to re-load cohesin and maintain cohesion during dictyate, a state in which oocytes are arrested for months in mouse and for decades in humans. This instead may not be necessary in spermatocytes, whose meiotic development is not arrested and first meiotic division takes place soon after prophase completion. Thus, cohesin loading factor Nipbl may no longer be required to load cohesin at chromosomal axes in late pachytene in spermatocytes and either dissociates from there or is relocated to chromocentres. Furthermore, since the timing of cohesin re-expression occurs simultaneously as the formation of crossovers, Nipbl bound to chromatin at axial elements of meiotic chromosomes might mediate genome-wide cohesin re-loading or might participate in the DSBs repair completion and creation of crossovers. This is at least true for yeast, whose Scc2 mediates global turnover and cohesion reinforcement in response to DSBs (Strom et al., 2004; Unal et al., 2004). Thus, formation and repair of meiotic DSBs can also have an impact on cohesin dynamics in pachytene. If cohesin re-loading and de novo distribution during pachytene are possible in mammalian oocytes, this could be mediated by Nipbl pool in nucleoplasm.

The question whether or not cohesin can be re-loaded after meiotic S phase has important medical applications since it has been suggested that gradual cohesin degradation significantly contributes to the high incidence of aneuploid eggs detected in older woman. To answer this issue I have deleted cohesin loading factor Nipbl after S-phase completion in mouse oocytes. These results are presented in Chapter 4.

3.3.4 Relocalisation of Nipbl to heterochromatin

I have observed that during diplotene in spermatocytes Nipbl re-localises from chromosomal axes to chromocenters. Although the reason for this re-location is unknown one can speculate that it is due to interaction with heterochromatin specific

proteins. Heterochromatin, important for chromosome structure and participating in gene regulation, accumulates heterochromatin binding proteins (HP1) and trimethylated histone H3 (H3K9me3). HP1 proteins have been characterized as enhancers in position effect variegation and heterochromatic gene silencing and activation (Lu et al., 2000; Shiv and Songtao, 2007). In animal cells they are recruited to pericentromeric heterochromatin by Suv39h histone methyltransferase (Guenatri et al., 2004; Bannister et al., 2001).

Nipbl has been shown to physically interact with HP1 through its chromoshadow domain (Lechner et al., 2000; Lechner et al., 2005) as well as with several histone deacetylases (Jahnke et al., 2008). In mitotic cells, HP1 dissociates from mitotic chromosomes and rebinds in telophase, a timing coinciding with Nipbl dynamics (Hirota et al., 2005). As shown by Visnes and colleagues, Nipbl co-localises with H3K9me3 in later stages of mouse meiotic prophase (Visnes et al., 2013). This implies that Nipbl might translocate from AE/LE to chromocenters by its binding affinity to HP1, but Nipbl function at these regions is currently unknown.

3.3.5 Nipbl and DSBs repair

It is well established that cohesin and Nipbl are important for homologous recombination-based DSBs repair. *scc-2(fq1)* mutants of *C.elegans* fail to load both, cohesins and synaptonemal complex components onto meiotic chromosomes. Since both are required for repair of Spo11-induced DSBs it is presumably their combined absence that impairs DSB break repair by homologous recombination. This results in the accumulation of Rad51 foci until diplotene in germ line indicating that the repair of meiotic DSBs is impaired and confirming the requirement of meiotic cohesin for DSB repair (Klein et al., 1999; Pasierbek et al., 2003; Lightfoot et al., 2011).

I hypothesized that Nipbl may bind in vicinity to DSBs to support the process of DNA repair. In order to test this, I examined the localisation of H2AX that becomes phosphorylated upon generation of DSBs and is well established DSB formation marker. In leptotene nuclei, when strong and chromosome-wide signal of γ -H2AX appears and marks numerous DSB sites, there is little evidence for co-localisation between these signals and those of Nipbl. During the course of the meiotic prophase, DSBs are gradually repaired and pan-nuclear staining of γ -H2AX largely disappears. In early pachytene, a sub-fraction of γ -H2AX signal persists on

chromosomal axes of autosomes in close proximity to Nipbl signals. This suggests that the cohesin loading activity of Nipbl may be involved in the processing of recombination intermediates taking place along the SC until mid-pachytene. The disappearance of γ -H2AX in mid-pachytene occurred highly coincidentally with that of Nipbl. Further, I examined the co-localisation of Nipbl with a marker for early recombination intermediates, Rad51. I found that Rad51 foci largely disappeared in early pachytene, which precedes the relocalisation of Nipbl. Finally, the co-localisation of Nipbl and a marker for crossovers, Mlh1 demonstrated that the disappearance of Nipbl from chromosomal axis in late pachytene correlates with the formation of crossovers. These results suggest that although Nipbl does not bind directly to the DSBs, its cohesin loading activity might contribute to the processing of DSBs and completion of their repair.

Interestingly, as reported in a recent study by Visnes and colleagues, spermatocytes with reduced expression level of Nipbl, did not exhibit impaired DSBs repair, however the repair was organized differently than in wild-type cells. Instead of taking place on chromosomal axes, DSBs were repaired on chromosomal loops. This change might be the effect of insufficient loading of cohesin subunits during meiotic prophase (Visnes et al., 2013). In fact, in spermatocytes with lower expression levels of meiosis-specific Smc1 β this reduction is associated with multiple irregular foci throughout nucleus in late prophase stages (Murdoch et al., 2013). Therefore to explore this further it would be interesting to test DSB repair ability of Nipbl-deleted oocytes.

3.3.6 Future directions

1) Characterization of Nipbl's role in gene expression regulation

Although Nipbl has been implicated in the control of gene expression, it is primarily associated with regulation of genes related to development. Even more elusive is Nipbl's role in genes regulation in meiotic cells. Therefore it would be interesting to investigate the global gene expression profiles of fully-grown Nipbl-deleted oocytes at GV-stage in comparison with control *Nipbl^{fl/fl}*. This can be executed by using DNA microarrays, such as Affymetrix oligo DNA arrays. However, a high number of oocytes or embryos required for this kind of experiments is often a limiting factor. For example, Hamatani and co-workers needed to use 500 preimplantation embryos for each RNA extraction performed in their study (Hamatani et al., 2004). However,

recently reported, improved RNA extraction and amplification techniques allow to reduce the number of oocytes required, to less than 60, making this type of experiments less technically challenging (Wang et al., 2004; Kurimoto et al., 2006). Alternatively, genes regulated by Nipbl during oocyte growth can be characterized by mRNA-seq using new generation sequencers (Tang et al., 2009). These experiments will allow improving our understanding of developmental gene regulation and molecular basis of Cornelia de Lange syndrome.

2) Binding of the other SMC complexes in Nipbl-deleted oocytes

Along cohesin, two additional classes of SMC-complexes, namely condensin and SMC5/6 complex, are present in eukaryotic cells. Condensin is required for chromosome condensation during mitosis, whereas SMC5/6 complex has been shown to be involved in multiple pathways including DNA damage repair and replication of repetitive sequences (DePiccoli et al., 2009). While Nipbl/Scs2 role as a cohesin loader in the mitotic cell cycle is well documented, its role in the loading of other SMC complexes remains speculative.

In *S. cerevisiae* the inactivation of Nipbl functional orthologs decreases condensin enrichment at normal binding sites and the degree of chromosome condensation indicating that Scs2 is involved in condensin loading (D'Ambrosio et al., 2008). Enrichment of a subfraction of SMC5/6 to its binding sites was also shown to be dependent on Scs2 (Lindroos et al., 2006). These observations suggest that Nipbl may mediate binding of all three classes of SMC complexes. In addition, a high degree of co-localisation between condensin and cohesin loader has been shown in yeast (D'Ambrosio et al., 2008). However, in *C. elegans* Scs2 deficiency does not affect the binding pattern of condensin nor the Smc5/6 complex (Lightfoot et al., 2011). Similarly in *Xenopus*, reduction of Nipbl/Scs4 activity has no impact on condensin loading (Gillespie and Hirano, 2004).

Little is known about localisation of condensin and Smc5/6 complex on mammalian meiotic chromosomes. Previous cytological characterisation in murine males revealed that condensin I is loaded onto chromosomes by prometaphase in both mitotically and meiotically dividing cells. Condensin, similarly to cohesin and Sycp3 was shown to form axial structure inside chromatids of condensed chromosomes from leptotene until pachytene and to largely co-localise with Nipbl/Scs4 in zygotene (Viera et al., 2007; Visnes et al., 2013). Therefore, these localisation studies

propose a role for Nipbl/Sccl complex as a condensin I loader in mammalian meiosis. The co-localisation experiments in the same study suggest however that the loading of Smc5/6 might be independent from Nipbl/Sccl in mouse spermatocytes, which is in contrast to previous studies in yeast (Lindroos et al., 2006). The analysis of spermatocytes from *Nipbl*^{+/-} animals, where Nipbl expression level is reduced by 30% conducted by Visnas and colleagues concluded that the reduction of Nipbl levels in these cells does not affect loading of any SMC-complexes (Visnas et al., 2013). However, to conclude the independent pathway in loading of condensin and SMC5/6 further immunostaining studies on meiotic cells where Nipbl has been deleted after S phase are necessary.

Chapter 4

Cohesin loading factor Nipbl expressed in primordial oocytes is sufficient for loading of cohesin in zygotes.

4.1 Introduction

In mammalian oocytes, meiotic recombination takes place before birth, during foetal development, after which oocytes arrest in a late stage of meiotic prophase, a stage equivalent to the mitotic G2 phase. The bi-orientation of chromosomes in meiosis I occurs only shortly before ovulation, a long period of time after chiasmata formation, which can be as long as several decades in humans. This means that chiasmata need to hold the homologs together throughout all this period. In case of failure, precocious loss of cohesion results in achiasmatic chromosomes, which are the major type of chromosome mis-segregation events during the first meiotic division (Hassold and Hunt, 2001). This suggests that faithful chromosome segregation depends on the stable maintenance of cohesin during the protracted meiotic prophase arrest in mammalian oocytes. The direct link between cohesin and aneuploid eggs was first suggested by the observation that mouse oocytes lacking meiotic cohesin subunits *Smc1 β* progressively lose chiasmata, which leads to the generation of aneuploid gametes (Hodges et al., 2005). In addition, recent cytological studies revealed that the amount of cohesin bound to mouse oocyte chromosomes is reduced with advanced maternal age (Chiang et al., 2010; Lister et al., 2010; Liu and Keefe, 2008). However, the reason why the levels of chromosome-bound cohesin decline during ageing is unknown.

Studies in mitotic mammalian and yeast cells showed that cohesin is very stable once involved in sister chromatid cohesion, and there is very little or no cohesin turnover after DNA replication (Gerlich et al., 2006; Haering et al., 2004). However, unlike rapidly proliferating mitotic cells, mammalian oocytes are characterized by slow cell cycle progression and extraordinarily long meiotic prophase arrest during which cohesion should be maintained. As shown in budding yeast, genome-wide post-replicative cohesin re-loading takes place after DNA damage is introduced, which suggest the presence of pathways in which cohesin can be re-established (Ström et al., 2007; Unal et al., 2007). In addition, the meiosis-specific *Smc1 β* subunit that participates in holding bivalents together in mouse oocytes, only associates with chromosomes after DNA replication (Hodges et al., 2005).

Loading of cohesin onto chromosomes in proliferating cells requires a conserved binary protein complex composed of *Nipbl* and *Mau-2* (Nasmyth, 2011). In addition to mitosis where inactivation of *Nipbl/Mau2* leads to reduced levels of chromatin-

bound cohesin and causes loss of cohesion (Seitan et al., 2006; Watrin et al., 2006), Nipbl/Mau2 is also proposed to be involved in cohesin loading during meiosis in plants, nematodes and *Coprinus* (Cummings et al., 2002; Gause et al., 2008; Lightfoot et al., 2011; Sebastian et al., 2009). However, these studies did not address the issue of whether the cohesion defect is caused by the inability in establishing cohesion during pre-meiotic S phase or the inability to maintain prolonged cohesion.

In Chapter 3 I demonstrated that Nipbl is expressed in mouse meiotic cells and, together with cohesin, is a likely component of the synaptonemal complex. This suggests that Nipbl might also be involved in cohesin loading during mammalian meiosis. Presuming that cohesion needs to be maintained by *de novo* cohesin loading during the prolonged meiotic prophase in mouse oocytes, inefficient cohesin loading activity might result in the reduction of chromosome-bound cohesin. Therefore, in this chapter I set out to test this hypothesis and examine the effect of *Nipbl* inactivation on chromosome cohesion and cohesin binding in mouse oocytes. Currently, it is unknown how mammalian development is affected by the loss of cohesin loading activity and further analysis of the role of Nipbl in the mammalian life cycle is limited due to the fact that only Nipbl heterozygous mutant mice (*Nipbl*^{+/-}) are available for experimental studies (Kawauchi et al., 2009). To bypass this, I employed a Cre-mediated knockout (conditional knockout) technique that enables cell-type specific inactivation of genes of interest. I used genetically modified mouse system, in which *Nipbl* gene deletion was imposed specifically at two time points of the meiotic prophase arrest of oocytes, through the expression of Cre recombinase. The expression of the *cre* transgene was driven by the *growth differentiation factor 9* (*Gdf-9*) promoter or *Zona pellucida 3* (*Zp3*) promoter (Lan et al., 2004; Lewandoski et al., 1997) that allowed me to inactivate *Nipbl* in oocytes enclosed within primordial follicles from postnatal day 3 or in primary follicles after entry into the growth phase, respectively. Importantly, the inactivation took place without affecting earlier DNA replication (E13) or homologous recombination (by birth). Both Cre transgene lines have been successfully used in previous studies (Kudo et al., 2006; Reddy et al., 2008). I have also established immortalized embryonic fibroblasts (iMEFs) from *Nipbl*^{fl/fl} embryos that upon *Nipbl* inactivation enabled me to study Nipbl function in an isolated, homogenous, and rapidly proliferating cell population as well as to identify the terminal cellular phenotype caused by Nipbl deficiency.

4.2 Results

4.2.1 Genetic inactivation of *Nipbl* in mouse oocytes.

In this study I have used mice with a targeted *Nipbl* locus, called *Nipbl^{fl/fl}*, in which two *loxP* sequences flank exon 2 of *Nipbl* (floxed allele). The elimination of exon 2 of *Nipbl* by Cre-mediated recombination leads to a frameshift with stop codons and creates a deletion allele (Δ allele). In order to conditionally inactivate *Nipbl^{fl/ox}* before and during oocyte growth we have used two deleter lines *Gdf-9-iCre* or *Zp3-cre*, respectively (Fig 4.1 A) (Lan et al., 2004; Lewandoski et al., 1997). *Zp3-cre* deletes a floxed region in oocytes enclosed within primary follicles, soon after entry into a growth phase, while *Gdf-9-iCre* does so in primordial oocytes. *Nipbl^{fl/fl}* females were crossed for two generations (F1 and F2) with males carrying Cre recombinase gene whose expression is driven either by *Zp3* or *Gdf-9* promoter. The first breeding step (F1) between the *Nipbl^{fl/fl}* females and Cre expressing males resulted in progeny that carry a single floxed allele and the Cre transgene (*Nipbl^{fl/+};Gdf-9-iCre* or *Zp3-cre*). In the second breeding step (F2) these mice were crossed with homozygous flox allele (*Nipbl^{fl/fl}*) females and progeny with four different genotypes were born (F3), including the desired conditional knockout *Nipbl^{fl/fl};Gdf-9-iCre* or *Nipbl^{fl/fl};Zp3-cre*, highlighted in a blue frame (Fig. 4.1 B). These experimental females were used for further experiments. In each generation, mouse genotypes were determined by PCR genotyping (Fig. 4.1 C). Thus, an appropriate breeding scheme allowed to obtain conditional knockout female mice designated *Nipbl^{fl/fl};Gdf-9-iCre* and *Nipbl^{fl/fl};Zp3-cre* where functional *Nipbl* gene is depleted in oocytes of primordial follicles (hereafter *Nipbl Δ^{Gdf9}* oocytes) and primary growing oocytes (hereafter *Nipbl Δ^{Zp3}* oocytes), respectively. Mice where *Nipbl* has been conditionally deleted in either dormant or growing oocytes in primordial or primary follicles respectively (*Nipbl^{fl/fl};Gdf-9-iCre* and *Nipbl^{fl/fl};Zp3-cre*) are phenotypically normal.

The efficiency of the Cre recombinase converting the *Nipbl^{fl/ox}* allele into a deleted allele was estimated by analysis of genotyping result. Genotyping of progeny showed that the *Nipbl^{fl}* allele is converted to *Nipbl Δ* with 100% efficiency as a consequence of Cre produced in oocytes from transgenes of *Zp3-cre* (n=228) and *Gdf-9-iCre* (n=146). This indicates that highly efficient Cre-mediated recombination in the female germ line is characteristic to both deleter lines. Ear snip DNA from 201 offspring of a cross between *Nipbl^{fl/+};Zp3-cre* females and wild-type *Nipbl^{+/+}* males was subjected to PCR analysis to determine the genotype of each animal. All

possible categories of progeny were obtained, however they did not follow Mendelian distribution. We identified 74 *Nipbl*^{Δ/+} out of a total of 201 offspring (37% versus the predicted 50%, $p=0.0002$) at 2-weeks-old. Reduced *Nipbl*^{Δ/+} offspring numbers are consistent with the previous observation that some animals heterozygous for a *Nipbl* gene trap allele (*Nipbl*^{564/+}) die before weaning age (Kawauchi et al., 2009). In contrast, we observed lower mortality in progenies from *Nipbl*^{fllox/+}; *Gdf-9-iCre* females (43% of total 95 pups, $p=0.18$).

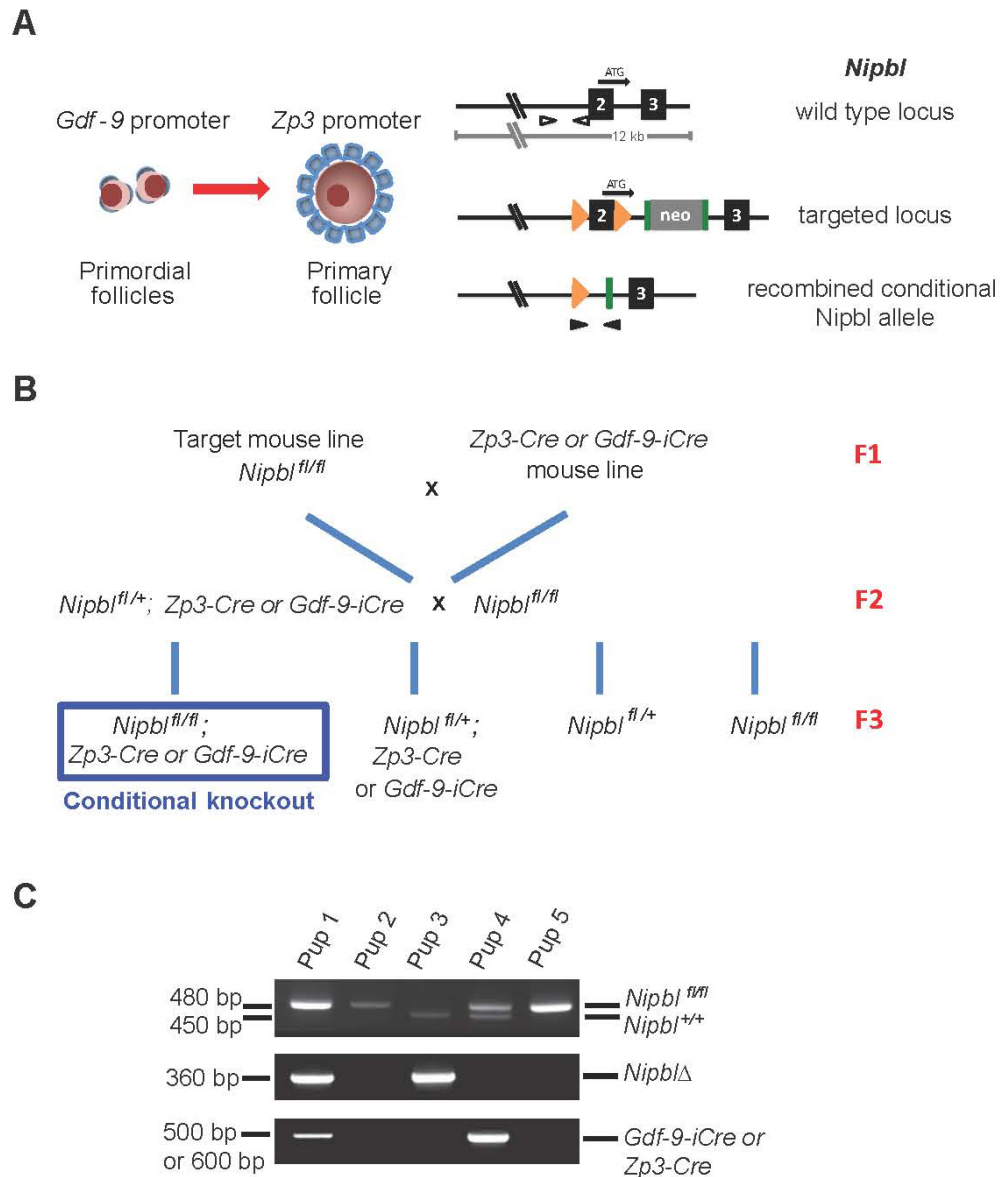


Figure 4.1 Genetic inactivation of *Nipbl* in mouse oocytes.

(A) Schematic diagram of gene targeting strategy. The deletion of exon 2 of *Nipbl* is mediated in oocytes of primordial follicles or in growing primary oocytes by GDF-9-iCre or Zp3-cre deleter line, respectively. In the targeted locus, exon 2 of *Nipbl* (black box 2) was flanked by loxP sites (orange triangles). The selection neo cassette (neo) is flanked by two Fip/Frt sites (green bars). The selection cassette was removed by Flpe-mediated recombination to obtain floxed alleles, and Cre-mediated recombination was used to generate Δ alleles. The excision occurs upon Gdf-9- or Zp3- driven Cre recombinase expression. Black arrows indicate the primers used for genotyping. (B) Mice mating scheme used to generate mice with oocyte-specific *Nipbl* gene inactivation. (C) A representative genotyping PCR result confirming the presence of *Nipbl* alleles and Cre or iCre transgene. PCR was carried out on genomic DNA isolated from ear snips of offspring. *Nipbl* flox primers pair distinguish *Nipbl* alleles that are wild-type (450 bp), recombined on one locus (450 bp and 480 bp) or recombined at both loci (480 bp). The deletion was confirmed by *Nipbl* Δ (360 bp). Presence of Zp3-cre or Gdf-9-iCre transgene was confirmed by PCR amplification product of 500 or 600 bp, respectively. Primer sequences are listed in Materials and Methods.

4.2.2 Depletion of functional *Nipbl* mRNA in *Nipbl*-deleted oocytes.

During growth mammalian oocytes store mRNA and proteins but once the growth is complete, a mature oocyte is transcriptionally quiescent and its maturation depends on accumulated maternal mRNA (Schmitt and Nebreda, 2002). In order to assess that the efficient elimination of *Nipbl* exon 2 occurs in *Nipbl* Δ^{Zp3} oocytes, the accumulation of *Nipbl* transcripts in fully-grown GV oocytes was tested by reverse transcription-quantitative PCR (RT-qPCR) assays (Fig. 4.2). RNA was isolated from three independent oocyte collections from *Nipbl*^{fl/fl};Zp3-cre and Cre-negative littermates, reverse-transcribed into cDNA and used for further amplifications. I have designed four sets of primers, whose locations are presented in Figure 4.2 A. A primer pair called Ex2 was designed to detect the presence of exon 2. Because the forward primer of this pair is located in the region of exon 2, the amplification of exon 2-deleted *Nipbl* transcripts does not take place. Three other primer sets were designed to test whether *Nipbl* Δ^{Zp3} oocytes accumulate the exon 2-deleted *Nipbl* transcripts instead of the intact ones. Therefore these assays were repeated using primer sets called A, B and AB for detecting transcripts for Nipbl A, Nipbl B and both isoforms, respectively. The specificity of amplification was confirmed by the presence of a single band of an expected size for each primer pair (Fig. 4.2 C). All the transcript levels were normalized against *Gapdh* mRNA. First, to confirm the efficient elimination of *Nipbl* exon 2, the accumulation of *Nipbl* transcript was examined using the Ex2 primer set. Relative quantifications using the 2- $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001) demonstrated that *Nipbl* Δ^{Zp3} oocytes contain only residual amount of Nipbl transcript (<5%) containing exon 2 when compared to control (*Nipbl*^{fl/fl}) oocytes (Fig. 4.2 B). To test whether *Nipbl* Δ^{Zp3} oocytes accumulate the exon 2-deleted *Nipbl* transcripts instead of the full-length ones, the assays were repeated with the combination of isoform-specific primer sets A, B and AB. These revealed that Nipbl transcript levels in *Nipbl* Δ^{Zp3} oocytes are 40-75% compared to those of controls (Fig. 4.2 B).

In summary, these results demonstrate that transcripts encoding functional *Nipbl* are efficiently eliminated during oocyte growth in *Nipbl* Δ^{Zp3} oocytes. Also, the qPCR assays suggested that the *Nipbl* gene is continuously transcribed during this developmental phase. It can be therefore concluded that in Cre-expressing oocytes, *Nipbl* exon 2 deletion and degradation of the wild-type Nipbl transcript occurs before germinal vesicle breakdown.

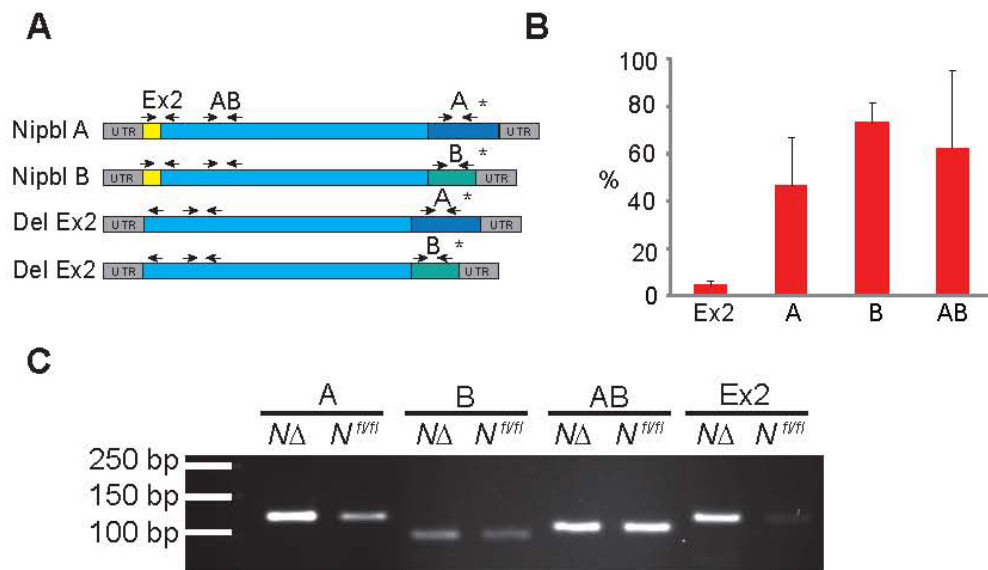


Figure 4.2 Functional *Nipbl* mRNA is not present in *Nipbl*-deleted oocytes.

(A) Positions of primers used to measure the abundance of mRNA transcripts. Yellow box, exon 2; UTR, untranslated region; asterisk, the stop codon. See Materials and Methods for primer sequences. **(B)** Depletion of functional *Nipbl* transcripts in *Nipbl*-deleted oocytes. Total RNA isolated from approximately 100 fully-grown *Nipbl*^{Δ^{Zp} and control *Nipbl*^{fl/fl} oocytes was reverse-transcribed and subjected to qPCR assays using primers designated in **A** and values normalized against the *Gapdh* transcript in each sample. Values obtained by each primer set from *Nipbl*^{Δ^{Zp} oocytes are presented as a percentage of that from *Nipbl*^{fl/fl} oocytes. Data are mean ± SD (n=3 independent experiments). **(C)** Gel electrophoresis of PCR products resulted from amplification of different parts of *Nipbl* transcript in mutant and control oocytes. A: *Nipbl* A (145 bp); B: *Nipbl* B (99bp); AB: *Nipbl* AB (114 bp); Ex2: *Nipbl* Ex2 (136 bp). Primer sequences are listed in Materials and Methods.}}

4.2.3 Inactivation of the *Nipbl* gene does not affect oocyte growth and maturation.

Next, I set out to examine meiotic maturation of *Nipbl* Δ^{Gdf9} oocytes. Meiotic maturation of oocytes is initiated by germinal vesicle breakdown (GVBD) followed by meiotic spindle assembly and consequent bi-orientation of bivalent chromosomes facilitated by chiasmata. If chiasmata are lost in the majority of chromosomes before resumption of meiosis as a result of defective crossing-over or sister chromatid cohesion, it is expected that those oocytes would be arrested in meiosis I and unable to extrude the first polar body (PB1), a phenotype observed in oocytes lacking *Mlh1* (Woods et al., 1999). *In vitro* maturation experiments revealed that *Nipbl* Δ^{Gdf9} oocytes extrude first polar body with a timing and efficiency similar to controls, between 6 and 7 hours after GVBD with the efficiency close to 80% (Fig. 4.3 A). This result shows that *Nipbl* inactivation does not affect the timing of meiosis I completion suggesting that functional *Nipbl* is dispensable for meiosis I.

Next, spindle formation and chromosome alignment were investigated in *Nipbl* Δ^{Gdf9} oocytes and compared to control *Nipbl*^{fl/fl} oocytes. Fully-grown, GV stage oocytes were isolated from the follicles and cultured *in vitro* for 6 h (metaphase I) and 18 h (metaphase II) after GVBD. Subsequently they were fixed and subjected to whole mount immunostaining with an antibody raised against α -tubulin. Immunofluorescence microscopy assessment revealed that bipolar spindle formation and chromosome alignment were also unaltered in *Nipbl* Δ^{Gdf9} oocytes (Fig. 4.3 B). These results suggest that meiosis I progression is unaffected by the absence of an active *Nipbl* gene during meiotic prophase arrest.

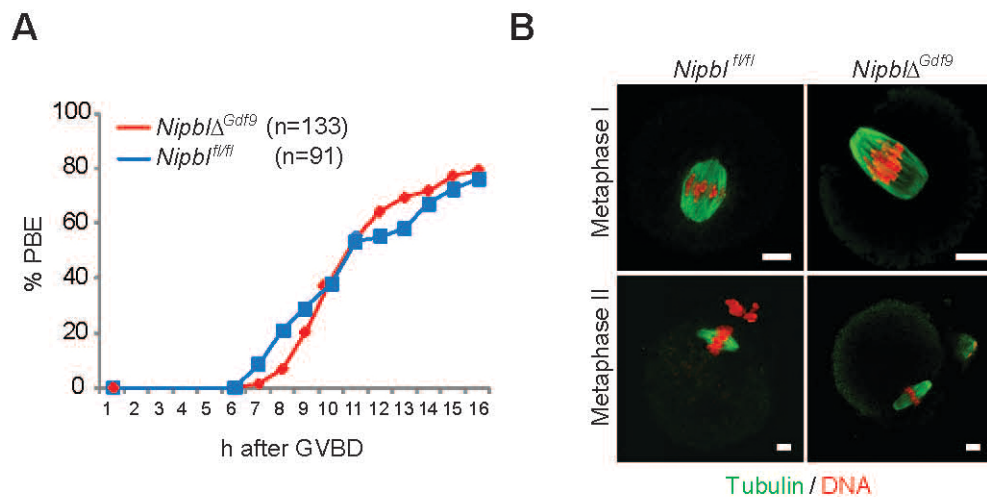


Figure 4.3 Inactivation of *Nipbl* does not affect oocyte growth and maturation.

(A) Unaltered kinetics of PB1 extrusion in *Nipbl* Δ^{Gdf9} oocytes during in vitro maturation culture. Cumulative numbers of *Nipbl* Δ^{Gdf9} and control oocytes that have undergone PB1 extrusion are presented as a percentage of total numbers of oocytes examined (n). **(B)** Normal spindle formation and chromosome alignment in *Nipbl* Δ^{Gdf9} oocytes. Confocal micrographs of *Nipbl* Δ^{Gdf9} and control oocytes fixed at 6 hr (metaphase I) and 18 hr (metaphase II) after GVBD, respectively. Microtubules were visualized by immunofluorescence staining with anti-tubulin (green) and DNA was counterstained with DAPI (red). Scale bar 20 μ m. At least 25 oocytes were analysed in each of three independent experiments.

4.2.4 *Nipbl* gene is dispensable for the maintenance of chiasmata and cohesion during meiotic prophase arrest and oocyte maturation.

The results demonstrating that the progression of meiosis I is unaffected by the inactivation of *Nipbl* gene during oocyte growth do not exclude a possibility that the deletion of *Nipbl* may result in achiasmatic chromosomes. As shown in studies employing *Sycp3*-depleted, XO oocytes or eggs from mice with naturally high frequency of numerical chromosomal aberrations, a small number of achiasmatic chromosomes can be bi-oriented and aligned without observable delay and with unaffected timing of first polar body (PB1) extrusion (Kouznetsova et al., 2007; LeMaire-Adkins et al., 1997; Sebestova et al., 2012). To test this hypothesis and identify whether there is any minor cohesion defect, I prepared chromosome spreads from *Nipbl* Δ^{Gdf9} and control *Nipbl*^{fl/fl} oocytes that were isolated from 5-8-week mice and cultured for 6 h (metaphase I) after GVBD. I scored their chromosomal configurations distinguishing between bivalents (four chromatids connected by chiasma and cohesion), univalents (two chromatids connected by centromeric cohesion) and single chromatids (no cohesion) (Fig. 4.4 B). Metaphase I chromosome spreads prepared from control oocytes contain only bivalents (n=450). Similarly, in spreads prepared from *Nipbl* Δ^{Gdf9} oocytes, only bivalents (n=760) were found, which suggests that the active *Nipbl* gene is dispensable for the maintenance of chiasmata and cohesion during meiotic prophase arrest and oocyte maturation (Fig. 4.4 A).

Taken together, *Nipbl*-deleted oocytes did not exhibit any abnormality during oocyte growth and maturation. These results suggest that either the Nipbl protein is stably maintained at levels sufficient for its role in the oocyte or it is functionally dispensable.

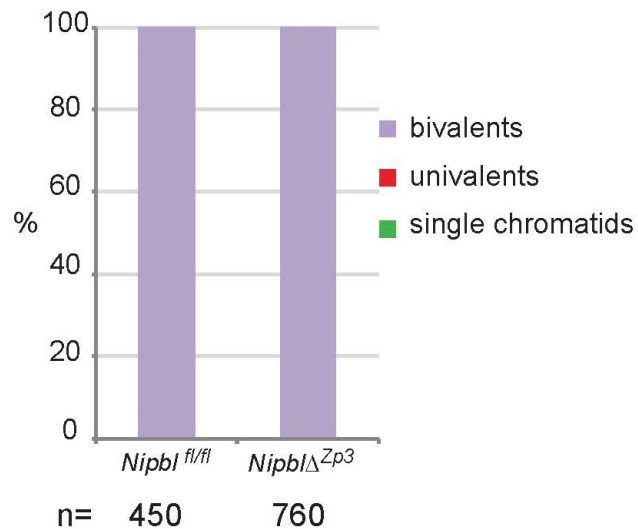


Figure 4.4 *Nipbl* gene is dispensable for the maintenance of chiasmata and cohesion during meiotic prophase arrest and oocyte maturation.

Oocytes isolated from 8 week-old *Nipbl^{fl/fl};Zp3-cre*, *Nipbl^{fl/fl};Gdf-9-iCre* and control (*Nipbl^{fl/fl}*) mice were cultured *in vitro* for 6 h (metaphase I) after GVBD. Chromosome spreads were fixed on glass slides and stained with DAPI. Frequencies of unpaired bivalents were scored. A total of 760 chromosomes in 87 oocytes from 14 *Nipbl^{ΔZp3}* females and total 450 chromosomes assessed in 53 oocytes from 11 control littermates were assessed. Only fully visible chromosomes were scored.

4.2.5 *Nipbl^{fl/fl};Gdf-9-iCre* and *Nipbl^{fl/fl};Zp3-cre* females are fertile.

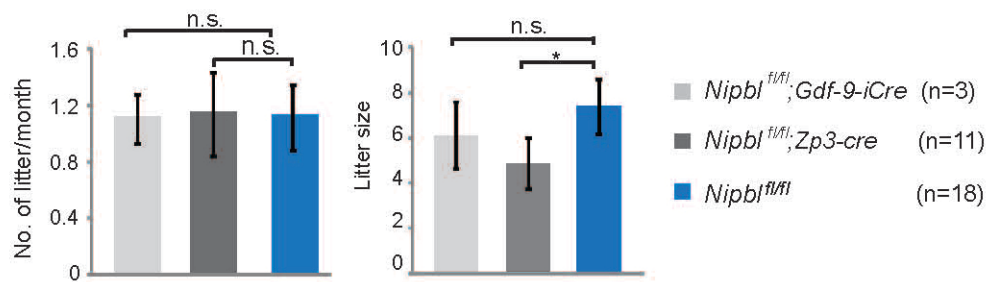
To test the reproductive capability of mutant mouse strains, *Nipbl^{fl/fl};Gdf-9-iCre* and *Nipbl^{fl/fl};Zp3-cre* females were bred with fertile *Nipbl^{+/+}* males. Over the course of breeding, eleven *Nipbl^{fl/fl};Zp3-cre* females produced an average of 20.3 pups per female from 46 litters (1.1 litters per month) with an average litter size of 4.9. Three *Nipbl^{fl/fl};Gdf-9-iCre* crosses produced an average of 46.3 pups per female from 20 litters (1.1 litters per month) with an average litter size of 6.1. These numbers were compared with eighteen control *Nipbl^{fl/fl}* mice that produced an average of 31 pups per female from 30 litters (1.1 litters per month) with an average litter size of 7.4 (Fig. 4.5 A). These results suggest that both mouse mutant strains are fertile and give birth with frequencies indistinguishable from control females, but produce smaller litter sizes, which translates to the reduction in cumulative number of offspring by approximately 34% for *Nipbl^{fl/fl}; Zp3-cre* and 18% for *Nipbl^{fl/fl};Gdf-9-iCre* per female when compared to *Nipbl^{fl/fl}* control females. Data have been summarized in Table 4.1.

The *Zp3-cre* transgene becomes active from growth stage of each oocyte in primary follicle. This means that the timing of floxed gene deletion spans over a period of just 2-3 weeks, when the oocyte grows, and the duration of this period is independent of maternal age. In contrast, the *Gdf-9-iCre* transgene is activated in dormant oocytes in primordial follicles from postnatal day 3, therefore the period of time when the active *Nipbl* gene is absent increases along with maternal age. Although *Nipbl^{fl/fl};Gdf-9-iCre* females retained fertility despite the loss of *Nipbl* soon after birth, it is possible that their oocytes may still be affected by a mild impairment of meiotic sister chromatid cohesion, which may become apparent only with increasing age. To test this, I examined whether the prolonged duration of *Nipbl* inactivation in dormant primordial oocytes affects fertility of *Nipbl^{fl/fl};Gdf-9-iCre* females. Breedings of three *Nipbl^{fl/fl};Gdf-9-iCre* females with wild-type *Nipbl^{+/+}* males were set up and continued until females reached the age of 6-7 months. I recorded their fertility and compared it to the fertility of four *Nipbl^{fl/fl}* and three *Nipbl^{fl/fl};Zp3-cre* females that were bred to *Nipbl^{+/+}* males for the same period of time. I observed that *Nipbl^{fl/fl};Gdf-9-iCre* females were fertile at least for 7 months, similarly to *Nipbl^{fl/fl};Zp3-cre* and control *Nipbl^{fl/fl}* females (Fig. 4.5 B). This suggests that the inactivation of the functional *Nipbl* gene soon after birth does not compromise fertility for a protracted period of time, in this case several months, which is comparable to the entire reproductive lifespan of mice.

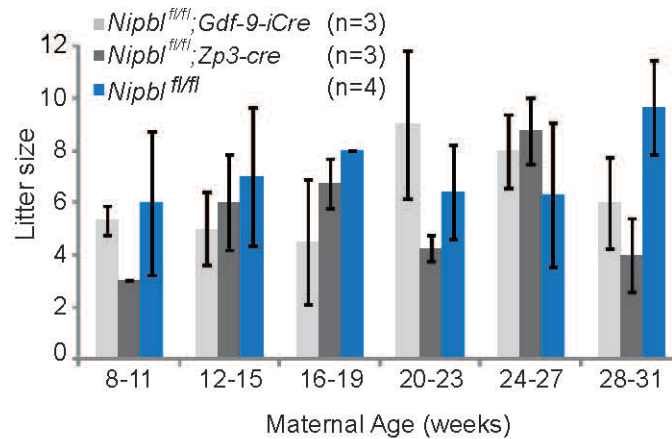
Next, I isolated oocytes from aged, 45-52-week-old, mutant and wild-type females, which were then cultured 6 h after GVBD (metaphase I). Total of 26 oocytes isolated from five *Nipbl*^{f/f}; *Gdf-9-iCre* females and 16 oocytes isolated from two *Nipbl*^{f/f} females were used for chromosome spreading. Chromosomal configurations were scored distinguishing between bivalents, univalents and single chromatids. While all metaphase I oocytes isolated from control mice contained only bivalents (n=380), 19% (5 out of 26) of oocytes isolated from mutant females were carrying univalents, pairs of sister chromatids not associated with the homologous pair. Surprisingly, each of these oocytes contained 40 univalents, indicating that all metaphase I bivalents lost chiasmata. Examples of oocytes' chromosome spreads containing set of 20 bivalents and these containing 40 univalents are presented in Figure 4.5 C.

This observation suggests that the inactivation of cohesin loading factor *Nipbl* for the length of the entire reproductive life after cohesion establishment during pre-meiotic S-phase, leads to the loss of cohesion and subsequently chiasmata at advanced age of female mice. As a result in some of mutant oocytes precocious chromosome segregation can be observed where all 20 bivalents separate into 40 univalents at metaphase I.

A



B



C

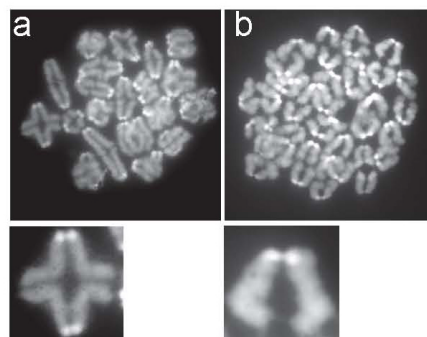


Figure 4.5 *Nipbl^{fl/fl};Gdf-9-iCre* and *Nipbl^{fl/fl};Zp3-cre* females are fertile.

(A) Mean numbers of litters per month (left). Mean litter sizes (right). Data are mean $\pm$ SEM. Asterisks indicate statistical significance (Student's t-test); n.s., no significant difference ($P > 0.05$); *significant difference ($P < 0.05$). Data for each graph are presented in Table 4.1. Note that reduction in mean litter sizes of *Nipbl^{fl/fl};GDF-9-iCre*, and *Nipbl^{fl/fl};Zp3-cre* females is consistent with the high mortality of *Nipbl^{Δ/+}* pups. (B) Mean litter sizes in relation to maternal ages. n, number of females used. (C) Examples of oocyte chromosome spreads isolated from 45-52-week-old females. (a) Only bivalents (four chromatids connected by chiasma and cohesion), (b) only univalents are present (two chromatids connected by centromeric cohesion).

Fertility of *Nipbl*^{f1/f1}, *Zp3-cre*, *Nipbl*^{f1/f1}, *Gdf-9-iCre* and *Nipbl*^{f1/f1} females

Genotype	n	Litters	Total Pups	Pups/Litter	Litters/Month
<i>Nipbl</i> ^{f1/f1} <i>Gdf-9</i>	3	20	122	6.1	1.1
<i>Nipbl</i> ^{f1/f1} <i>Zp-3</i>	11	46	224	4.9	1.1
<i>Nipbl</i> ^{f1/f1}	18	107	795	7.4	1.1

Table 4.1 Details of breeding studies.

4.2.6 Inactivation of *Nipbl* in neonatal oocytes does not hinder mitotic cohesin loading in zygotes.

The developmental capability of progenies from *Nipbl^{fl/fl};Gdf-9-iCre* females mated with wild-type males suggests that the Nipbl protein synthesized before *Nipbl* gene deletion in primordial follicles is sufficient to support cohesin loading until it becomes available from the paternal *Nipbl⁺* allele, before the zygotic gene activation (ZGA) occurs. Since zygotic genome activation takes place at the 2-cell stage in the mouse (Schultz, 2005), loading of cohesin in the fertilized eggs must be dependent on maternally accumulated proteins and proteins synthesized from maternally derived mRNAs. A study from Tachibana-Konwalski and co-workers showed that Rad21-containing, but not Rec8-containing cohesin complex is present on chromosomes in mouse zygotes and its artificial destruction at metaphase induces sister chromatid segregation (Tachibana-Konwalski et al., 2010). This suggests that *de novo* loading of the Rad21-containing cohesin in zygotes is essential for normal embryonic development. Therefore, I set out to investigate whether Nipbl-deleted eggs can load mitotic cohesin (Rad21) in zygotes generated by natural fertilization, before zygotic gene activation at the 2-cell stage (Fig. 4.6).

For this experiment, three-to-four week old *Nipbl^{fl/fl};Gdf-9-iCre* females were superovulated and subsequently mated with wild-type *Nipbl^{+/+}* fertile males. Zygotes of desired pronuclear stages were collected and cultured for further investigation. The stages of cell cycle phases in zygotes corresponded to the times following superovulation of females and were determined by the distance between two pronuclei, where P1-2 stage is equivalent to G1-phase, P3-P4 to S phase and P5 stage to G2 phase (Fig. 4.7 A) (Adenot et al., 1997). In order to examine chromosomal binding of Rad21 and Nipbl on pronuclei of zygotes at different developmental stages after natural fertilization, I prepared nuclear spreads and performed immunofluorescent staining (Fig. 4.7 B). Rad21 and Nipbl can be observed on chromosomes in both male and female pronuclei already in G1 phase (PN1-2 stage) in *Nipbl^{+/+}* zygotes (Fig. 4.7 B). In a more advanced developmental stage of the zygote, at the P5 stage (G2 phase), I found that the chromosomal binding of Rad21 is not compromised in fertilized *Nipbl*-deleted eggs (Fig 4.6 B). This suggests that *de novo* loading of the Rad21-containing mitotic cohesin complex can be supported by the Nipbl protein expressed around the time of birth in primordial oocytes.

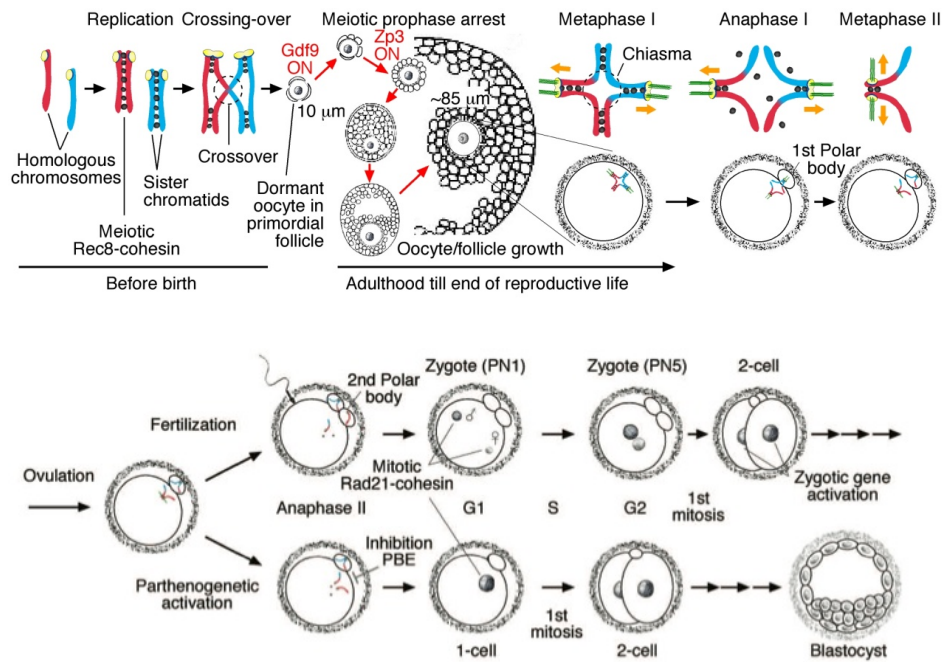
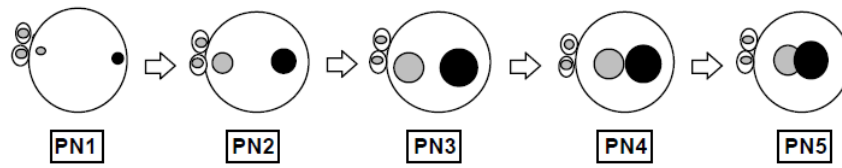


Figure 4.6. Schematic of meiotic progression of oocytes, fertilization of eggs, the first cleavage and parthenogenetic activation of eggs in mammals.

After mitotic proliferation, the entire population of female germ cells synchronously enters meiosis by replicating DNA and establishing sister chromatid cohesion mediated by Rec8-containing cohesin. In mice, this occurs within the embryonic ovaries 13-14 days after conception (E13-14). Immediately after replication during early meiotic prophase, homologous recombination creates crossovers by the time of partum (E19) and oocytes enter cell cycle arrest in dictyate, a late stage of meiotic prophase. Primordial follicles are established between postnatal day 1-3 (PD1-3) by enclosure of the oocyte by a few pregranulosa cells. Cre recombinase driven by the Gdf9 promoter recombines the loxP sites between PD3 and 3 weeks. The vast majority of primordial follicles are lost (atresia) and only the surviving dormant oocyte pool contributes to fertility. After the sexual maturation of females, subsets of oocytes are periodically selected and grow in accordance with the differentiation of pregranulosa to granulosa cells, with their proliferation inducing follicular expansion, called recruitment. Cre driven by the Zp3 promoter becomes active in growing oocytes. Fully-grown oocytes included in the antral follicles acquire competence to exit meiotic prophase arrest, and meiotic resumption is manifested by the germinal vesicle breakdown (GVBD). Bi-orientation of bivalent chromosomes whose four chromatids are connected by cohesin and a chiasma, the consequence of crossover, is established at metaphase I. Chiasma resolution is facilitated by dissociation of cohesin at chromosome arms in anaphase I, which creates univalent chromosomes containing two chromatids connected by cohesin at centromeres. Extrusion of the first polar body (PB1) marks completion of meiosis I, with oocytes then arresting again in metaphase II. Following ovulation, eggs are fertilized by sperm in the oviduct, which triggers exit from meiosis II to form zygotes containing two pronuclei. After a short G1-phase zygotes start the first round of mitosis, whose progression can be staged by the distance between two pronuclei (PN1-5 stages). The mitotic cohesin complex containing Rad21 is bound to G1-pronuclei in zygotes and is responsible for functional cohesion. Active transcription to accumulate maternal mRNA is arrested upon completion of oocyte growth. Following maturation, fertilization and early embryonic cleavage depend on stored proteins and translation of maternal mRNAs until transcription resumes (zygotic gene activation) in the 2-cell stage in mice. Mouse eggs can be chemically activated and parthenogenetically develop into blastocysts in vitro, though taking longer (ca. 5 days) than in utero (3 days).

A



B

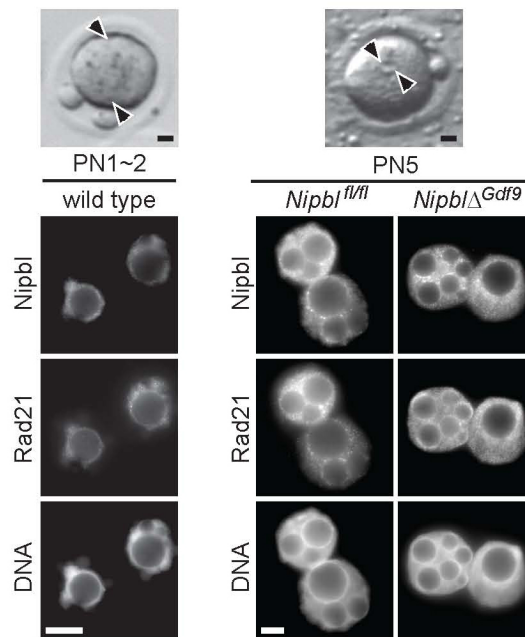


Figure 4.7 Inactivation of Nipbl before oocyte growth does not interfere with mitotic cohesin loading in zygotes.

(A) Male and female pronuclei are represented by dark and grey circles, respectively. The size of pronuclei and position within zygotes changes along with advancing pronuclear stages (PN). During PN1-2, equivalent to G1 phase, small pronuclei are first located at the periphery of the embryo, which is followed by the increase in size and migration towards the center of the embryo. During PN3-4 equivalent to S phase, enlarged pronuclei migrate towards the center of the zygote and position in close proximity to each other. At PN5, equivalent to G2 phase, large central pronuclei are apposed (Adapted from Adenot et al., 1997). (B) Loading of Rad21-containing mitotic cohesin in *Nipbl^{ΔGdf9}* eggs naturally fertilized with *Nipbl⁺* sperm. Representative bright field images of zygotes at pronuclear stage 1-2 (PN1-2, G1 phase) and stage 5 (PN5, G2 phase) are shown at the top with positions of pronuclei indicated (arrowheads). Immunofluorescence micrographs of pronuclei chromosome spreads stained with mouse monoclonal anti-Rad21 and rat monoclonal anti-Nipbl. Scale bar 5 μ m. Experiment has been repeated 3 times.

4.2.7 Parthenogenetic development of Nipbl-deleted blastocysts

Observation of the long term stability of Nipbl and cohesin loading activity in early zygotes led me to examine further for how long Nipbl expressed in pre-growth-stage oocytes can support cell cleavage in preimplantation embryos. To address this we employed the technique of parthenogenesis allowing us to activate unfertilized eggs from *Nipbl^{fl/fl};Gdf-9-iCre* females and monitor early embryogenesis in culture. This process is presumably entirely dependent on the cohesin loading activity of Nipbl synthesized before gene deletion in primordial oocytes in the neonatal ovary. Following superovulation of *Nipbl^{fl/fl};Gdf-9-iCre* and control *Nipbl^{fl/fl}* females, *Nipbl Δ^{Gdf9}* and control eggs arrested in metaphase II were parthenogenetically activated by ethanol treatment. The second polar body extrusion was inhibited by incubation in actin polymerisation inhibitor, Cytochalasin B. Since in mouse, parthenotes develop to blastocyst stage within five days of *in vitro* culture (Kono et al., 2004), we further cultured parthenogenetically activated *Nipbl Δ^{Gdf9}* and control eggs, up to five days after the activation (Fig. 4.6).

In our experiments, we experienced relatively low blastocyst developmental rate. Only 17% (33 blastocysts out of 198 activated eggs in 7 independent experiments) of control eggs from *Nipbl^{fl/fl}* females developed into blastocysts. We believe this is due to strain background, since 60% of parthenogenetically activated eggs from another mouse strain with a different genetic background (65 blastocysts out of 109 activated eggs in 7 independent experiments) developed into blastocysts in our hands. Eggs from *Nipbl^{fl/fl};Gdf-9-iCre* females developed into blastocysts with an average of 14% efficiency (11 blastocysts out of 78 activated eggs, 5 independent experiments), which is similar to control eggs from *Nipbl^{fl/fl}* females. This suggests that eggs in which *Nipbl* has been inactivated just after birth are still able to undergo early embryonic cleavage and form blastocysts. Representative bright-field pictures of mutant and control blastocysts are presented in Figure 4.8 A.

Next, we investigated blastomeres in experimental and control parthenotes. These were fixed five days after activation and their mitotic spindles and chromosomes were visualised (Fig. 4.8 A). Immunofluorescence staining revealed that *Nipbl*-deleted parthenotes contain a high fraction of blastomeres undergoing mitosis with massive chromosome misalignment (84%, n=12 mitotic cells in 11 parthenotes), while only a third of control *Nipbl^{fl/fl}* blastocysts showed this defect (30%, n=10 mitotic cells in 12 parthenotes) (Fig. 4.8 B). The examples of correctly aligned and

misaligned chromosomes on the mitotic spindle found in parthenotes are magnified (Fig. 4.8 A). These results demonstrate the blastomeres in mutant parthenotes have significant sister chromatid cohesion defects.

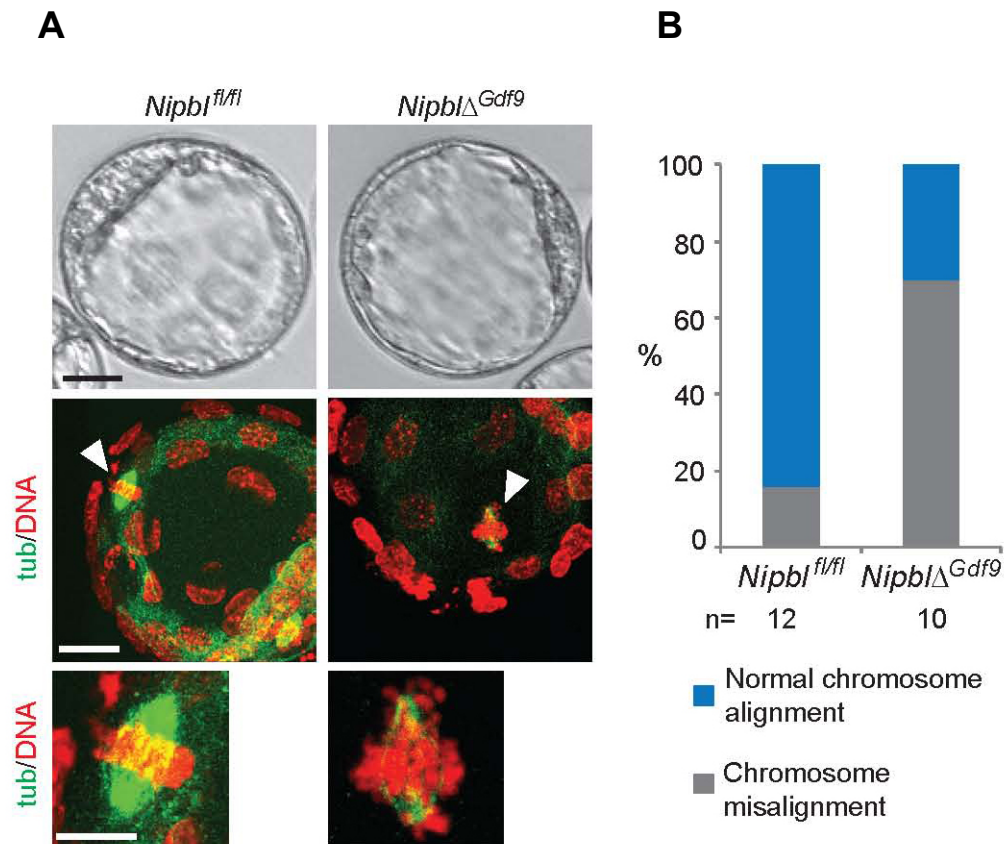


Figure 4.8 Nipbl synthesized in primordial follicles is able to support development of parthenotes.

Eggs from superovulated *Nipbl^{fl/fl};Gdf-9-iCre* and control *Nipbl^{fl/fl}* females were collected, parthenogenetically activated by a short incubation in 7% ethanol and cultured in vitro. **(A)** Representative bright-field (top) and confocal micrographs of *Nipbl Δ ^{Gdf9}* and control parthenotes that developed into blastocysts. Microtubules were visualized by immunofluorescence staining with an anti-tubulin (green) and DNA was counterstained with DAPI (red). Mitotic blastomeres containing the spindle with aligned (left) and misaligned (right) chromosomes are magnified at the bottom. Scale bars 25 μ m and 5 μ m in magnified images. **(B)** High incidences of chromosome misalignment in mitotic blastomeres of *Nipbl Δ ^{Gdf9}* parthenogenetic blastocysts. Experiments in **A** were performed parthenogenetic blastocysts developed from *Nipbl Δ ^{Gdf9}* and control eggs and scored for chromosome alignment. Numbers of mitotic blastomeres examined are indicated (n). This experiment has been conducted by Dr Shoma Nakagawa.

4.2.8 Depletion of Nipbl in embryonic fibroblasts leads to deferred cell death associated with nuclear abnormality

Further, I asked whether the stability of Nipbl is specific to oocytes arrested in meiotic prophase. To address this I established immortalized embryonic fibroblasts (iMEFs) from *Nipbl^{f1/f1}* embryos by prolonged passaging and culture *in vitro*. Next, I generated a lentivirus that expresses Cre recombinase fused with EGFP (Lv-Cre-EGFP), which allows eliminating the floxed allele. Any possible effect of lentiviral infection not specific to floxed allele deletion was controlled with EGFP expression lentivirus (Lv-EGFP), which does not have Cre activity (Ahmed et al., 2004). Through viral titration I found that 15 μ l of either Lv-EGFP or Lv-Cre-EGFP is required to infect ~80% of 1.5×10^5 cells iMEFs. To enrich Cre-EGFP and control EGFP expressing iMEFs populations I used the fact that upon Lv-Cre-EGFP or Lv-EGFP intergration with cell nucleus, these cells start to express EGFP. This feature allowed me to employ fluorescence-activated cell sorting (FACS) at three days post-infection (3 dpi) to collect only EGFP-positive cells, which were further cultured. Genotyping of iMEFs confirmed that Lv-Cre-EGFP infection converted *Nipbl^{f1/f1}* allele to *Nipbl ^{Δ/Δ}* by three days post-infection (3 dpi) (Fig. 4.9). I noticed that *Nipbl^{f1/f1}* iMEFs expressing EGFP alone (iMEFs^{EGFP}) proliferated as efficiently as uninfected iMEFs, while the cell number of those expressing Cre-EGFP (iMEFs^{Cre}) appeared to stay almost unchanged until six days post-infection (6 dpi), then gradually decreased and no iMEFs^{Cre} survived by fourteen days post-infection (14 dpi) (5 independent experiments). I used the bromodeoxyuridine (BrdU) nuclear incorporation assay to measure the fractions of proliferating iMEFs at 9 dpi after 16 h of BrdU treatment. I found that the fraction of BrdU-positive cells were much smaller in iMEFs^{Cre} (30%) than in the control iMEFs^{EGFP} (85%), suggesting that Nipbl is essential for cell proliferation (Fig. 4.10 A). Next, I measured the Nipbl protein level by Western blot in iMEFs^{Cre} cells in comparison with control iMEFs^{EGFP} and untreated iMEFs at 5 and 10 days after infection. I found that at five days post-infection (5 dpi) Nipbl protein levels did not decrease in iMEFs^{Cre} cells when compared to control iMEFs^{EGFP} and untreated iMEFs cells. Only ten days after infection (10 dpi) a decrease in protein level of Nipbl was observed in iMEFs^{Cre} (Fig. 4.10 B). These results demonstrate a slow depletion of Nipbl protein in iMEFs^{Cre} that can be attributed to the stability of Nipbl protein that was synthesized before *Nipbl* inactivation.

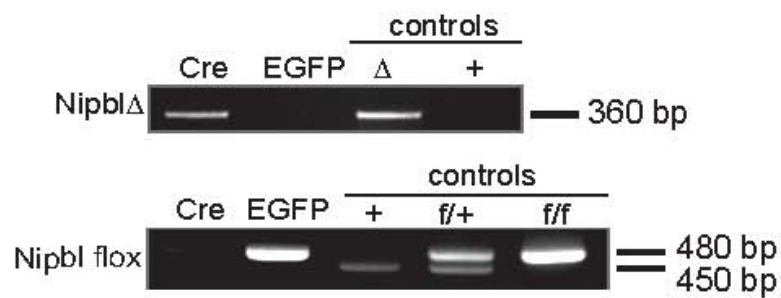


Figure 4.9 Efficient conversion of *Nipbl^{fl/fl}* to *Nipbl^{Δ/Δ}* in iMEFs following infection with lentiviruses expressing Cre recombinase.

Nipbl^{fl/fl} iMEFs were infected with Cre-EGFP expression viruses (Cre) or control viruses expressing EGFP alone and sorted by FACS for GFP signals at 3 dpi. Genotyping was performed on sorted cells using primers to detect the *Nipbl^Δ* and *Nipbl^{fl}* alleles. DNA isolated from ear notches (from *Nipbl^{+/+}*, *Nipbl^{fl/+}* and *Nipbl^{fl/fl}* mice) were used as controls. Note that the primer set for *Nipbl^{fl}* allele also amplifies smaller fragment for *Nipbl⁺* allele than *Nipbl^{fl}*. The sizes of amplified products are indicated. Cre, iMEFs^{Cre}; EGFP, iMEFs^{EGFP}.

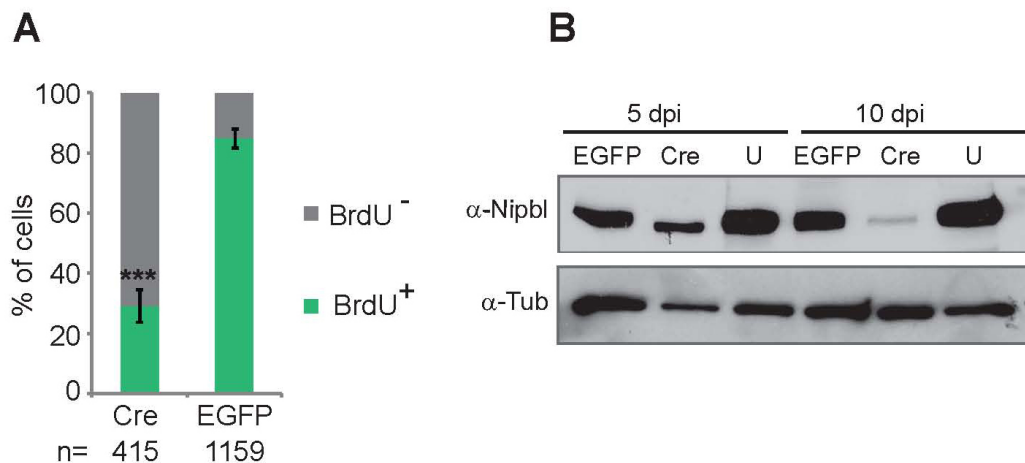


Figure 4.10 Slow decrease in Nipbl protein levels after *Nipbl* inactivation.

(A) Presence of a small but significant proportion of proliferating *Nipbl* ^{Δ/Δ} iMEFs at 9 dpi. *Nipbl* ^{Δ/Δ} and *Nipbl*^{*fl/fl*} iMEFs at 9dpi were cultured for 16 h in BrdU-containing medium. Cells were fixed in culture dishes and subjected to immunofluorescent staining with mouse monoclonal anti-BrdU antibody. Fractions of BrdU positive cells were scored against total numbers of cells marked by DAPI staining. Data are mean $\pm$ SD (n=3 independent experiments). Numbers of cells scored are indicated (n). Asterisks indicate statistical significance (Student's t-test). *** p=0.00107. **(B)** Slow depletion of Nipbl in *Nipbl* ^{Δ/Δ} iMEFs. Western blot analysis of Nipbl protein in total cell lysates of control virus-infected *Nipbl*^{*fl/fl*} iMEFs (EGFP), *Nipbl* ^{Δ/Δ} iMEFs (Cre) and uninfected iMEFs *Nipbl*^{*fl/fl*} (U) prepared on 5 and 10 dpi using anti-Nipbl A (KT55) antibody. Anti-Tubulin was used as a loading control.

Next, I prepared nuclear spreads from iMEFs^{Cre} and control iMEFs^{EGFP} cells at nine days post-infection (9 dpi) and examined their morphology. I found that iMEFs^{Cre} exhibited aberrant morphology with different nuclear appearances (Fig. 4.11 A). Further, I scored the frequency of abnormal nuclear morphology in iMEFs^{Cre} and control iMEFs^{EGFP} cells at nine days post-infection (9 dpi) and found that 58% of iMEFs^{Cre} (118 out of 203 nuclei observed) exhibited aberrant nuclei, which is in contrast to only 10% (23 out of 232 nuclei observed) of such nuclei in iMEFs^{EGFP}. Since abnormal nuclear morphology is often a consequence of chromosome mis-segregation during cell division, I examined metaphase chromosome spreads from iMEFs^{EGFP} and iMEFs^{Cre}. I found that at nine days post-infection (9 dpi) 50% of iMEFs^{Cre} (26 out of 52 metaphase cells observed) had lost sister chromatid cohesion, which contrasts to the absence of such chromosomes in iMEFs^{EGFP} (0 out of 50 metaphase cells observed) (Fig. 4.11 B). These results suggest that inactivation of *Nipbl* in fibroblasts leads to slow reduction in proliferation and eventual cell death with nuclear abnormality likely due to chromosome missegregation during mitosis. In addition, the fact that *Nipbl*-deleted fibroblasts could survive up to 2 weeks after *Nipbl* gene inactivation suggests a slow turnover of Nipbl protein, which is consistent with my observation that Nipbl protein synthesized before *Nipbl* gene deletion in neonatal ovary is stably maintained in the oocyte.

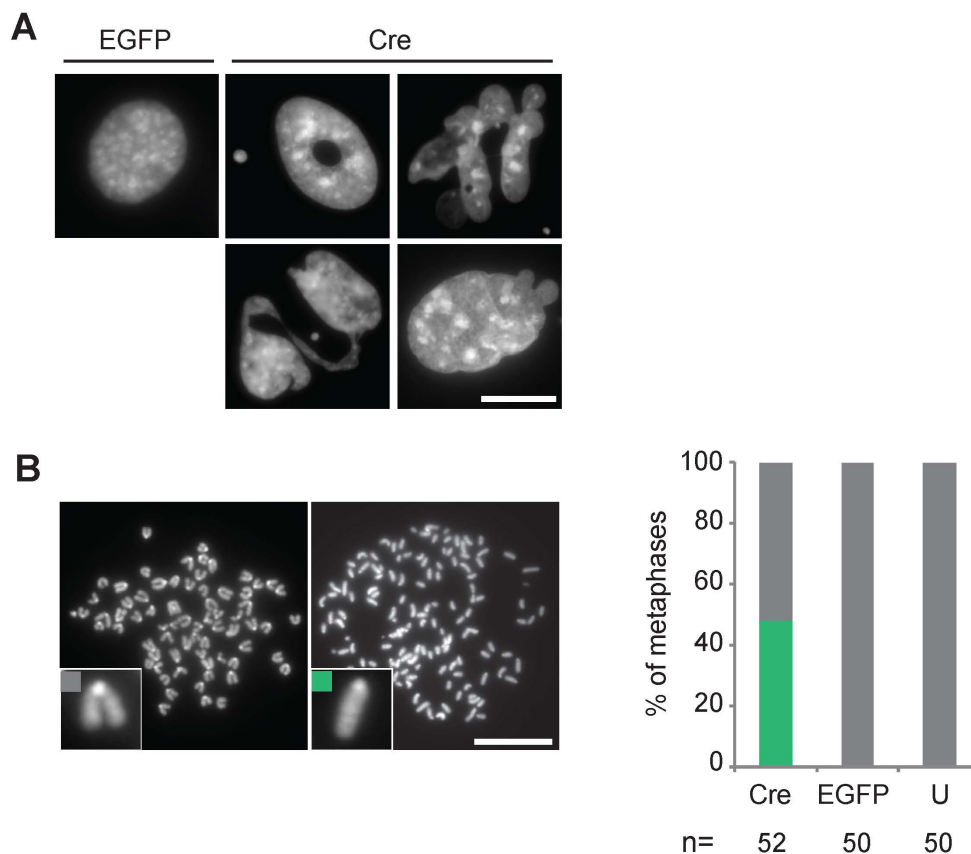


Figure 4.11 Lentivirus mediated Nipbl-depletion results in aberrant nuclear morphology and precocious chromosome segregation in iMEFs^{Cre}.

(A) Images of DAPI-stained nuclei at 9 dpi with normal appearance in *Nipbl^{f/f}* iMEFs infected with control viruses compared to those with representative abnormal morphologies in *Nipbl^{Δ/Δ}* iMEFs. Scale bar 10 μ m. (B) (Left) Example images of DAPI-stained mitotic chromosomes with or without sister chromatid cohesion at centromeres found in control-infected *Nipbl^{f/f}* iMEFs, or *Nipbl^{Δ/Δ}* iMEFs, respectively. Scale bar 10 μ m. (Right) High incidences of cohesion loss in *Nipbl^{f/-}* iMEFs. Graph shows the proportions of mitotic chromosome spreads without sister chromatid cohesion (green) per total mitotic chromosomes at 9 dpi in *Nipbl^{f/-}*, control virus-infected iMEFs *Nipbl^{f/f}* (EGFP) and uninfected iMEFs *Nipbl^{f/f}* (U) iMEFs. Numbers of cells with mitotic chromosomes observed are indicated (n).

4.3 Discussion

4.3.1 Summary of observations

In the previous chapter I have demonstrated that cohesin loading factor Nipbl is a component of chromosomal axis, from the start of its formation in leptotene in meiocytes of both sexes. However, I observed that Nipbl remains at chromosomal axis in oocytes until later stage (dictyate) than in spermatocytes. Thus, this localisation suggests that cohesin re-loading might be taking place on chromosomal axes. Cohesin replenishment could presumably be involved in crossover formation or maintenance of chiasmata in mouse oocytes. As shown by numerous studies depletion of Nipbl orthologs in both animal and plant models result in defects in sister chromatid cohesion, nuclear division and transcriptional dysregulation of multiple genes, including meiotic cohesin subunits. Furthermore impaired synaptonemal complex assembly and DNA repair defects have been observed (Lin et al., 2011; Sebastian et al., 2009; Seitz et al. 1996; Cummings et al. 2002). In fruit fly haploinsufficiency of Nipbl causes premature disassembly of the SC (Gause et al. 2008), but does not affect chromosome segregation or fertility. In worm, loading of cohesin but not other SMC complexes to chromosome axes is impaired when *Scs2* is mutated, leading to cytological defects and failure to repair meiotic DSBs (Lightfoot et al. 2011).

During mammalian oogenesis meiosis is initiated during foetal development. Female germ cells replicate their DNA and establish sister chromatid cohesion mediated by Rec8-containing cohesin. Directly after replication, during early meiotic prophase, crossovers are created by homologous recombination and oocytes enter cell cycle arrest in dictyate, a late stage of meiotic prophase. After the sexual maturation of females, oocytes can re-enter meiosis in a response to hormonal signals during the menstrual cycle, which promotes first meiotic division and ovulation. Since cohesion is established during DNA replication in germ cells of embryonic ovary and meiotic prophase arrest can last for several decades in humans, this means that cohesin must persist on DNA and mediate cohesion for extraordinarily long period of time. It was postulated that in a consequence of oocyte ageing during meiotic prophase arrest, the frequency of chromosome missegregation significantly increases at both meiotic divisions. Presumably the gradual loss of cohesin that leads over time to impaired cohesion is the main contributor to the high incidence of aneuploid oocytes detected in older women (Hassold and Hunt, 2001; Hassold et al., 2001). In support of this, oocytes deficient in meiosis specific cohesin subunit *Smc1 β* are sterile due to

high aneuploidy rate, a phenotype that deteriorates with age of female mice (Mann et al., 2005). It has been also observed that the levels of Rec8 are reduced in age-dependent manner in both mouse and humans (Chiang et al., 2010; Garcia-Cruz et al., 2010; Lister et al., 2010). These studies indicate that a minimum amount of cohesin associated with chromosomes is required for cohesion and to prevent aneuploidy. Consequently, this demonstrates that cohesin dynamics in meiosis has major medical importance.

Therefore, cohesin re-loading in meiosis has been proposed as a key factor contributing to age-related aneuploidy. The goal of this study was to examine the possibility of cohesin turnover during protracted meiotic prophase in mouse oocytes. By employing Cre-loxP system, conditional allele of *Nipbl* was successfully inactivated specifically in dormant primordial or growing oocytes. This thesis employs the first inducible mammalian model for *Nipbl* gene inactivation and presents consequences of homozygous inactivation of *Nipbl* gene in the mouse oocytes arrested in meiotic prophase. Here, I show that although the efficient depletion of functional *Nipbl* transcripts has taken place, *Nipbl*-deleted oocytes underwent meiotic maturation with unaffected chiasmata and cohesion. *Nipbl*-deleted eggs were fertile with successful *de novo* loading of mitotic cohesin upon fertilization, and developed into blastocysts upon parthenogenetic activation. However, these blastocysts contained a high proportion of mitotic cells with misaligned chromosomes. These data suggest that *Nipbl* is extremely stable in the oocyte, consistent with our observation of *Nipbl-deleted* embryonic fibroblasts that could survive up to 2 weeks with slow depletion of *Nipbl* protein, until their eventual death with abnormal nuclear morphologies likely caused by precocious loss of sister chromatid cohesion. Also, it was observed that aged mutant females (48-55 weeks-old) produced oocytes that had precociously lost cohesion in all twenty bivalents at metaphase I, producing eggs with 40 univalents. Since this phenotype has not been observed in younger females this suggest the late onset of cohesion defects upon *Nipbl* inactivation, consequently implying stable maintenance of *Nipbl* protein and cohesin-loading activity in both oocytes and fibroblasts.

4.3.2 Cohesin reloading during meiotic prophase

In the course of my PhD two other groups have addressed the issue of cohesin replenishment in oocytes during the arrest. Both studies fail to find the evidence supporting hypothesis of cohesin turnover in mouse oocytes. In the first study, Revenkova and co-authors showed that homozygous conditional inactivation of

Smc1 β in primordial follicles, therefore after crossover formation, did not result in premature loss of cohesion and these mice remained fertile for more than 8 months. This implies the lack of *de novo* cohesin loading in meiotically arrested oocytes enclosed in primordial follicles and proposes the dispensability of *de novo* synthesis of *Smc1 β* protein during meiotic prophase (Revenkova et al., 2010a). This also suggests that meiotic cohesin is sufficiently stable to mediate cohesion during the lifespan of foetal oocytes and that either little or no turnover takes place in oocytes until fertilization. In other study, Tachibana-Konwalski and colleagues used fully-grown GV-stage oocytes from transgenic mice producing TEV-cleavable Rec8 and injected them with wild-type Rec8 and subsequently with TEV protease. Rec8 synthesized after pre-meiotic S phase did not provide functional cohesion and did not prevent bivalent breakdown. This suggests the absence of cohesin turnover or renewal in meiotic prophase-arrested oocytes or failure of this cohesin to become cohesive (Tachibana-Konwalski et al., 2010). Thus, these studies propose that the cohesin complex loaded and engaged in cohesion during DNA replication is not replenished throughout the protracted period of meiotic prophase arrest, which might be as long as reproductive lifespan of female mouse. This fits well with the hypothesis that the age-related increase of aneuploidy in human eggs might be due to progressive decay of cohesin bound to chromosomes. Also, this predicts dispensability of cohesin loading factor *Nipbl* in mammalian oocytes. On the other hand, since both studies examined oocytes already arrested in meiotic prophase, these observations do not rule out the possibility that cohesin is replenished on chromosomal axes in early prophase, before arresting. In fact, my study showed that *Nipbl* localises to axial element throughout meiotic prophase suggesting that cohesin turnover might take place during this time.

However, a recent study by Visnes and colleagues demonstrated that *Nipbl*^{+/-} mouse spermatocytes, which have reduced level of *Nipbl* protein, did not exhibit loss or reduction of any SMC complex bound to chromosomal axes when compared to wild-type spermatocytes. Thus, this shows that although *Nipbl* gene dosage is reduced, the chromatin association for the majority of SMC complexes remains normal. Indeed, as recently reported, the cohesin loading defects in *Nipbl*^{+/-} cells are limited only to a few specific loci in neurons but bulk cohesin loading and robust cohesion all along the chromosome is otherwise unperturbed in *Nipbl*^{+/-} cells (Remeseiro et al. 2013). Thus, although partial *Nipbl* deficiency affects DNA repair and results in gene expression dysregulation, the localisation of SMC complexes appears unaltered. This is consistent with observations from other organisms where different minimal

concentrations of cohesin are required for distinct cohesin functions. As suggested by Heidinger-Pauli and co-workers, chromosome condensation and DNA repair are more sensitive to decreasing cohesin levels and become compromised by levels reduced to 30% of wild-type levels. This is unlike sister-chromatid cohesion and chromosome segregation, which remain unaffected even when cohesin levels are reduced to 13% of wild-type levels (Heidinger-Pauli et al. 2010). Taking this into consideration, in my study I have deleted both alleles of *Nipbl* specifically in primordial or growing oocytes and investigated their consequences.

I found that although *Nipbl* gene has been successfully inactivated in oocytes included in neonatal primordial follicles or in the adult growing follicles, these *Nipbl*-deleted oocytes underwent normal cell cycle progression with unaffected cohesion. This is in accordance with studies by Revenkova (2010b) and Tachibana-Konwalski (2010) and proposes the lack of cohesin turnover in oocytes arrested in meiotic prophase. However, irrespectively of the presence or absence of cohesin reloading during meiosis until anaphase II, cohesin has to be loaded *de novo* in fertilized eggs in order to support correct chromosome segregation during the first zygotic cleavage. Recent study demonstrated a rapid switch of cohesin subunits between meiosis and mitosis. While meiotic cohesion is mediated by cohesin complexes containing Rec8 subunit, the first mitotic cell cycle in zygotes is supported by cohesin complexes containing mitotic Rad21 subunit (Tachibana-Konwalski et al., 2010). I hypothesized that since zygotic gene expression commence at two-cell stage in mouse embryos, the loading of Rad21-containing cohesin in those embryos must rely on maternally stored *Nipbl* protein or synthesized from maternally stored *Nipbl* transcripts. As demonstrated by my results *Nipbl*-deleted eggs successfully loaded Rad21-containing cohesin. In addition, *Nipbl* protein accumulated before *Nipbl* inactivation as early as in primordial oocytes in neonatal ovary is sufficient to support the development of blastocysts generated from parthenogenetically activated *Nipbl*-inactivated eggs. However, these blastocysts contained high fraction of mitotic cells with misaligned chromosomes. This is possibly due to exhaustion of accumulated *Nipbl* protein pool in these cells.

Although *Nipbl*^{f/f};Gdf-9-iCre females maintained the reproductive capability for 8 months, detailed analysis of chromosomal content of their eggs revealed precocious segregation of all bivalents at metaphase I in some oocytes. Two scenarios can potentially result in the presence of bivalents at metaphase I (i) impaired crossovers formation or (ii) chiasmata breakdown. Since *Nipbl* gene has been deleted after

crossover formation in oocytes, the possibility of impaired crossover formation due to insufficient levels of cohesin seems unlikely. Thus, precocious chiasmata breakdown can be interpreted as a consequence of decrease in cohesin level, a potential effect of *Nipbl* deletion in primordial oocytes. Since very low levels of cohesin have been showed to be sufficient for cohesion (Heidinger-Pauli et al., 2010) this implies a dramatic drop in chromatin-bound cohesin. However, this phenotype has been observed only in sporadic oocytes of *Nipbl^{fl/fl};Gdf-9-iCre* females. Theoretically, the presence of univalents at metaphase I in *Nipbl* Δ oocytes could be a long-term result of *Nipbl* inactivation. Would their ratio increase together with mouse female age? Is there diversity in level of chromatin-bound cohesin among oocytes that renders them higher resistance to consequences of drop in cohesin levels? These questions still remain unanswered and require further studies.

Taken together, these results suggest that *Nipbl* synthesized in dormant primordial oocytes is stably maintained at least in majority of oocytes. Also, it is sufficient for supporting not only oocyte maturation and development but also cohesin loading in zygotes and several rounds of cell division of parthenogenetically activated eggs. So, if *Nipbl* is present in oocytes why does cohesin re-loading is suppressed during prolonged meiotic prophase in oocytes? This can be explained by three hypothetical scenarios. Firstly, cohesin may be loaded onto chromatin by *Nipbl* in a form preventing it from taking part in functional cohesion e.g enabling the acetylation of its Smc3 subunit that is a known prerequisite for enduring sister chromatid cohesion (Gerlich et al., 2006; Ben-Shahar et al., 2008). Further, even though *Nipbl* is present in cells its cohesin loading activity may be suppressed by currently unknown mechanism. Alternately, chromatin unbound cohesin complex is modified in a manner that prevents it from being loaded in oocytes during this stage.

As demonstrated in Chapter 3, *Nipbl* was not detected on chromosomes after dictyate stage. However, its localisation in the oocytes still remains unclear. The possible explanation is that since *Nipbl* has been shown to translocate from chromosomes to cytoplasm upon mitotic entry (Watrin et al., 2006), possibly also in oocytes *Nipbl* is maintained in chromatin-unbound form after dictyate stage. Together, these results suggest exceptionally stable nature of *Nipbl* in mammalian oocytes, which is proposed by recent studies also for the cohesin complex. Consequently, this proposes the presence of a subset of proteins in oocytes that are

stable for extended period of time that can be as long as the entire reproductive lifespan of females.

4.3.3 Delayed cell death of Nipbl-deficient fibroblasts with cohesion defect

I further set out to investigate whether the stable maintenance of Nipbl-dependent cohesin loading is specific only for oocytes. Therefore, I generated highly proliferating *Nipbl*^{fl/fl} immortalized mouse embryonic fibroblast (iMEFs) cell line and inactivated *Nipbl* by virus-mediated Cre expression. A previous study where Nipbl has been knockdown in HeLa cells by siRNA demonstrated that the reduction of chromatin-bound cohesin and consequent precocious loss of cohesion in the majority of cells took place within 72 h after the treatment (Watrin et al., 2006). In contrast, I observed that Nipbl-deficient iMEFs could survive up to two weeks in culture. However, the cell number of *Nipbl*^{-/-} iMEFs remained unchanged for six days after Cre-mediated *Nipbl* inactivation and BrdU incorporation assays demonstrated that 30% of cells were still proliferating six days after Cre expression. This suggests that *Nipbl*^{-/-} iMEFs die in an asynchronous manner, which may be the result of different levels of Nipbl protein accumulation in the cells when *Nipbl* is deleted. Near the end of the cell culture a high fraction of cells exhibited abnormal nuclear morphologies, which is a hallmark of defective chromosome segregation. Therefore, these results indicate that precocious loss of cohesion in the absence of cohesin loading activity is the most probable reason for cell death. However, the observation that *Nipbl*^{-/-} iMEFs cells can survive up to 14 days in culture suggest that Nipbl-mediated cohesion loading activity is also relatively stable in mitotically proliferating cells.

The relative stability of Nipbl-dependent cohesin loading can be explained by two hypothetical scenarios. Firstly, since it is not known how much of cohesin loading complex is needed for cohesin loading, it is possible that cells accumulate excess of Nipbl protein that can support many rounds of cohesin loading and cell division cycles. This hypothesis would support observation of asynchronous cell death of *Nipbl*^{-/-} iMEFs, where the level of Nipbl accumulation may vary cell by cell. Alternately, Nipbl protein may not be degraded in an active destruction mechanism because it is required in DNA damage repair processes (Oka et al., 2011; Ström et al., 2004; Unal et al., 2004). Taken together, these results propose the stable maintenance of Nipbl protein and cohesin loading activity in both mitotic and meiotic cells, at least in oocytes and fibroblasts.

4.3.4 Future directions

1) Damage-induced cohesin re-loading in mouse oocytes

In mitosis, the role of cohesin in DNA repair is well established. It has been showed that in yeast and in human cells, genome-wide post-replicative cohesin loading is induced by DNA double strand breaks (DSBs), which implies the presence of post-replicative cohesin replenishment pathway (Ström et al., 2007; Unal et al., 2007). This damage-induced cohesin re-loading also requires Scc2 (Unal, 2004; Ström, 2004) and becomes cohesive (Unal et al., 2007). This is supposed to facilitate DSB repair by keeping sister chromatids in close proximity, allowing repair by sister chromosome recombination. An interesting question to answer would be whether damage-induced cohesin re-loading take place in oocytes and whether it is mediated by Nipbl? This could be achieved by analyzing repair of irradiation-induced DSB in *spo11;Nipbl* double mutants oocytes.

2) Role of Mau2 in mammalian meiosis

Cohesin loading complex, also known as collerin, consists of a large, highly conserved Nipbl (also known as Scc2, delangin or adherin, the functional homolog of budding yeast Scc2), which has been a subject of this thesis and a less well-conserved smaller protein called Mau2 or Scc4. As shown by Visnes and co-workers Nipbl and Mau2 co-localise throughout meiotic prophase, which suggests that they do not perform independent functions during mammalian meiosis, at least not as can be cytologically detectable (Visnes et al., 2013). These observations have also been confirmed in immunostainings performed in our lab (data not presented here). In yeast *S. cerevisiae*, inactivation of Scc4 specifically during meiosis leads to a near complete failure to undergo meiosis and only a small fraction of cells were able to complete first meiotic division. This is alike to Scc2 meiotic null strain (Lin et al. 2011). Very little is known about Mau2/Scc4 role in mammalian oocytes. Is their function redundant together with Nipbl? To answer this Mau2 conditional inactivation system in mouse oocytes is required to allow thorough functional analysis.

Chapter 5

The cell cycle ubiquitin ligase Anaphase-Promoting Complex (APC/C) is essential for maintaining the survival of dormant oocytes in primordial follicles.

5.1 Introduction

Mammalian oocytes initiate meiosis during foetal development but soon after birth, they become arrested at a late stage of meiotic prophase, in a dictyate stage. Meiotic prophase is a stage equivalent to G2-phase of mitotic cells. However, in contrast to mitotic G2 phase, meiotic prophase arrest can be sustained for a prolonged period of time, the shortest until first ovulation and the longest until menopause. Since no cellular proliferation or differentiation is observed in these oocytes, they are considered to be dormant. By paracrine stimuli dormant oocytes enter the growth phase, during which they synthesize proteins required for meiotic resumption and early embryonic development, however these growing oocytes are still arrested in meiotic prophase. When the growth phase is completed and granulosa cells form multiple layers, they are called antral follicles. Oocytes in such follicles obtain competence to resume meiosis. A surge of luteinizing hormone (LH) promotes meiotic resumption of fully-grown oocytes. The resumption of meiosis is associated with the breakdown of germinal vesicle (GVBD).

As described in Chapter 1, maintenance of meiotic prophase arrest in fully-grown oocytes is dependent on inhibitory phosphorylation of Cdk1, whilst the resumption of meiosis, similarly to G2/M transition of mitosis, is triggered by upregulation of Cdk1 activity (Dorée and Hunt, 2002). Cdk1 activity is tightly regulated by the cAMP-PKA-Wee1-Cdc25 pathway (Duckworth et al., 2002). Recent studies in mouse oocytes suggested that, Cdk1 activity is also downregulated by APC/C^{Cdh1}-mediated cyclin B1 degradation, which suppresses meiotic resumption at late meiotic prophase (Reis et al., 2006).

At the initiation of mitotic anaphase APC/C targets cyclin B1 and securin for degradation. Cyclin B1 destruction downregulates Cdk1 activity, while degradation of securin frees separase that subsequently leads to chromosome segregation during anaphase. The activity of APC/C is suppressed during mitotic G2-phase by binding of an inhibitor Emi1. In contrast, as suggested by recent studies, APC/C is active in fully-grown meiotically competent oocytes and is involved in targeted degradation of nuclear cyclin B1 leading to down-regulation of Cdk1 activity (Thornton and Toczyski, 2006). This was further investigated in a study by Reis and co-workers (2006) in which they showed that *in vitro* culture of fully-grown GV oocytes with the knockdown of Cdh1 (one of the co-activators of APC/C) in the presence of cAMP analogues, leads to precocious meiotic resumption. This

suggests that APC/C^{Cdh1} is active before GVBD (Reis et al., 2006). However, two *in vivo* studies demonstrated that although APC/C activity has been depleted during oocyte growth, these mice were able to produce fully grown meiotically competent oocytes (McGuinness et al., 2009; Holt et al., 2011), suggesting that APC/C is dispensable for the meiotic prophase arrest of oocytes during oocyte growth. Taken together, these studies suggest that although APC/C is unessential for most of the time during oocyte growth, it becomes active possibly only shortly before GVBD. Precocious activation of Cdk1 in oocytes that have not achieved full size or did not acquire competence to mature would cause adverse consequences leading to loss of oocytes, clinically recognised as premature ovarian failure (POF). Therefore, in order to understand the mechanism of the long meiotic prophase arrest maintenance, it is essential to identify the precise timing of APC/C activation in oocytes. In this study I employed mice with targeted *Apc2* locus encoding essential for APC/C's activity cullin domain and conditionally inactivated APC/C in dormant, non-growing primordial follicles and in growing primary oocytes using two deleter Cre lines described in Chapter 4. The consequences of inactivation of *Apc2* on meiotic prophase resumption and oocyte growth were further investigated.

5.2. Results

5.2.1 Genetic inactivation of *Apc2* in mouse oocyte.

I used mice with targeted a *Apc2* locus, called *Apc2^{fl/fl}*, whose exons 2 to 4 are flanked by *loxP* sites (*floxed* allele). Deletion of exons 2 to 4 of the *Apc2* locus by cre-mediated recombination creates a deletion allele (Δ allele) that is a loss-of-function allele (Wirth et al., 2004) (Fig. 5.1 A). In order to conditionally inactivate the *Apc2* subunit specifically in oocytes of primordial follicles or in growing primary follicles, *Apc2^{fl/fl}* females were crossed for two generations (F1 and F2) with males carrying *Cre* recombinase gene whose expression is driven by the *Zp3* or *Gdf-9* promoter respectively (Fig. 5.1 B) (Lan et al., 2004; Lewandoski et al., 1997). The first breeding step (F1) between the *Apc2^{fl/fl}* females and *Cre* expressing males resulted in progeny that carry a single floxed allele and the *Cre* vector (*Apc2^{fl/+};Gdf-9-iCre* or *Zp3-cre*). In the second breeding step (F2) these mice were crossed with homozygous flox allele (*Apc2^{fl/fl}*) females and the progeny with four different genotypes were born (F3) including the desired conditional knockout *Apc2^{fl/fl};Gdf-9-iCre* or *Zp3-cre*, highlighted in a blue frame (Fig.5.1 B). These experimental females were used for further experiments. In each generation, mouse genotypes were determined by genotyping (Fig. 5.1 C). This allowed us to obtain conditional knockout female mice designated *Apc2^{fl/fl};Gdf-9-iCre* and *Apc2^{fl/fl};Zp3-cre* where APC/C activity is depleted in oocytes of primordial follicles and primary growing oocytes, respectively. McGuinness and co-workers reported that *Apc2^{fl/fl};Zp3-cre* females failed to become pregnant and *Apc2 Δ^{Zp3}* oocytes do not extrude the 1st polar body (complete meiosis I) that suggest that APC/C is essential for the first meiotic division (McGuinness et al., 2009). I confirmed that *Apc2 Δ^{Zp3}* oocytes do not extrude 1st polar body when cultured *in vitro* (n=43, 0% PBE, in 2 independent experiments), in contrast to control *Apc2^{fl/fl}* oocytes (n=46, 73% PBE, in 2 independent experiments).

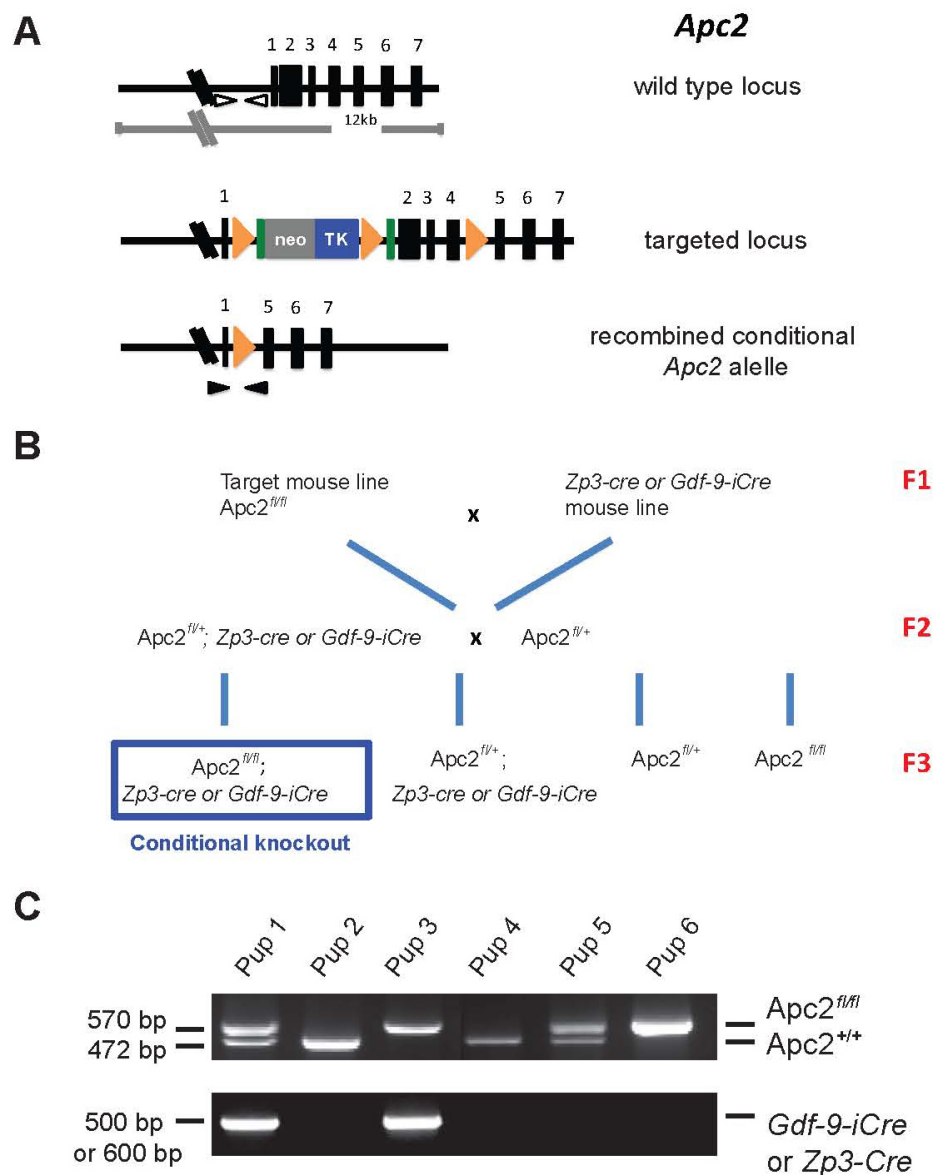


Figure 5.1 Genetic inactivation of *Apc2* in mouse oocyte.

(A) Schematic diagram of targeting strategy for generating *Apc2*-deleted alleles. In the targeted locus exons 2-4 (black boxed) encoding *Apc2* subunit were flanked by loxP sites (orange triangles). The selection neo-TK cassette (neo-TK) was flanked by two FRT sites (green bars). The selection cassette was removed by Flpe-mediated recombination to obtain floxed alleles, and Cre-mediated recombination was used to generate Δ alleles (Wirth et al., 2004). The excision occurs upon *Gdf-9*- or *Zp3*- driven Cre recombinase expression. Black arrows indicate the primers used for genotyping. (B) Mating scheme to create experimental *Apc2* conditional knockout female mice by Cre/loxP recombination. Two rounds of breeding were required to produce knockout mice with oocyte-specific depletion of APC/C activity. (C) Result of representative genotyping PCR on genomic DNA isolated from mouse ear notch from breeding represented in part A. Deletion of *Apc2* from one allele or both allele by *Gdf-9-iCre* or *Zp3-Cre* is demonstrated by genotyping of pup 1 or pup 3. Primer pairs amplify the product of about 472 bp from the wild-type allele and 570 bp of the recombined allele. The presence of *Gdf-9 iCre* or *Zp3-Cre* transgene corresponds to 500 bp or 600 bp amplification band respectively. Primer sequences are listed in Materials and Methods.

5.2.2 Depletion of APC/C activity in growing oocytes accelerates GVBD.

Maintenance of high levels of cyclic adenosine monophosphate (cAMP) in oocytes is required for inhibiting the activation of maturation-promoting factor (MPF) and MAPK signaling and sustaining meiotic arrest (Mehlmann et al., 2002). *In vivo*, LH surge activates phosphodiesterase (PDE3A) that reduces cAMP levels within the oocytes and initiates the pathways triggering meiotic resumption (GVBD) (Richard et al., 2001). Before the LH surge, PDE3A activity is inhibited in oocytes by the inflow of cyclic guanosine monophosphate (cGMP) from the cumulus cells via gap junctions (Vaccari et al., 2009). Milrinone is a phosphodiesterase-3 inhibitor able to inhibit the action of PDE3 and thus prevents degradation of cAMP. It is routinely used to inhibit meiotic maturation of oocytes cultured *in vitro*.

Reis and co-workers observed that GVBD in wild-type oocytes is inhibited by a 50 μ M concentration of milrinone, while Cdh1-depleted oocytes undergo GVBD at the same inhibitor concentration within 24 h of incubation (Reis et al., 2006). Under physiological conditions, GVBD takes place between 60 and 120 min after oocytes are isolated from the follicles in media with no PDE inhibitor. We conceived a possibility that testing the efficiency of GVBD after 24 h of oocyte culture in the presence of inhibitor may be biased by secondary effects of the Cdh1 depletion in the oocytes. Therefore, I set out to investigate how activity of the APC/C complex affects GVBD efficiency in the presence of PDE inhibitor within shorter timespan of 2 h of culture. IBMX, another PDE inhibitor, raises the intracellular level of cAMP (Magnusson et al. 1977).

We hypothesized that the sensitivity of GVBD to different concentrations of IBMX should reflect APC/C activity that downregulates Cdk1 activity during GVBD. Oocytes in which APC/C activity has been depleted during the oocyte growth (*Apc2 Δ^{Zp3}*) and control oocytes (*Apc2^{fl/fl}*) were denuded of their cumulus cells and cultured in medium. Control oocytes have undergone GVBD within between 30 and 120 min when cultured in a medium that does not contain IBMX. Next we examined the effect of IBMX on GVBD in these oocytes. Oocytes were cultured in medium supplemented with IBMX at 3, 6 or 12 μ M concentration. I found that the fractions of oocytes that have undergone GVBD between 30 and 120 min were reduced in a rate correlating to the dosage of IBMX concentration. At 120 min, 18% of oocytes cultured in 12 μ M of IBMX have undergone GVBD, 39% in 6 μ M, 58% in 3 μ M and 80% in IBMX-free medium (Fig. 5.2 A). Next, I cultured *Apc2 Δ^{Zp3}* oocytes in inhibitor-

free medium and observed that, in striking contrast to control oocytes, 60% of them had undergone GVBD within 30 min, suggesting that GVBD is accelerated in *Apc2Δ^{Zp3}* oocytes in comparison to control oocytes. When *Apc2Δ^{Zp3}* oocytes were cultured in media containing different doses of IBMX, I found that the efficiency of GVBD in *Apc2Δ^{Zp3}* oocytes is again reduced in the IBMX dosage dependent manner. IBMX concentration of up to 12 μM is not sufficient to inhibit GVBD within 30 min of incubation and *Apc2Δ^{Zp3}* oocytes have undergone GVBD in a dose-dependent manner (12 μM, 3%; 6 μM, 20%; 3 μM, 42% and in IBMX-free medium, 58%) (Fig. 5.2 B). However, when the fractions of oocytes that underwent GVBD in the same concentration of IBMX in a specific time were compared between *Apc2Δ^{Zp3}* and control oocytes, I found that *Apc2Δ^{Zp3}* oocytes underwent GVBD more efficiently than control oocytes. This result demonstrates that *Apc2Δ^{Zp3}* oocytes are more resistant to the GVBD-inhibitory effect of IBMX than control oocytes and GVBD is accelerated in comparison to control oocytes, which suggests that the activity that drives GVBD is accumulated at higher levels in *Apc2Δ^{Zp3}* than control oocytes.

I sought the IC₅₀ of IBMX (IC_{50_{IBMX}}) in inhibiting GVBD within 2 h of oocyte culture. Non-linear regression analysis on the set of data obtained from scoring GVBD efficiency of *Apc2Δ^{Zp3}* and control oocytes in different IBMX concentrations at 120 min of culture was used to calculate IC₅₀ of IBMX (IC_{50_{IBMX}}) required to inhibit GVBD. The value of IC_{50_{IBMX}} of *Apc2Δ^{Zp3}* oocytes is equal to 5.5 μM and 2.5 μM for control oocytes (Fig. 5.2). Thus, the depletion of APC/C activity in *Apc2Δ^{Zp3}* decreases their sensitivity to IBMX by 120% when compared to control oocytes.

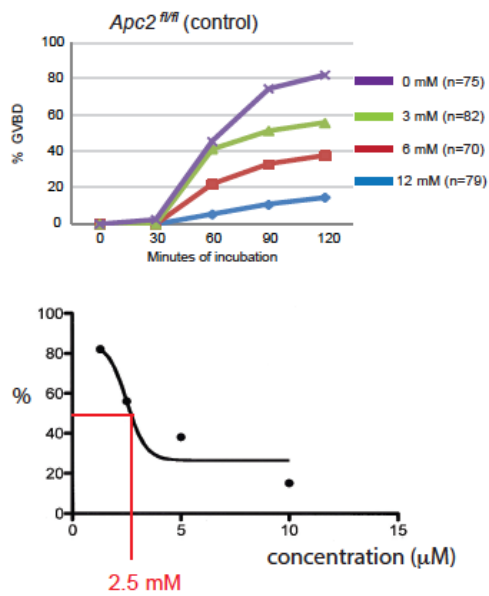
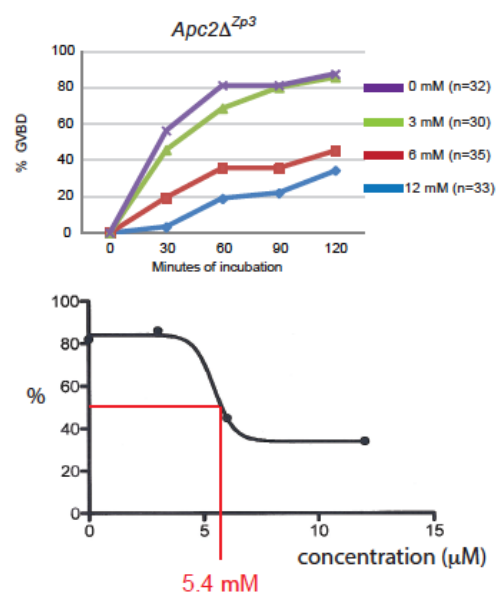
A**B**

Figure 5.2 Depletion of APC/C activity in *Apc2Δ^{Zp3}* decreases their sensitivity to IBMX.

Fully grown GV-stage oocytes from 3–4-week-old PMSG-primed *Apc2^{fl/fl};Zp3-cre* females (**A**) and control littermates *Apc2^{fl/fl}* (**B**) were collected 48 hours after the injection, divided into groups and cultured in 3 different concentrations of IBMX: 12 μ M, 6 μ M, 3 μ M or in inhibitor-free medium (0 μ M). Resumption of meiosis visualized as GV breakdown (GVBD) of mutant and control oocytes was monitored every 30 min for 2 h. Graphs represent the kinetics of GVBD of mutant and control oocytes in the presence of different concentrations of IBMX. GVBD is expressed as a cumulative percentage of the total number of oocytes observed. Numbers of oocytes (n).

Since GVBD is promoted by the Cdk1 activity, increased competence of *Apc2Δ^{Zp3}* oocytes in undergoing GVBD than control oocytes may be due to upregulated Cdk1 activity upon meiotic resumption. If this is true, the efficiency of GVBD in *Apc2Δ^{Zp3}* oocytes should be more resistant to inhibition of Cdk1 activity than that of control oocytes.

In order to specifically and rapidly inactivate Cdk1 I have used RO-3306, Cdk1-specific inhibitor which binds to ATP-binding pocket of Cdk1 (Vassilev et al., 2006). Although a variety of chemical inhibitors of Cdk1 are available, a number of them is likely to affect other kinases within and possibly outside of the Cdk family. For example, Roscovitine have been reported to block in addition to Cdk1/2 also Cdk7 and Cdk9 (Alvi et al., 2005).

I sought IC₅₀ of RO3306 (IC₅₀_{RO3306}) in inhibiting GVBD within 2 h. *Apc2Δ^{Zp3}* and control (*Apc2^{fl/fl}*) oocytes were denuded of their cumulus cells and cultured in medium supplemented with RO3306 at 0.6, 1.3, 2.5, 5 or 10 μM. Both *Apc2Δ^{Zp3}* and control *Apc2^{fl/fl}* oocytes were incubated in RO3306-free media. GVBD was scored at 30 min intervals for a period of 2 h (Fig. 5.3). In control *Apc2^{fl/fl}* oocytes the process of GVBD was inhibited for the first 30 min of culture, regardless of the presence of RO3306 inhibitor. Within further 90 min, *Apc2^{fl/fl}* control oocytes have undergone GVBD at rates that are correlated to RO3306 concentrations, but GVBD was completely suppressed by 10 μM of RO3306. At 120 min none of oocytes cultured in 10 μM have undergone GVBD, 4% in 5 μM, 60% in 2.5 μM, 80% in 1.3 μM and 90% in 0.6 μM and RO3306-free medium (Fig. 5.3 A). This is in contrast to *Apc2Δ^{Zp3}* oocytes, in which a concentration of RO3306 up to 2.5 μM is not sufficient to inhibit GVBD within first 30 min of culture (Figure 5.3 B). At 120 min 8% of oocytes cultured in 10 μM of RO3306 have undergone GVBD, 80% in 5 μM, 92% in 2.5 μM, 95% in 1.3 μM and 100% in 0.6 μM and RO3306-free medium (Fig. 5.3 A). This suggests that both *Apc2Δ^{Zp3}* mutant and *Apc2^{fl/fl}* control oocytes have undergone GVBD at 120 min of culture at rates that are correlated to RO3306 concentration. My results shows that *Apc2Δ^{Zp3}* oocytes are more resistant to the GVBD-inhibitory effect of RO3306 than control oocytes, which suggests that the Cdk1 activity is accumulated at higher levels in *Apc2Δ^{Zp3}* than control oocytes.

I have used non-linear regression analysis on the data set data obtained from scoring GVBD efficiency of *Apc2Δ^{Zp3}* and control oocytes in different RO3306

concentrations at 120 min of culture to calculate IC₅₀ of RO3306 to inhibit GVBD (Figure 5.3). The value of IC_{50_{RO3306}} of *Apc2Δ^{zp3}* oocytes is equal to 5.6 μM and 2.8 μM for control oocytes. Thus, the depletion of APC/C activity in *Apc2Δ^{zp3}* increases the activity of Cdk1 by 100% when compared to control oocytes.

Taken together, these data suggest that Cdk1 activity is more upregulated in fully-grown GV *Apc2Δ^{zp3}* oocytes than those of controls, which defines the role of APC/C in downregulating Cdk1 activity at the stage more precisely than previous studies.

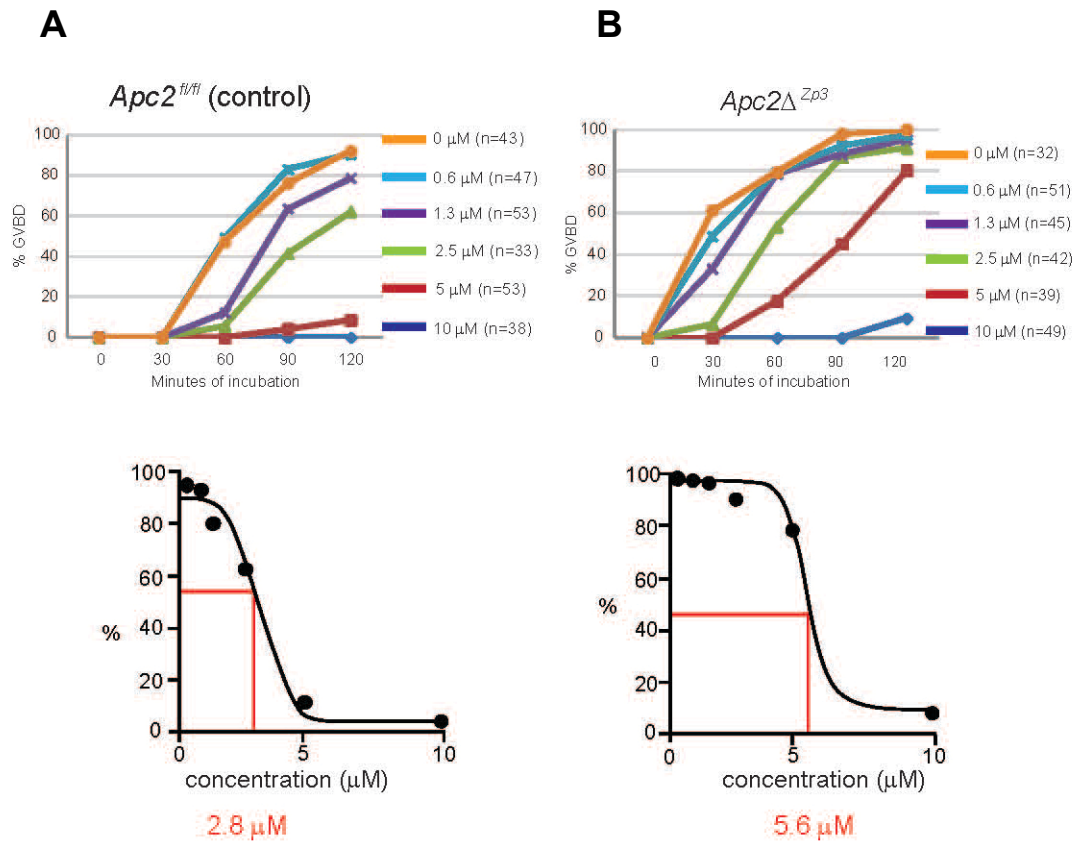


Figure 5.3 Cdk1 upregulation in *Apc2Δ^{Zp}* oocytes.

Fully grown GV-stage oocytes from 3–4-week-old PMSG-primed *Apc2^{fl/fl};Zp3-cre* (A) females and control littermates *Apc2^{fl/fl}* (B) were collected 48 hours after the injection, divided into groups and cultured in 5 different concentrations of Cdk1 inhibitor: 10 μM, 5 μM, 2.5 μM, 1.3 μM, 0.6 μM in medium or in inhibitor-free medium (0 μM). Resumption of meiosis visualized as GV breakdown (GVBD) of mutant and control oocytes was monitored every 30 min for 2 h. Graphs represent the kinetics of GVBD of mutant and control oocytes in the presence of different concentrations of Cdk1-inhibitor plotted against the time. GVBD is expressed as a cumulative percentage of the total number of oocytes observed. Numbers of oocytes (n).

5.2.3 *Gdf-9-iCre* deleter line deletes floxed alleles in dormant oocytes of primordial follicles from the postnatal day 3.

The results mentioned in 5.2.2 as well as previous studies suggested that the APC/C activity in fully-grown oocytes has a negative effect on GVBD by downregulating Cdk1 activity. This led us to ask a question whether the APC/C plays an essential role for suppressing premature GVBD in dormant oocytes within primordial follicles that are arrested for protracted period of time. To inactivate APC/C during meiotic prophase arrest of dormant oocytes enclosed in primordial follicles, we employed *Gdf-9-iCre* deleter line. This transgene line was generated by Austin Cooney's group and enables deletion of floxed genomic regions in the dormant oocytes of primordial follicles (Lan et al., 2004).

We presumed that due to ovarian heterogeneous follicular content the expression of *GDF-9-iCre* does not take place simultaneously in all oocytes enclosed in primordial follicles. Therefore, I set out to assess the timing and kinetics of Cre-mediated loxP recombination in the mouse ovary. We employed mouse reporter strain *Rosa26^{Stop-YFP/Stop-YFP}* (described in Materials and Methods) that was crossed with transgenic males carrying a *Gdf-9-iCre* transgene. From the resulting progeny *Gdf9-iCre;Rosa26^{Stop-YFP/Stop-YFP}* females were used for further experiments. In their ovaries YFP was expressed only after *GDF-9* driven iCre-mediated excision of loxP-flanked Stop cassette has taken place (thereafter called *Gdf9-iCre;Rosa26^{YFP/YFP}*). Ovaries of *Gdf9-iCre;Rosa26^{YFP/YFP}* mice were collected at postnatal days 1 (PD1), 3 (PD3), 5 (PD5), 15 (PD15), 21 (PD21) and 35 (5 weeks), fixed and sectioned. Tissue sections were then subjected to chromogenic immunostaining with anti-GFP antibody in order to detect cre recombinase activity. Ovaries from *Rosa26^{Stop-YFP/Stop-YFP}* littermates were used as negative controls for the staining. The representative ovarian sections are presented in Figure 5.4 A. Oocytes of different developmental stages positive for immunostaining were scored (Fig. 5.8 B). As expected, no staining was observed in oocytes of *Rosa26^{Stop-YFP/Stop-YFP}* control females at the age of 1–35 days after birth. Also, I observed no staining at PD1 in *Gdf9-iCre;Rosa26^{YFP/YFP}* ovary (Fig. 5.4 A: a, a'). At PD3, I scored that 40% of primordial follicles, 39% of transitional and 90% of primary and bigger follicles were GFP positive in *Gdf9-iCre;Rosa26^{YFP/YFP}* mice (Fig. 5.4 A: b, b'; black arrow, oocytes positive for GFP staining; white arrow, oocytes negative for GFP staining). At PD5, 48% of primordial, 66% of transitional and 89% of primary and bigger follicles were GFP positive (Fig. 5.4 A: c, c'). At PD15 anti-GFP staining was observed in 67% of

primordial, 70% of transitional and 95% of primary and bigger follicles (Fig. 5.4 A: d, d'). At PD21, positive staining was detected in 82% of primordial, 89% transitional and 100% of primary and bigger follicles in *Gdf9-iCre;Rosa26^{YFP/YFP}* ovaries (Fig. 5.4 A:e, e'). At 5 weeks of age 90% of primordial, 95% of transitional and 100% of primary and bigger were GFP-positive (Fig. 5.4 A: f, f'). Anti-GFP staining was specific and limited only to oocytes at all follicular stages.

These results demonstrate that *Gdf-9* driven *iCre*-mediated deletion initiates at postnatal day 3. However, the deletion does not take place simultaneously in all primordial follicles. *iCre* is activated in all dormant oocytes of primordial follicles within 5 weeks of birth.

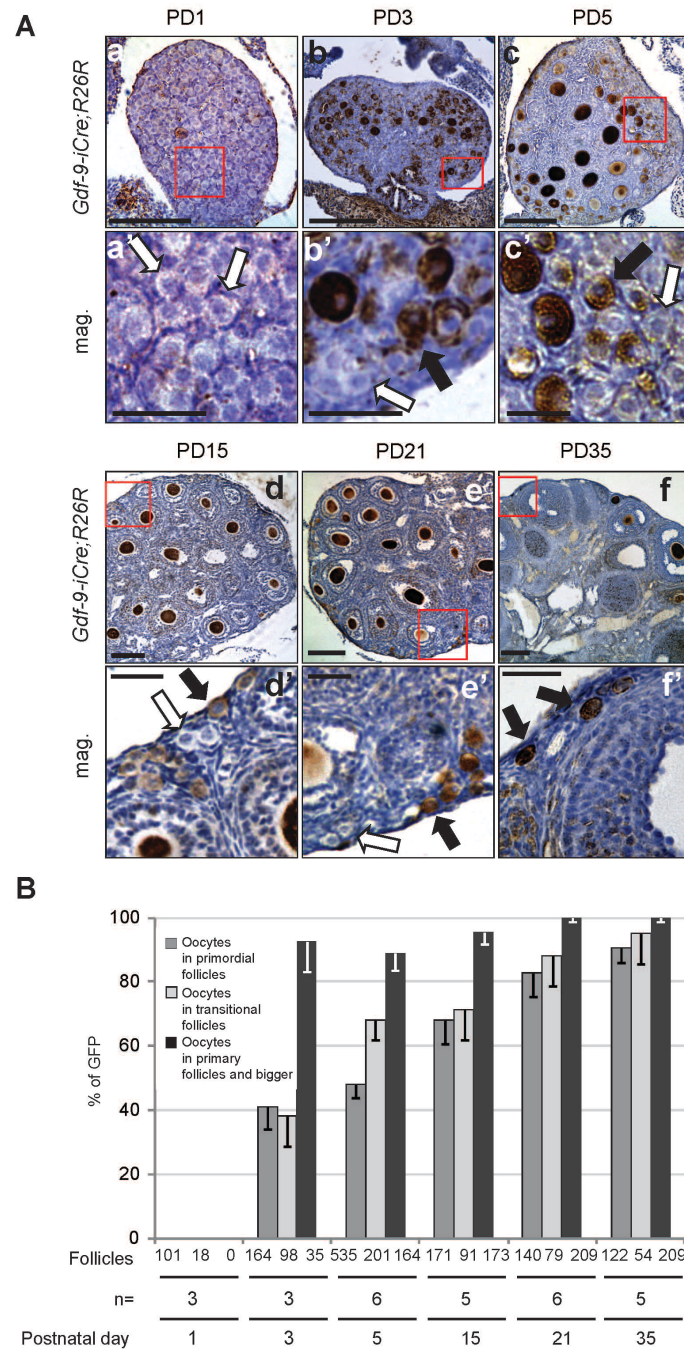


Figure 5.4 Monitoring of *Gdf-9-iCre* mediated deletion.

(A) Ovaries from (a) Day 1 (PD1), (b) Day 3 (PD3), (c) Day 5 (PD5), (d) Day 12 (PD12), (e) Day 24 (PD24) and (f) Day 35 (5w) of *Gdf9-iCre;Rosa26^{YFP/YFP}* mice were collected, fixed and sectioned. Tissue sections were then immunostained with rabbit polyclonal anti-GFP antibody, a horseradish peroxidase-conjugated secondary antibody, and the stain developed with diaminobenzidine (DAB) and counterstained with haematoxylin. The brown staining represents the DAB-chromogen staining (black arrow). Oocytes with no staining are indicated by white arrow. One ovary from each mouse was used for scoring. Scale bars: 200 μ m (a, b, c, d, e, f) and 50 μ m for magnified images (a', b', c', d', e', f'). (B) GFP-positive oocytes in primordial, transitional or primary follicles and bigger follicles were scored and are expressed as a % of the total number of this type of follicles per ovary. Error bars were calculated using Wald's test. Follicles, numbers of follicles scored; n, number of mice used.

5.2.4 APC/C inactivation in dormant primordial follicles by *Gdf9-iCre* line leads to primordial follicle loss.

I made the use of oocytes whose *Apc2* gene had been conditionally inactivated in dormant primordial follicles (*Apc2^{fl/fl};Gdf9-iCre*) and examined whether *Apc2*-inactivation has any influence on ovarian folliculogenesis. I found that *Apc2^{fl/fl};Gdf9-iCre* females fail to become pregnant when kept with wild-type males for 3 months or more. Next, I examined ovarian follicular complement in *Apc2^{fl/fl};Gdf9-iCre* female mice. Ovaries from 3- (PD3), 5- (PD5), 7- (PD7), 15- (PD15), 21-day-old (PD21), 5-, 8- and 13-week-old *Apc2^{fl/fl};Gdf9-iCre* and control *Apc2^{fl/fl}* littermates were dissected out, fixed and cut into serial sections, stained with haematoxylin and eosin and observed under the microscope (Fig. 5.5). Numbers of primordial and activated follicles in ovaries from *Apc2^{fl/fl};Gdf9-iCre* and control *Apc2^{fl/fl}* females were scored (Fig. 5.6). According to the monitoring study of the *Gdf9-iCre* expression (Result 5.2.3), Cre-mediated recombination is initiated in dormant oocytes of primordial follicles at the postnatal day 3 in mouse. Therefore, the first wave of postnatal follicular development was analysed at postnatal day 3 (PD3) in *Apc2^{fl/fl};Gdf9-iCre* and control *Apc2^{fl/fl}* females. At this stage, only fully formed primordial follicles that contain small oocytes surrounded by flattened pre-granulosa cells were scored, while oocytes still enclosed in cysts were excluded from the scoring because *Gdf9-iCre* has not been activated during this stage, as described in 5.2.3. The numbers of primordial follicles cells were similar between *Apc2^{fl/fl};Gdf9-iCre* and control *Apc2^{fl/fl}* ovaries (Fig. 5.5), indicating that comparable numbers of follicles are formed in the control and mutant ovaries. Also, the numbers of activated transitional follicles, in which some of pre-granulosa cells have undergone cuboidalization, are similar at PD3 (Fig. 5.6). Although no apparent morphological difference was observed in *Apc2^{fl/fl};Gdf9-iCre* and control *Apc2^{fl/fl}* ovaries two days after iCre activation, at PD5, careful follicle counting revealed the decline in the primordial follicles number by 30% in the *Apc2^{fl/fl};Gdf9-iCre* ovaries when compared to those of *Apc2^{fl/fl}* control mice (Fig. 5.6). This suggests that the depletion of APC/C activity in primordial follicles have a rapid effect on follicular survival, thus some of the primordial follicles deprived of APC/C activity at PD3 are already depleted between PD3 and PD5. Note that the number of primordial follicles found in the ovary at PD5 is significantly higher than at PD3. This is because primordial follicle formation, which includes enclosure of oocytes by pre-granulosa cells takes place 3-5 days after birth in the mouse. At postnatal day 7 (PD7) ovaries from both genotypes appeared similar in size and both contained primordial, transitional and

primary follicles with enlarged oocytes surrounded by one layer of cuboidal granulosa cells (Fig. 5.5). Accordingly, the percentage of primordial follicles in *Apc2^{fl/fl};Gdf-9-iCre* ovaries at PD7 was lower by 48% than that in *Apc2^{fl/fl}* control ovaries (Fig. 5.6). Also, the total number of activated follicles in *Apc2^{fl/fl};Gdf-9-iCre* ovary was reduced by 43%. By day 21 (PD21) and 35 (5w), ovaries from both genotypes maintained similar size and *Apc2^{fl/fl};Gdf-9-iCre* appeared normal in morphology (Fig. 5.5), however the numbers of dormant primordial follicles and activated follicles continued to decrease. In PD21 *Apc2^{fl/fl};Gdf-9-iCre* ovary the numbers of primordial follicles were reduced by 54% in comparison to those in control ovaries. Also, the total number of activated follicles was reduced by 40% (Fig. 5.6). At postnatal day 35 (5 weeks), at the time of the sexual maturity onset, no primordial follicles were identified in *Apc2^{fl/fl};Gdf-9-iCre* ovaries (Fig. 5.6), whereas 58% of the follicles in control ovaries were still at the primordial stage (Fig. 5.6). Thus, the deletion of *Apc2* gene from primordial follicles mediated by *Gdf-9-iCre* led to complete primordial follicle depletion within 32 days of *Cre* recombinase activation. Figure 5.7 represents the kinetics of primordial follicle loss in *Apc2^{fl/fl};Gdf-9-iCre* ovary in comparison to control *Apc2^{fl/fl}*. From the age of 8 weeks, *Apc2^{fl/fl};Gdf-9-iCre* ovaries became smaller than the control ones (Fig. 5.6). The presence of corpora lutea (CL), temporary endocrine structures developed from the remnants of ovulated follicles during luteal phase of the menstrual cycle suggest that *Apc2^{fl/fl};Gdf-9-iCre* females ovulate, similarly to control females (Fig. 5.6, CLs are marked by asterisks). At 13 weeks, ovarian morphology is largely changed in *Apc2^{fl/fl};Gdf-9-iCre* females; ovaries were significantly smaller and rounded, which is in contrast to control *Apc2^{fl/fl}* ovaries that contained CL (Fig. 5.6, CLs are marked by asterisks) and follicles at various developmental stages (Fig. 5.6).

The observation of the presence of growing follicles in mutant *Apc2^{fl/fl};Gdf-9-iCre* ovaries led us to ask whether the oocytes enclosed in these follicles did not undergo *cre*-mediated *Apc2* inactivation. In order to test this, I have isolated and cultured these oocytes and I found that they do not extrude 1st polar body (PB1) when cultured *in vitro* (n=36, 0% PBE, in 2 independent experiments), a phenotype observed when APC/C activity is deleted in growing oocytes (McGuinness et al., 2009). As shown by monitoring study *Gdf-9-iCre* deleter line deletes floxed alleles in dormant oocytes of primordial follicles from the postnatal day 3, therefore the presence of growing follicles in *Apc2^{fl/fl};Gdf-9-iCre* ovaries suggest the presence of specific time frame within meiotic prophase arrest in dormant oocytes when these

oocytes are sensitive to the lack of APC/C activity. When APC/C inactivation takes place during this time, primordial oocytes are depleted.

Taken together, these results demonstrate that at postnatal day 3, control mouse ovary contains approximately 1700 fully formed primordial follicles and 1200 activated transitional follicles, whilst a high number of oocytes are present as syncytia. At PD5, many syncytia are invaded by pregranulosa cells and new primordial follicles are formed, thus a higher number of primordial follicles was scored in comparison to PD3. From the PD5 stage onwards, the number of primordial follicles decreases steadily, as some of them get activated and enter the pool of activated follicles or undergo atresia and are quickly removed from the ovary. Some of the primordial follicles stay arrested and can follow the aforementioned fate later during the reproductive lifespan. The numbers of dormant, primordial follicles and activated follicles were scored until five weeks of age, when on average, control mouse ovaries contain 1000 primordial follicles and 700 activated follicles. Mouse ovaries, in which *Apc2* has been inactivated specifically in primordial oocytes at PD3, had similar numbers of primordial follicles to control ovary at PD3. However, within two days after *Apc2* inactivation, at PD5, numbers of both dormant and activated follicles were reduced by 33% and 46%, respectively, in comparison to controls. This suggests that depletion of APC/C activity in primordial follicles has a rapid effect on follicular survival. Further on, APC/C inactivation in primordial follicles triggers their accelerated depletion in *Apc2^{fl/fl};Gdf-9-iCre* ovaries that leads to complete exhaustion of primordial follicles pool before adulthood, within 5 weeks after birth. At 5 weeks of age mutant ovaries contain approximately 300 activated follicles that are lost within the next three weeks, at 8 weeks of age. The depletion kinetics for *Apc2* depleted primordial follicles follows the kinetics observed for iCre activation in *Gdf9-iCre;Rosa26^{YFP/YFP}*.

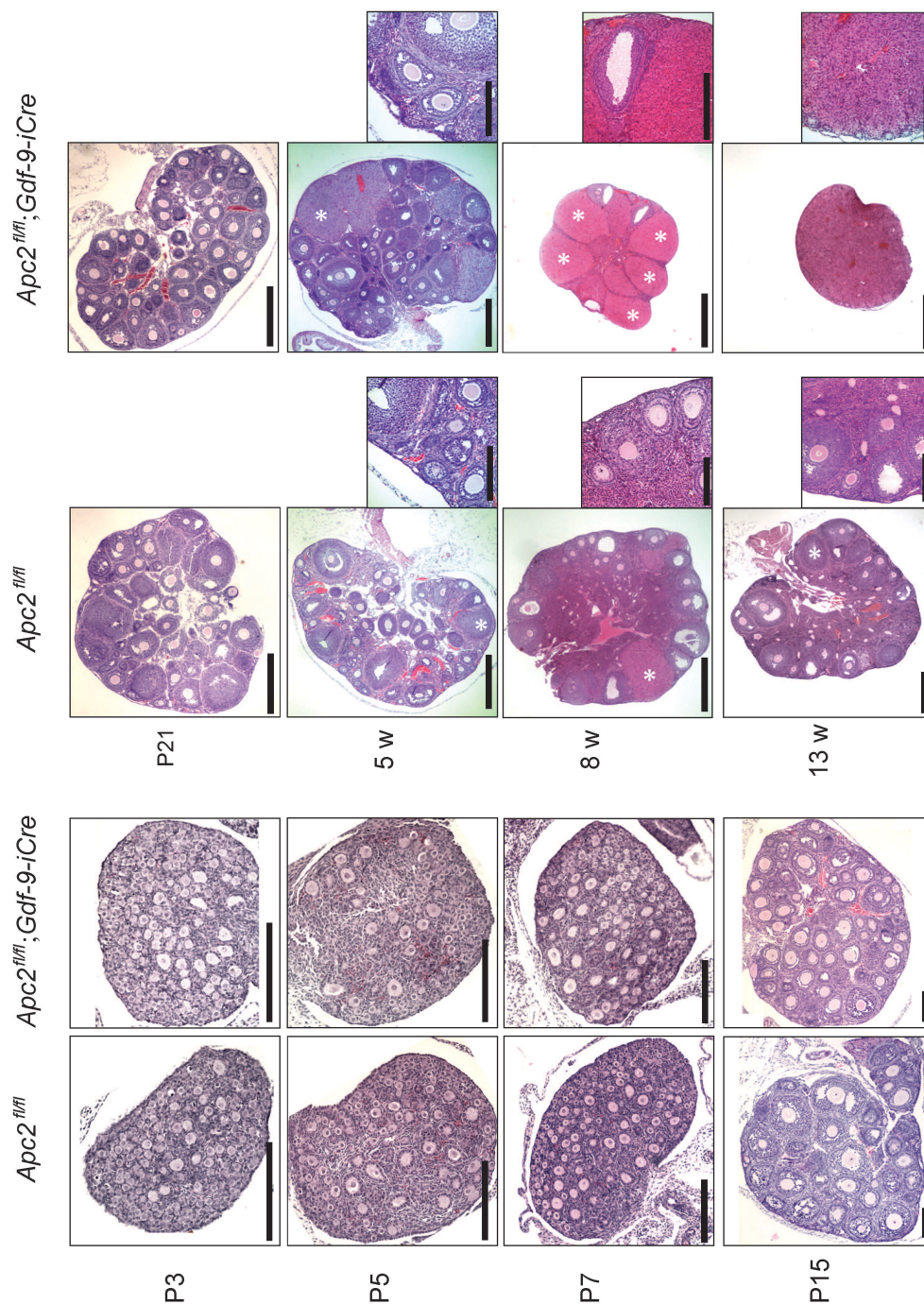


Figure 5.5. Ovarian morphology in *Apc2^{fl/fl}*; *Gdf-9-iCre* and control *Apc2^{fl/fl}* female mice. Ovaries from 3- (PD3), 5- (PD5), 7- (PD7), 15- (PD15), 21-day-old (*Apc2^{fl/fl}*; *Gdf-9-iCre*) and control *Apc2^{fl/fl}* littermates were isolated, fixed, cut into serial sections and stained with haematoxylin and eosin. For each genotype and each age, ovaries from 3-6 mice were used. Representative sections are shown. Corpus luteum is marked by asterisk. Scale bars: (P3-P15) 200 μ m and (P35-13 weeks) 500 μ m. mag., Scale bar of magnified images 200 μ m.

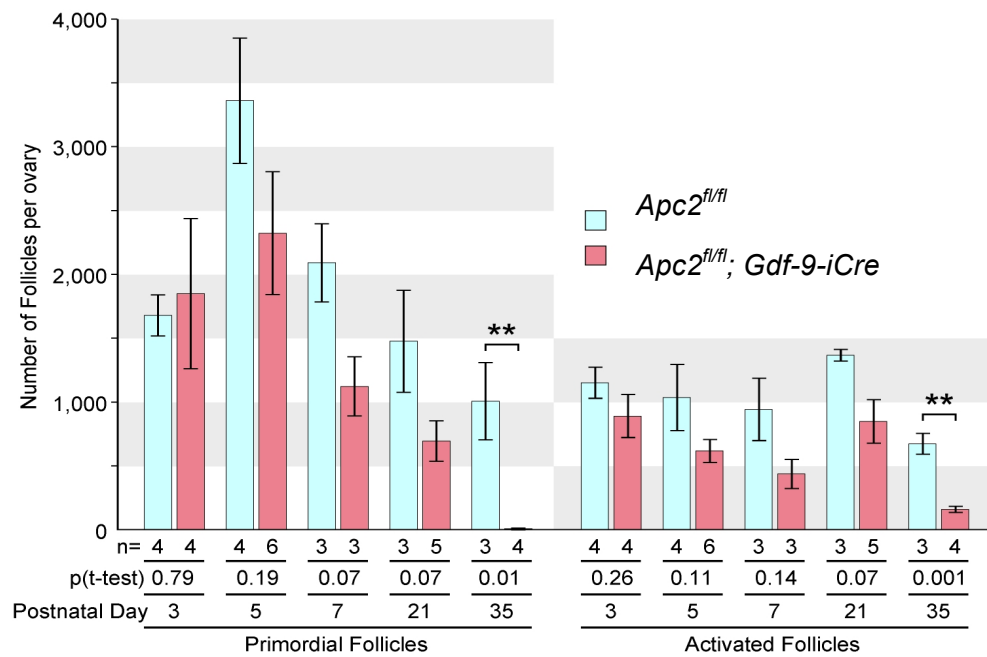


Figure 5.6 Quantification of ovarian follicles in $Apc2^{fl/fl}; Gdf-9-iCre$ and control $Apc2^{fl/fl}$ mice.

Ovaries from 3- (PD3), 5- (PD5), 7- (PD7), 21-day-old (PD21), 5-, 8- and 13-week-old $Apc2^{fl/fl}; Gdf-9-iCre$ and control $Apc2^{fl/fl}$ littermates were collected, sectioned and stained with haematoxylin and eosin. Numbers of primordial and activated follicles in ovary (mean $\pm$ SEM) in $Apc2^{fl/fl}; Gdf-9-iCre$ and control $Apc2^{fl/fl}$ at different ages were counted. The numbers of mice used for each genotype and each age are indicated (n). One ovary from each mouse was used. ** high statistical significance ($p < 0.01$).

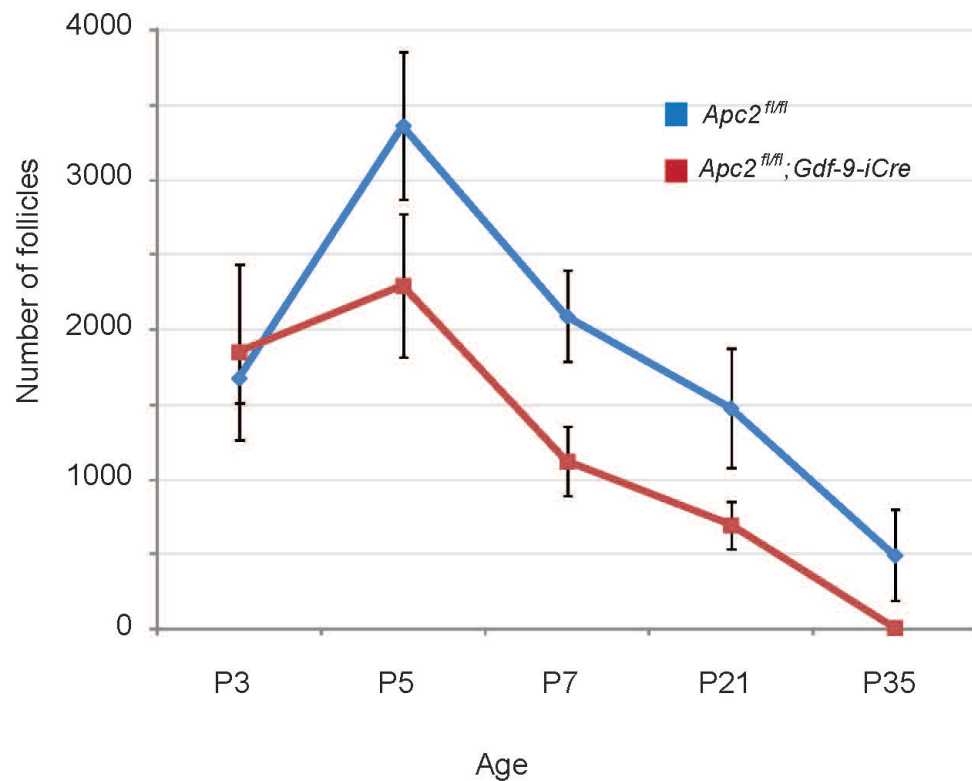


Figure 5.7 Kinetics of primordial follicle loss in $Apc2^{fl/fl};Gdf-9-iCre$ and control $Apc2^{fl/fl}$ ovary.

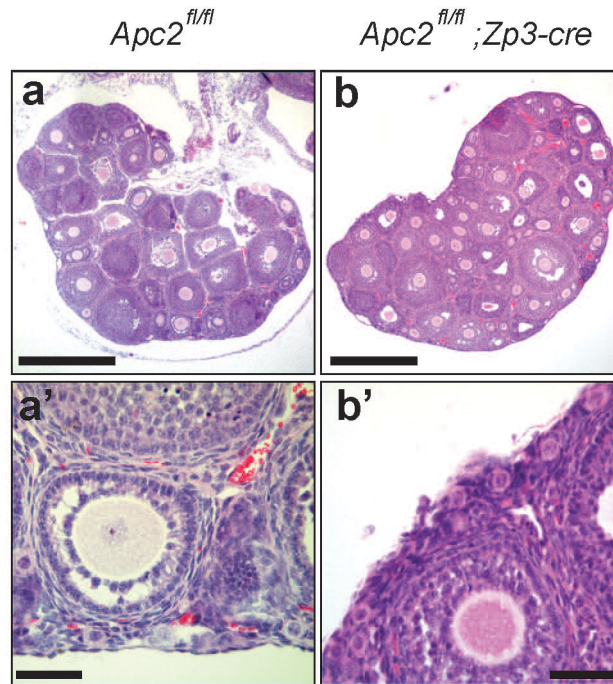
The total numbers of primordial follicles in the ovaries from 3- (PD3), 5- (PD5), 7- (PD7), 21-day-old (PD21), 5-week-old $Apc2^{fl/fl};Gdf-9-iCre$ and control $Apc2^{fl/fl}$ females were counted on serial histological sections. No primordial follicle was found in the mutant ovary at the beginning of adulthood, i.e. 5 weeks.

5.2.5 Follicular growth is largely unaffected by deletion of APC/C activity in growing oocytes by *Zp3-cre*.

As reported by McGuinness and co-workers, depletion of APC/C activity in the oocytes during their growth prevents them from anaphase I onset and leads to infertility (McGuinness et al., 2009). There is no significant reduction in total numbers of fully-grown oocytes isolated from an ovary from adult *Apc2^{fl/fl};Zp3-cre* females compared to those from control *Apc2^{fl/fl}* females. However, much higher frequencies of fully-grown oocytes without GV are isolated from *Apc2^{fl/fl};Zp3-cre* ovaries than controls where such oocytes are present in small numbers (N. Kudo, personal communication). The high incidence of GV loss is attributed to the high Cdk1 activity promoting GVBD in *Apc2^{fl/fl};Zp3-cre* oocytes, as described in 5.2.2. These observations suggest that oocyte growth is largely unaffected by the loss of APC/C activity during this stage, however folliculogenesis in the ovaries of *Apc2^{fl/fl};Zp3-cre* has not been studied precisely. Therefore, we generated female mice where *Apc2* gene was inactivated specifically in growing oocytes in primary follicles by Cre expression driven from *Zp3* promoter (*Apc2^{fl/fl};Zp3-cre*) and examined the effect of APC/C activity depletion on follicular development. Ovaries from 21-day-old *Apc2^{fl/fl};Zp3-cre* and control *Apc2^{fl/fl}* littermates were dissected out, fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, cut into 6 μ m-thick serial sections, stained with haematoxylin and eosin and observed under the microscope (Fig. 5.8 A). Numbers of dormant, primordial and activated follicles in ovary in *Apc2^{fl/fl};Zp3-cre* and control *Apc2^{fl/fl}* were scored (Fig. 5.7 B).

The morphology of *Apc2^{fl/fl};Zp3-cre* ovaries appeared normal when compared to control *Apc2^{fl/fl}* ovaries (Fig. 5.8 A), and the scoring of follicles revealed that similar numbers of primordial, primary, secondary, and early antral follicles are present in both ovaries (Fig. 5.8 B). This result demonstrates that the follicular complement is not influenced in the ovaries whose growing oocytes in primary follicles have lost the APC/C activity. This confirms previous results and suggests that APC/C is dispensable during oocyte growth.

A



B

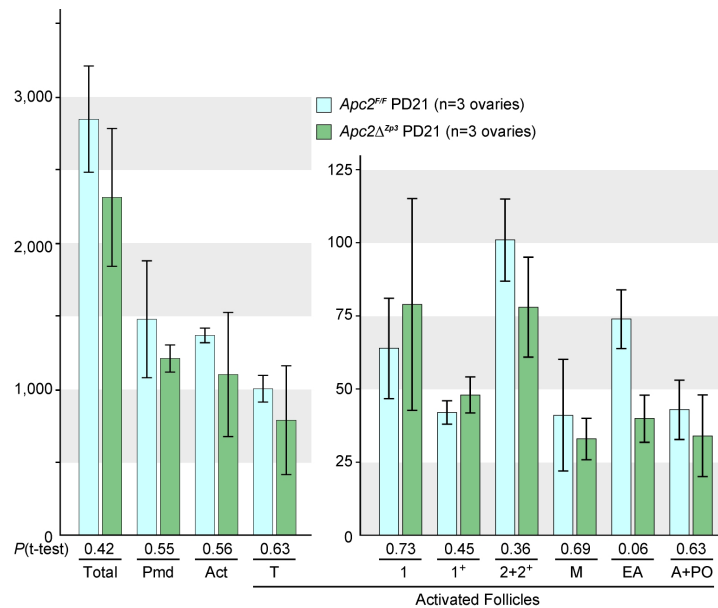


Figure 5.8 Follicular growth is largely unaffected by deletion of APC/C activity in growing oocytes by *Zp3-cre*.

(A) Representative sections of ovaries from 21-day-old (PD21) *Apc2^{fl/fl};Zp3-cre* and control *Apc2^{fl/fl}* littermates. Ovaries were fixed, sectioned and stained with haematoxylin and eosin. Scale bars: 500 μ m and 200 μ m in magnified images. **(B)** Numbers of different types of follicles per ovary (mean $\pm$ SEM) including numbers of primordial and activated follicles (mean $\pm$ SEM) were counted. One ovary from each mouse was used. n, the numbers of mice used for each genotype. Pmd, oocyte in primordial follicle; T, oocyte in transitional follicle; 1, primary follicle; 1+, primary plus follicle; 2+2+ combined secondary and secondary plus follicles; M, multilayer follicle; EA, early antral follicle; A+PO, antral follicle and pre-ovulatory.

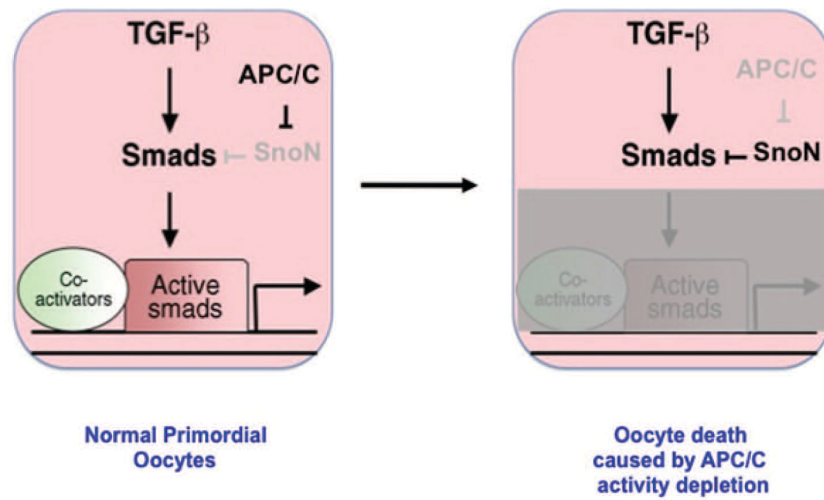
5.2.6 Investigating ovarian targets of APC/C.

Results described in 5.2.4 demonstrated that upon *Apc2* inactivation dormant primordial oocytes are completely depleted before adulthood, within 5 weeks after birth, suggesting that the APC/C activity is required for the survival of primordial oocytes. Since APC/C substrates are degraded by the proteasome, it is conceivable that the loss of oocytes by APC/C inactivation is mediated by upregulation of APC/C substrate(s). Because TGF- β superfamily is one of the major players involved in follicular development, we hypothesized that upregulation of SnoN, an APC/C substrate and a negative regulator of TGF- β superfamily-Smad signaling, leads to follicular loss (Fig 5.9 A). I presumed that deletion of SnoN will rescue dormant primordial follicles loss observed in ovarian phenotypes in the *Apc2^{fl/fl};Gdf-9-iCre* females.

To test this hypothesis, *SnoN^{L/L}* mouse strain was bred with *Apc2^{fl/fl};Gdf-9-iCre* mice, and after multiple breeding rounds experimental double knockout *Apc2^{fl/fl};SnoN^{L/L};Gdf-9-iCre* mice were obtained. Total of five double knockout *Apc2^{fl/fl};SnoN^{L/L};Gdf-9-iCre* females from three independent breeding pairs were sacrificed at five weeks of age. This time point was used because the complete primordial follicles depletion in *Apc2^{fl/fl};Gdf-9-iCre* mice takes place at 5 weeks of age, therefore this time point is the most useful to assess the SnoN deletion rescue ability. The ovarian follicular content of *Apc2^{fl/fl};SnoN^{L/L};Gdf-9-iCre* was compared to follicular contents of *Apc2^{fl/fl}* and *Apc2^{fl/fl};Gdf-9-iCre* at the same age (Fig. 5.9 B).

I observed that ovaries from *Apc2^{fl/fl};SnoN^{L/L};Gdf-9-iCre* females did not contain any primordial follicles, similarly to *Apc2^{fl/fl};Gdf-9-iCre* (Fig. 5.9 B) while *Apc2^{fl/fl}* control ovaries contained on average 1000 primordial follicles. This indicates that the deletion of *SnoN* does not prevent primordial follicle loss caused by the depletion of APC/C activity, therefore it is likely that loss of dormant oocytes by APC/C inactivation is not through upregulation of SnoN.

A



B

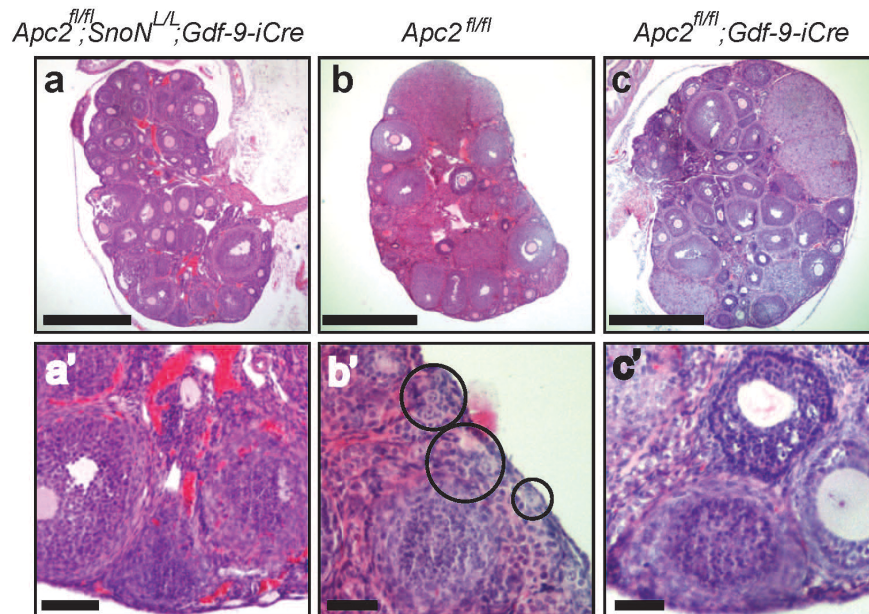


Figure 5.9 Inactivation of SnoN in APC/C-deleted primordial follicles does not prevent their loss.

(A) A hypothetical model of regulatory mechanism of the primordial follicle maintenance by SnoN in normal follicles or upon *Apc2* inactivation that leads to upregulation of SnoN and sequesters TGF- β superfamily signalling required for primordial oocyte maintenance. (B) Representative sections of ovaries from 5-week-old (PD35) *Apc2^{fl/fl}; SnoN^{L/L}; GDF-9-iCre* and control *Apc2^{fl/fl}* mice. Ovaries were fixed, sectioned and stained with haematoxylin and eosin. Primordial follicles are encircled. Scale bar: a, b, c 500 μ m and magnified sections: a', b', c' 50 μ m.

Next, we hypothesized that together with the deletion of APC/C activity, the upregulation of p53 occurs. p53 is a member of family of tumor suppressors implicated in regulation of apoptosis in germ cells. To test this hypothesis, $p53^{neo/neo}$ mouse strain was bred with $Apc2^{fl/fl};Gdf-9-iCre$ mice, and after multiple breeding rounds experimental double knockout $Apc2^{fl/fl};p53^{neo/neo};Gdf-9-iCre$ mice were obtained. Total of three double knockout $Apc2^{fl/fl};p53^{neo/neo};Gdf-9-iCre$ females from two independent breeding pairs were sacrificed at five weeks of age. Similarly to experiment presented in Figure 5.10, this time point has been used because the complete primordial follicles depletion in $Apc2^{fl/fl};Gdf-9-iCre$ mice happens at 5 weeks of age, therefore this time point is the most useful to assess the $p53^{neo/neo}$ deletion rescue ability. The ovarian follicular content of $Apc2^{fl/fl};p53^{neo/neo};Gdf-9-iCre$ was compared to follicular contents of $Apc2^{fl/fl}$ and $Apc2^{fl/fl};Gdf-9-iCre$ at the same age (Fig. 5.10).

I observed that ovaries from $Apc2^{fl/fl};p53^{neo/neo};Gdf-9-iCre$ females did not contain any primordial follicles, similarly to $Apc2^{fl/fl};Gdf-9-iCre$, while $Apc2^{fl/fl}$ control ovaries contained on average 1000 primordial follicles (Fig. 5.10). This indicates that the depletion of p53 activity does not prevent primordial follicle loss caused by the depletion of APC/C activity, therefore it is likely that the loss of dormant oocytes by APC/C inactivation is not through upregulation of p53.

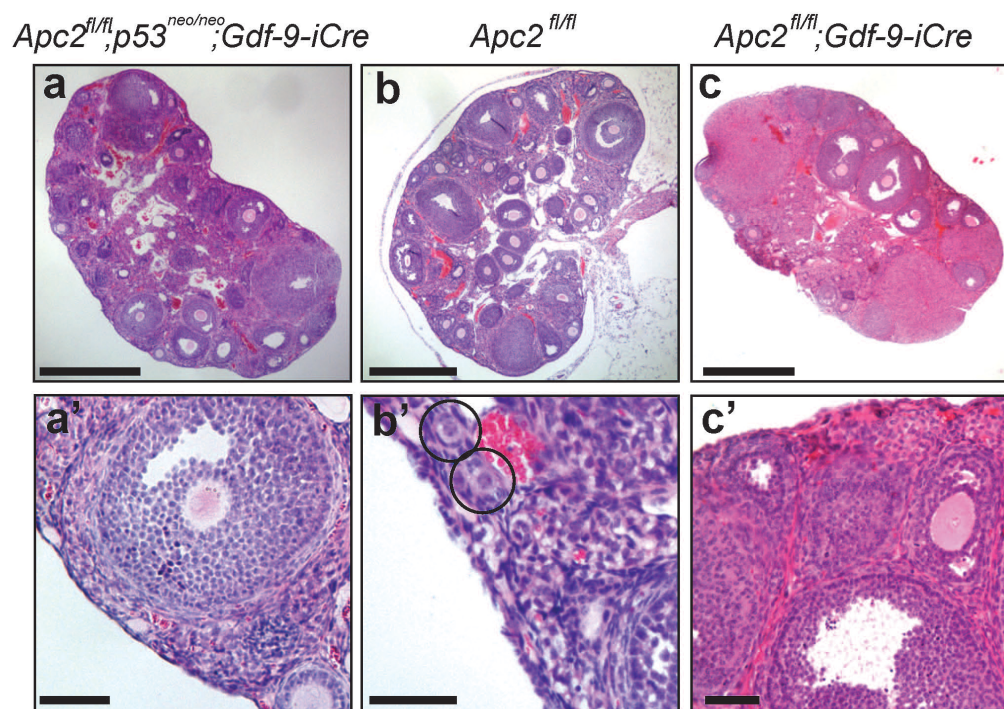


Figure 5.10 Inactivation of p53 in APC/C-deleted primordial follicles does not prevent their loss.

Representative sections of ovaries from 35-day-old (PD35) *Apc2^{fl/fl};p53^{neo/neo};Gdf-9-iCre*, *Apc2^{Δ^{Gdf-9}}* and control *Apc2^{fl/fl}* mice. Ovaries were fixed, sectioned and stained with haematoxylin and eosin. Primordial follicles are encircled. Scale bar: a, b, c 500 μ m and magnified sections: a', b', c' 50 μ m.

5.3 Discussion

5.3.1 Summary of observations

Following mitotic proliferation, mammalian female germ cells initiate meiosis by DNA replication and crossing-over during embryogenesis. Before birth, oocytes become dormant as they arrest in meiotic prophase, a stage equivalent to G2-phase. Shortly after birth each oocyte is enclosed by several somatic cells, called pre-granulosa cells and forms primordial follicles. The numbers of primordial follicles are greater than oocytes ovulated throughout reproductive life. This is because primordial follicles are progressively lost through atresia, while their surviving pool must be maintained as long as reproductive lifespan to supply fertile ova. In adulthood, a subset of primordial follicles is periodically recruited for oocyte growth and granulosa cell expansion in a process of follicular activation. Upon hormonal stimuli fully-grown oocytes finally resume meiosis and are ovulated. Precise regulatory mechanisms for controlling the fates of oocytes in primordial follicles, namely survival, death and differentiation, are still largely unknown.

An unusual feature of female germ cells is that they are arrested in G2-phase for an extremely extended period of time. The maintenance of the cell cycle arrest of oocytes is therefore an important aspect of follicular survival. Recent studies proposed the involvement of the anaphase-promoting complex/cyclosome (APC/C), a cell cycle ubiquitin ligase complex, in down-regulating the cyclin-dependent kinase activity in fully-grown oocytes. As previously suggested APC/C becomes active during meiotic prophase in oocytes, however the precise timing of APC/C activation is still uncertain. In order to address this issue in this study, mice with targeted *Apc2* locus encoding essential for APC/C's activity cullin domain were employed. This allowed me to conditionally inactivate APC/C in dormant, non-growing primordial follicles and in growing primary oocytes using two deleter Cre lines, *Gdf-9-iCre* and *Zp3-cre* respectively.

The examination of ovarian follicular content of *Apc2^{fl/fl};Zp3-cre* mice previously performed in our lab demonstrated an absence of metaphase II oocytes, which is consistent with the previous observation that *Apc2Δ* oocytes are arrested at metaphase I in *in vitro* culture experiment (McGuinness et al., 2009). In addition, the fraction of scored metaphase I oocytes at every stage of antral differentiation within mutant ovaries was significantly higher than in controls (18% versus 4%,

respectively) suggesting that the timing of M-phase entry is advanced in the absence of APC/C activity in growing oocytes. This suggests that the deficiency in APC/C activity in growing oocytes triggers precocious meiotic resumption, which is agreement with previous studies (Holt et al., 2011; McGuinness et al., 2009). Altogether these studies suggest that APC/C is active before the meiotic resumption (GVBD).

In my study I found that upon APC/C inactivation oocytes of primordial follicles, these cells were completely depleted before adulthood, within 5 weeks of birth. This suggests that the APC/C activity is required for the survival of primordial oocytes and probably involved in some unknown regulatory mechanism essential for primordial follicle maintenance.

5.3.2 APC/C and control of meiotic resumption timing in oocytes

It is well established that at metaphase-to-anaphase transition in mitotic cells, active APC/C targets cyclin B1 for degradation and consequently down-regulates the activity of Cdk1. Therefore, next we asked through what mechanism APC/C controls the timing of meiotic resumption (or GVBD) in oocytes. A study by Reis and co-workers (2006) demonstrated that as a result of knockdown of APC/C's co-activator Cdh1, Cdk1 is upregulated at prometaphase stage, suggesting that APC/C^{Cdh1} is also active during prometaphase after GVBD (Reis et al., 2006). Since Cdk1 activity is downregulated before GVBD this led us to hypothesize that APC/C might also control meiotic resumption by downregulating Cdk1 activity through cyclin B1 targeted degradation. To test this idea I aimed to assess Cdk1 activity in fully-grown *Apc2*-deleted oocytes before GVBD using a chemical compound RO3306 able to inhibit Cdk1 activity. I showed that in *Apc2*Δ^{Zp3} oocytes Cdk1 activity is more upregulated when compared to control oocytes, which correlates with the elevated levels of cyclin B1 in response to *Apc2*-deletion. Therefore here for the first time I showed that APC/C activity in fully-grown oocytes has a negative effect on GVBD by downregulating Cdk1 activity. This has been also shown by other in vivo mouse model, where upon conditional deletion of APC/C's co-activator Fzr1, known to be involved in APC/C activation during meiotic prophase arrest, in growing and mature oocytes these oocytes exhibited a higher propensity to undergo GVBD even in follicular environment rich in cAMP, which was associated with elevated levels of cyclin B1 (Holt et al., 2011). In addition, authors showed that levels of Cdc25B, a potential substrate of APC/C^{Cdh1} and phosphatase of Cdk1-cyclin B1 have not been

altered in these oocytes, suggesting that meiotic prophase arrest depends primarily on APC/C-cyclin B1 pathway rather than phosphorylation status of Cdk1. However, my result does not exclude a possibility that APC/C regulates some other factor(s) in addition to cyclin B1 in order to regulate meiotic resumption.

In fact, two other types of cyclins: A2 and B2 have been also shown to participate in Cdk1 activation during the exit from meiotic prophase arrest (Satyanarayana and Kaldis, 2009). While cyclin B1 has been shown to be essential from early development, cyclin B2's function is redundant with other cyclins such as cyclin B1 in somatic cells (Brandeis et al., 1998). Cyclin B2 is expressed in mouse oocytes (Chapman and Wolgemuth, 1993; Ledan et al., 2001) and a recent *in vitro* study by Gui and Homer reported that lack of cyclin B2 prevents the activation of Cdk1 during meiosis resumption in mouse oocytes (Gui and Homer, 2013). They showed that depletion of Hec1, a subunit of the Ndc80 complex leads to compromised M phase entry in mouse oocytes. This is presumably because of Hec1 functions in protecting cyclin B2 from APC/C^{Cdh1}-mediated destruction. They suggest that wild-type levels of cyclin B1 are not sufficient for meiotic resumption in the absence of cyclin B2, implying that cyclin B2 and B1-dependent Cdk1 activities might be additive for entry into M phase (Homer, 2013; Gui and Homer, 2013). Interestingly, also in *Xenopus* eggs equal amounts of Cdk1 bound to cyclin B1 and B2 have been purified (Gautier et al., 1990). Therefore, these observations propose a significant role for cyclin B2 in Cdk1 activation during meiotic resumption in mouse oocytes. Another known APC/C^{Cdh1} substrate, cyclin A2, has been also involved in G2/M transition in oocytes (Touati et al., 2012). Both cyclin A isoforms (A1 and A2) expressed in mammals can bind and activate Cdk1, however while knockout of cyclin A1 does not affect mouse development, its absence impairs male meiosis (Liu et al., 1998). In contrast, cyclin A2 is essential for survival (Murphy, 1997) and somatic cells lacking cyclin A2 have compromised G2/M transition (Fung et al., 2007; Gong et al., 2007). Recently reported study revealed that injection of anti-cyclin A antibody delays meiosis resumption in mouse oocyte indicating that cyclin A2 plays an important role in control of G2/M transition (Touati et al., 2012). Thus, cyclin A and cyclin B2 also appear to contribute in Cdk1 activation at GVBD of mouse oocytes.

5.3.3 The role of APC/C in dormant oocytes of primordial follicles

Considering the importance of APC/C's activity in controlling meiotic resumption in fully-grown oocytes I have further asked whether APC/C plays an essential role for

suppressing premature meiotic resumption also in dormant oocytes within primordial follicles. I analysed the consequences of the inactivation of *Apc2* allele within primordial follicles and observed the depletion of entire pool of primordial follicles within 5 weeks after birth that eventually resulted in premature ovarian failure (POF) in young adulthood (8 weeks). Since APC/C activity has never been examined before in dormant oocytes enclosed in primordial follicles, my results propose that APC/C is active in primordial follicles and plays a significant role in the survival of dormant primordial oocytes. Oocyte loss in *Apc2^{fl/fl};Gdf-9-iCre* ovaries follows the Cre activation kinetics, suggesting that oocytes are depleted very quickly after *Apc2* inactivation.

Interestingly, although all primordial follicles were depleted within 5 weeks of *Apc2*-deletion, a number of growing follicles are present in *Apc2^{fl/fl};Gdf-9-iCre* ovaries. By isolation and *in vitro* culture I showed that these oocytes do not complete meiosis I, a phenotype observed when APC/C activity is depleted during oocyte growth (McGuinness et al., 2009), therefore I excluded the possibility that these oocytes 'escaped' the Cre-mediated *Apc2* inactivation. Since Cre activation kinetics demonstrated that *Gdf-9* promoter is active exclusively in dormant oocytes within primordial follicles from postnatal day 3, this suggest the presence of a stage within long dormant state of oocytes enclosed in primordial follicles when oocyte survival is dependent on APC/C. Thus, my discovery proposes that APC/C might be required for defining the final number of primordial follicle pool that will serve as a reserve throughout reproductive life of a female.

5.3.4 Mechanism of primordial follicle loss

Further, I set out to understand the underlying mechanism of primordial oocyte loss caused by *Apc2* deletion. Since APC/C substrates are degraded by proteasome, I hypothesized that the loss of dormant oocytes upon APC/C inactivation might be mediated by upregulation of APC/C substrate(s). A list of potential substrates, proteins containing KEN- or D-box, which is recognized by APC/C is provided in Table 5.1.

Name	Function	Cell cycle phase
APC/C-Cdc20 and –Cdh1 substrates		
Cyclin A	S-phase and G2/M driver	Prometaphase
Cyclin B	G2/M driver	Early anaphase
Securin	Chromosome segregation inhibitor	Early anaphase
Deoxythymidylate kinase	dTTP production	
APC/C-Cdc20 substrates		
Cdc25 A	Cell cycle phosphatase	
p21Waf1	Cdk inhibitor	
Nek2A	Centrosome regulator	Prometaphase
Id-1	DNA binding protein inhibitor	Dendrite morphogenesis
APC/C–Cdh1 substrates		
Cdc20	APC/C co-activator	Late anaphase
Cdh1	APC/C co-activator	Late anaphase
Cdc6	DNA replication	
Thymidine kinase 1	dTTP production	
Geminin	DNA replication inhibitor	Late anaphase
FoxoM1	S-phase and M-phase progression transcription factor	
Tome-1	Mitotic entry	
Polo-like kinase 1	Mitotic kinase	Late anaphase
Aurora kinase A	Mitotic kinase	Late anaphase
Aurora kinase B	Mitotic kinase	Late anaphase
Kif22	Spindle regulator	
Ckap2	Spindle regulator	
Tpx2	Spindle regulator	
Rsc1	Chromosome segregation	
SgoL1	Chromosome cohesion	Late anaphase
Anillin	Cytokinesis	
SKp2	F-box in SCF complex	
UbcH10	Ubiquitin-conjugating enzyme E2	
Pfkfb3	Glycolytic enzyme	Metabolism and survival
Id-2	DNA-binding protein inhibitor	Axon growth + patterning
SnoN/SkiL	TGF- β signalling	Axon growth + patterning

Table 5.1 Substrates of mammalian APC/C.

As described before, APC/C activity in fully-grown oocytes has a negative effect on meiotic resumption by downregulating Cdk1 activity, mediated by cyclin B1 targeted degradation, but other cyclins or molecules might also be involved. It is unknown whether Cdk1 hyperactivation is also a reason for dormant oocytes loss. We conceived that since oocytes enclosed in primordial follicles are in a quiescent state, the APC/C's targets that are potentially upregulated in its absence might not be involved in cell cycle progression.

We employed double knockout strategy and simultaneously deleted APC/C and SnoN in dormant oocytes of primordial follicles. SnoN, a negative regulator of TGF- β superfamily signalling, inhibits activity of Smad signalling via direct binding to Smad2/3 in both cytoplasm and nucleus and by inhibiting their association with Smad4 prevents Smad2/3 from nuclear translocation and activation of downstream target genes (He et al., 2003; Luo, 2004; Stroschein et al., 1999). TGF- β stimulation induces rapid degradation of SnoN allowing the formation of transcriptionally active Smad complex, which then can activate the expression of TGF- β (Stroschein et al., 1999). Unfortunately, SnoN inactivation did not prevent primordial follicle loss caused by the depletion of APC/C activity, therefore it is likely that the loss of dormant oocytes by APC/C inactivation is not through upregulation of SnoN. A recent study provide additional evidence that the regulation of SnoN might not be essential for dormant oocyte survival since Smad4 depletion in dormant oocytes within primordial follicles only mildly reduces overall fertility of mice (Li et al., 2012).

Further, we employed the same strategy to simultaneously inactivate p53 and APC/C in primordial follicles. The peak in p53 expression during mouse embryogenesis correlates with germ cell formation which is associated with high rate of cellular death (Moallem and Hales, 1998), therefore we conceived that p53 is a likely candidate to be upregulated in the absence of APC/C activity in primordial follicles. Unfortunately, p53 inactivation did not prevent primordial follicle loss caused by the depletion of APC/C activity. A recent study proposed Tap63, another member of p53 family of proteins, to be the master regulator of apoptosis in primordial follicles during chemotherapy-induced loss of ovarian reserve (Kim et al., 2013).

5.3.5 Future directions

1) Primordial oocyte-specific targets of APC/C

Because APC/C is an ubiquitin ligase that targets specific substrates for degradation by proteasome it can be presumed that the upregulation of dormant oocyte-specific targets leads to their death. Identification of primordial oocyte-specific targets of APC/C poses a challenge for the future research. However, By comparing kinetics of Cre activation and primordial follicle loss in *Apc2* ovary I concluded that primordial follicles are eliminated very quickly from the ovary after APC/C inactivation. This poses a technical challenge when aiming at understanding the molecular mechanism leading to primordial follicle loss. Therefore, in the future an *ex vivo* experimental system should be developed in order to study primordial oocyte-specific targets of APC/C.

Chapter 6

General Discussion

6.1 Mechanisms maintaining the dormancy and survival of mammalian primordial follicles

The pool of dormant primordial follicles in the ovary is the source of all developing follicles and fertilizable eggs that will support female fertility throughout the reproductive life span. Primordial follicles are formed perinatally (depending on the species) and at birth they are destined to follow one of three fates: to remain in a non-growing quiescent state, to be recruited into the growing follicle pool or to undergo atresia directly from the dormant state. Since the unbalance of the above leads to accelerated loss of oocytes, clinically recognised as premature ovarian failure (POF), the understanding of the molecular mechanisms and molecules involved in the control of dormancy, survival and activation of primordial follicles has significant clinical applications.

In recent years the knowledge of regulatory systems governing dormancy, survival or activation of mammalian primordial follicles has been enhanced mainly through generation and characterization of genetically modified mouse models. A number of studies demonstrated that in addition to TGF- β superfamily, also PI3K signalling pathway is involved in controlling the fate of mammalian primordial follicles. As introduced in Chapter 1, the maintenance of a quiescent state of dormant oocytes is mediated by PTEN, Tsc1/2, Foxo3a, p27, Foxl2 and AMH. Deletion of some of them in mouse models leads to premature irreversible activation of primordial follicle pool and their consequent exhaustion within a few weeks. This group of suppressors of follicular activation is accompanied by molecules involved in the maintenance of primordial follicles survival. Inactivation of Pdk1 or rpS6 in primordial follicles leads to loss of primordial follicles directly from the dormant state resulting in accelerated ovarian ageing and POF (Reddy et al. 2008; Reddy et al., 2009a, 2009b; Castrillon et al., 2003). Altogether these factors in a synergistic and coordinated way maintain primordial follicles in a dormant state.

In my study I have observed that upon APC/C inactivation in dormant oocytes, all primordial follicles are lost within five weeks, while complete follicular depletion takes place within eight weeks. Therefore, here for the first time I show that APC/C activity is indispensable for maintaining survival of primordial follicles, which in turn is crucial for determining the duration of female fertility. Because of the similarity to ovarian phenotypes observed upon inactivation of Pdk1 and rpS6 I propose that APC/C is a new primordial follicles survival 'maintainer' (Fig. 6.1).

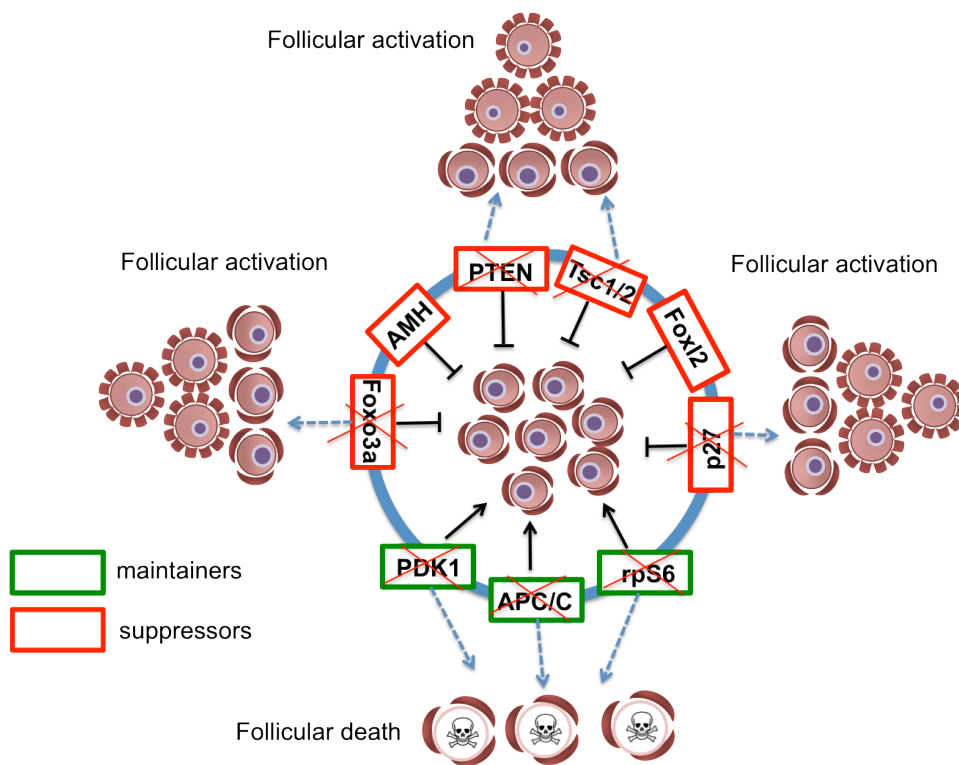


Figure 6.1 APC/C as a new factor contributing to maintenance of primordial follicles survival.

Inactivation (marked with an X) of 'supressors' (highlighted in red frames) such as PTEN, Tsc1, Tsc2, Foxo3a or p27 (marked with an X) leads to all primordial follicles being prematurely activated, suggesting that the activity of these molecules is necessary to maintain the quiescence of primordial follicles. In the absence of 'maintainers' (highlighted in green frames), molecules such as Pdk1, rpS6 or proposed APC/C, primordial follicles are prematurely lost directly from their dormant state (Adapted and modified from Reddy et al., 2009b).

6.2 Roles of *Nipbl* in mouse oocytes

Aneuploidy constitutes a major health problem because it is a direct cause of genetic abnormalities in embryos, spontaneous abortions and congenital diseases such as Down's syndrome. Over 90% of aneuploidy arises in oocytes and the errors frequency is positively correlated with maternal age in humans (Hassold and Hunt, 2001). A number of studies provided the direct evidence that achiasmatic chromosomes that have lost cohesion precociously affect the accurate segregation of homologous chromosomes during meiosis I and sister chromatids during meiosis II (Hodges et al., 2005; Liu and Keefe, 2008). In addition, chromosome-bound cohesin decay has been proposed to be a predominant mechanism of human age-related aneuploidy (Chiang et al., 2010; Lister et al., 2010). This hypothesis assumes insufficient or lack of turnover of proteins in the cohesin complex after cohesion is established during S phase in the foetal ovary. Indeed, even though meiosis-specific cohesins are transcribed during oocyte growth in the adult ovary, there is no indication that functional cohesin complex is produced (Revenkova et al., 2010a; Revenkova et al., 2010b). Further, despite the lack of transcription of *Smc1 β* in growing oocytes, chromosome segregation occurs normally, suggesting that cohesin initially loaded during S phase is sufficient for accurate chromosome segregation (Revenkova et al., 2010a). Also, TEV-induced destruction of cohesion mediated by TEV-cleavable Rec8 in mature GV oocytes was not prevented by ectopic expression of wild-type Rec8 transgene during oocyte growth (Tachibana-Konwalski et al., 2010). Thus, together these studies propose that the cohesion established during foetal development is necessary and sufficient, suggesting very stable nature of the cohesin complex.

In my study I showed that despite efficient depletion of functional transcripts of cohesin loading factor *Nipbl* in mouse oocytes, these oocytes successfully underwent meiotic maturation and their chromosomes maintained chiasmata and cohesion. Even more, eggs whose *Nipbl* has been inactivated at primordial stage were fertile with successful *de novo* loading of mitotic cohesin upon fertilization. *Nipbl* protein accumulated before gene inactivation was sufficient to support cohesin loading during egg development into blastocysts upon parthenogenetic activation. Although these blastocysts contained a high fraction of mitotic cells with misaligned chromosomes, these data suggest that *Nipbl* is very stable in oocytes. This is in agreement with observation that *Nipbl*-deleted embryonic fibroblasts could survive

up to 2 weeks with slow depletion of Nipbl protein until their death, which was associated with abnormal nuclear morphologies likely due to precocious loss of sister chromatid cohesion. Thus, the study presented in this thesis proposes for the first time a stable nature of cohesin loading factor Nipbl in mammalian oocytes, which has been recently proposed also for the cohesin complex. Together, these observations suggest the presence of a subset of proteins in oocytes that are stable enough to last the entire reproductive lifespan of females.

However, the stable nature of these proteins constitutes a challenge when studying the consequences of Nipbl functional loss. Therefore, to study the effects of Nipbl deficiency approaches that directly inactivate proteins should be applied. For example, Tachibana-Konwalski and co-workers engineered TEV-cleavable Rec8 proteins that allow for direct inactivation of Rec8 protein upon injection with TEV protease. Alternately, auxin-inducible degron system discovered in plants has been recently shown to allow rapid degradation of target proteins in many non—plant species, including mammalian cells (Nishimura et al., 2009; Tachibana-Konwalski et al., 2010)

Nipped-B, a fruit fly homologue of Nipbl was isolated as a gene involved in regulation of long-range activation of the *cut* and *ubx* genes encoding homeobox proteins. Haploinsufficiency of human *NIPBL* causes a developmental multi-organ disease called Cornelia de Lange syndrome (Wallace and Felsenfeld, 2007). These observations proposed that Nipbl homologues are involved in gene expression through their function in cohesin loading. Cohesin complexes co-localise with CTCF, a regulator of enhancer-promoter interactions, and have been shown to regulate transcription through specific organisation of chromatin loops in somatic cells (Parelho et al., 2008; Stedman et al., 2008; Wendt et al., 2008). Currently it is unknown what genes are regulated by Nipbl. The analysis of global gene expression profile of Nipbl-depleted oocytes at fully-grown GV stage in comparison to control oocytes would provide insight into developmental gene regulation and molecular basis for Cornelia de Lange Syndrome.

Appendix I

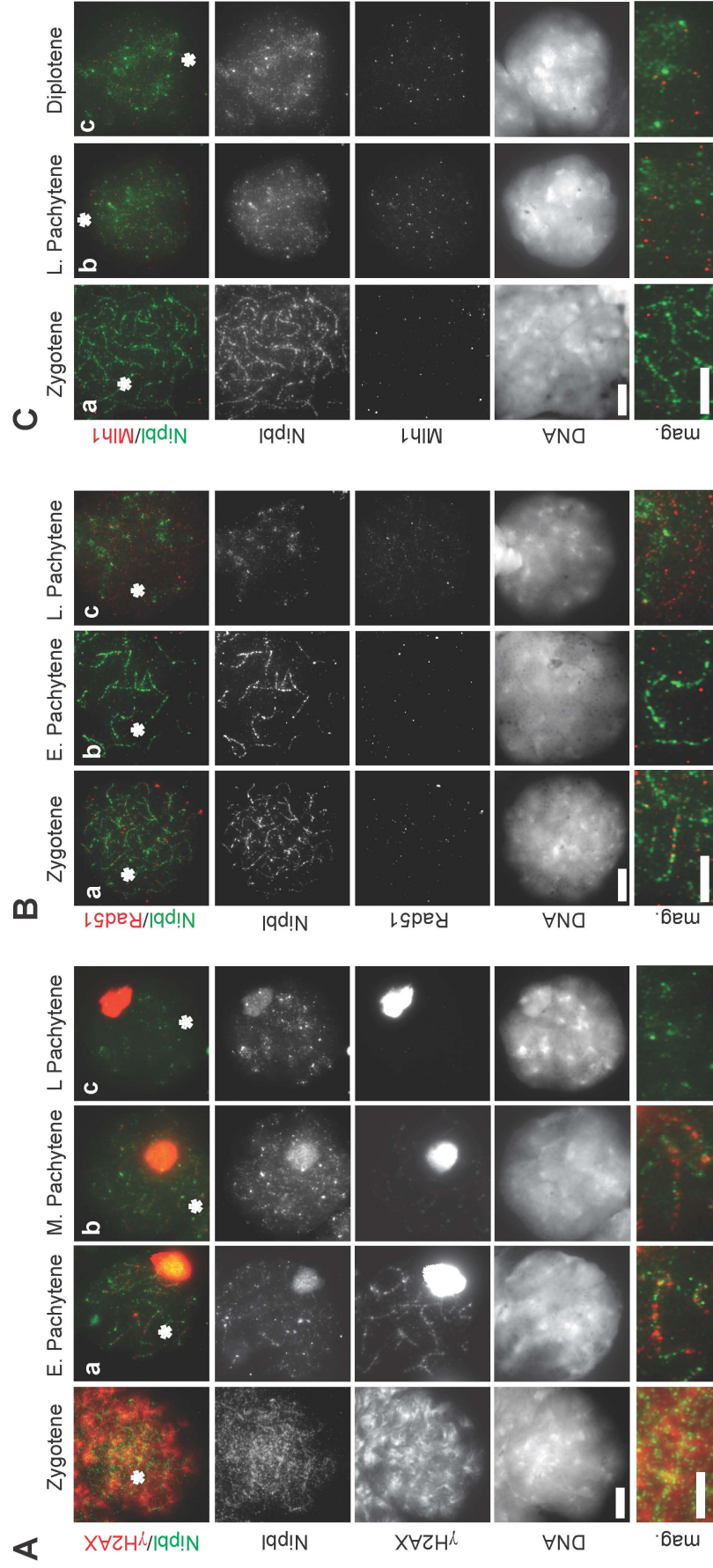


Figure 1 - 1 Temporal correlation between the re-localisation of Nipbl B and other meiotic prophase events during spermatogenesis.

Chromosomal spreads from testicular cells were immunofluorescently labelled with rat monoclonal antibody against Nipbl B (KT55) and marker antibodies: (A) rabbit polyclonal anti- γ -H2AX antibody marking double strand breaks (DSBs). Note that anti- γ -H2AX highly accumulates at the sex body during the stages shown. a: Zygote, b: Early Pachytene, c: Mid-Pachytene, d: Late Pachytene (B) mouse monoclonal anti-Rad51 antibody marking the early recombination intermediates. a: Zygote, b: Early Pachytene, c: Late Pachytene (C) mouse monoclonal anti-Mlh1 antibody marking the appearance of recombination nodules. a: Zygote, b: Early Pachytene, c: Diplotene. DNA was counterstained with DAPI and merged images are shown (γ -H2AX, Rad51 and Mlh1 are in red and Nipbl is in green). In colour merge images co-localisation of red and green signals appear yellow. The regions indicated by asterisks in a, b, c are magnified (bottom); E., early; M., mid; L., late. Scale bar 5 μ m.

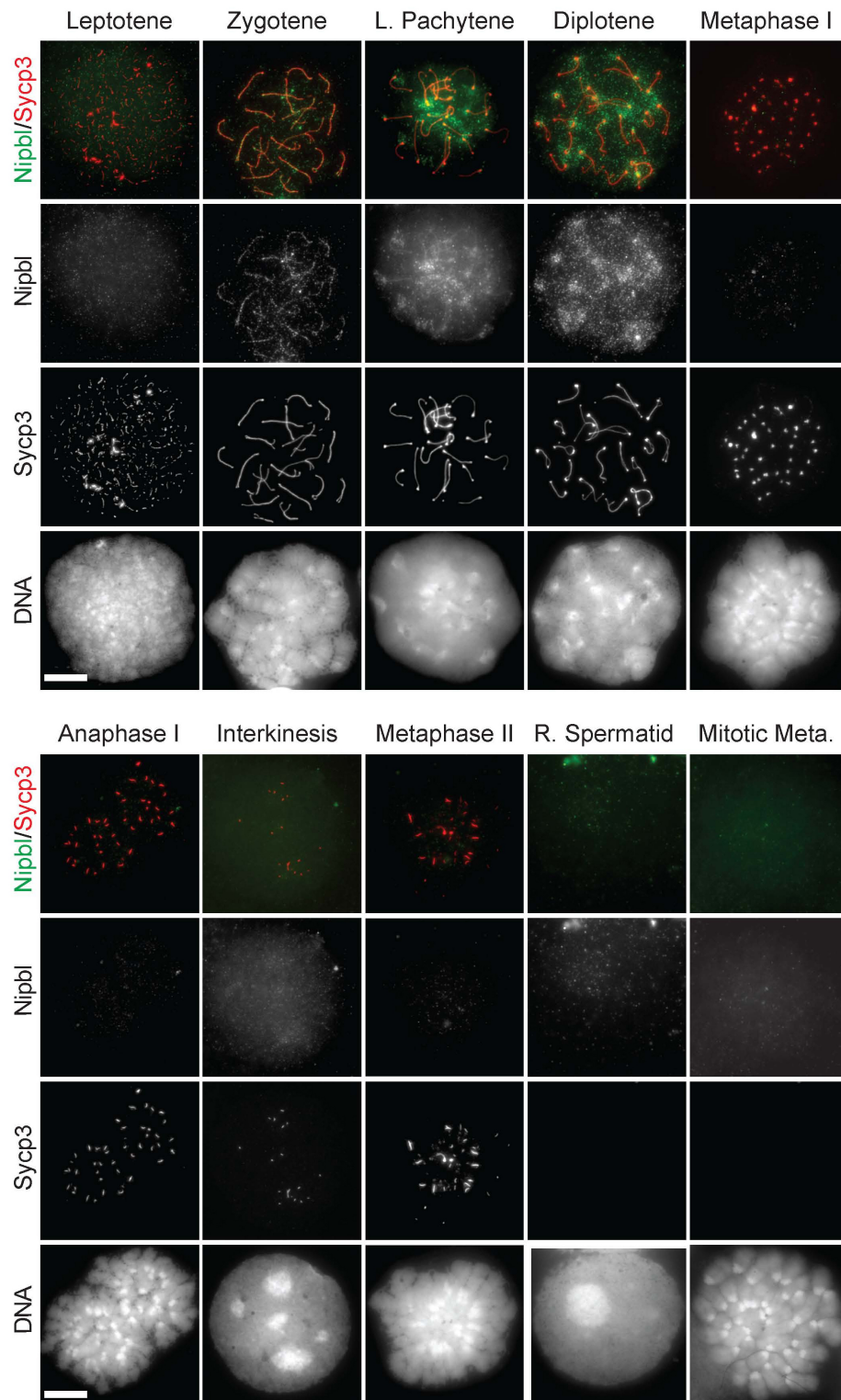


Figure 1 - 2 Immunofluorescence images of spermatocyte chromosomes stained with anti-Nipbl KT55 and anti-Sycp3 antibodies.

Chromosome spreads from testicular cells were subjected to immunofluorescent staining with rat monoclonal antibody against Nipbl B (KT55) and anti-Sycp3 mouse monoclonal antibody. Sycp3 marks the AE/LE of synaptonemal complex. DNA was counterstained with DAPI and merged images are shown. L., late; R., round. Meta., Metaphase. Scale bar 5 μ m.

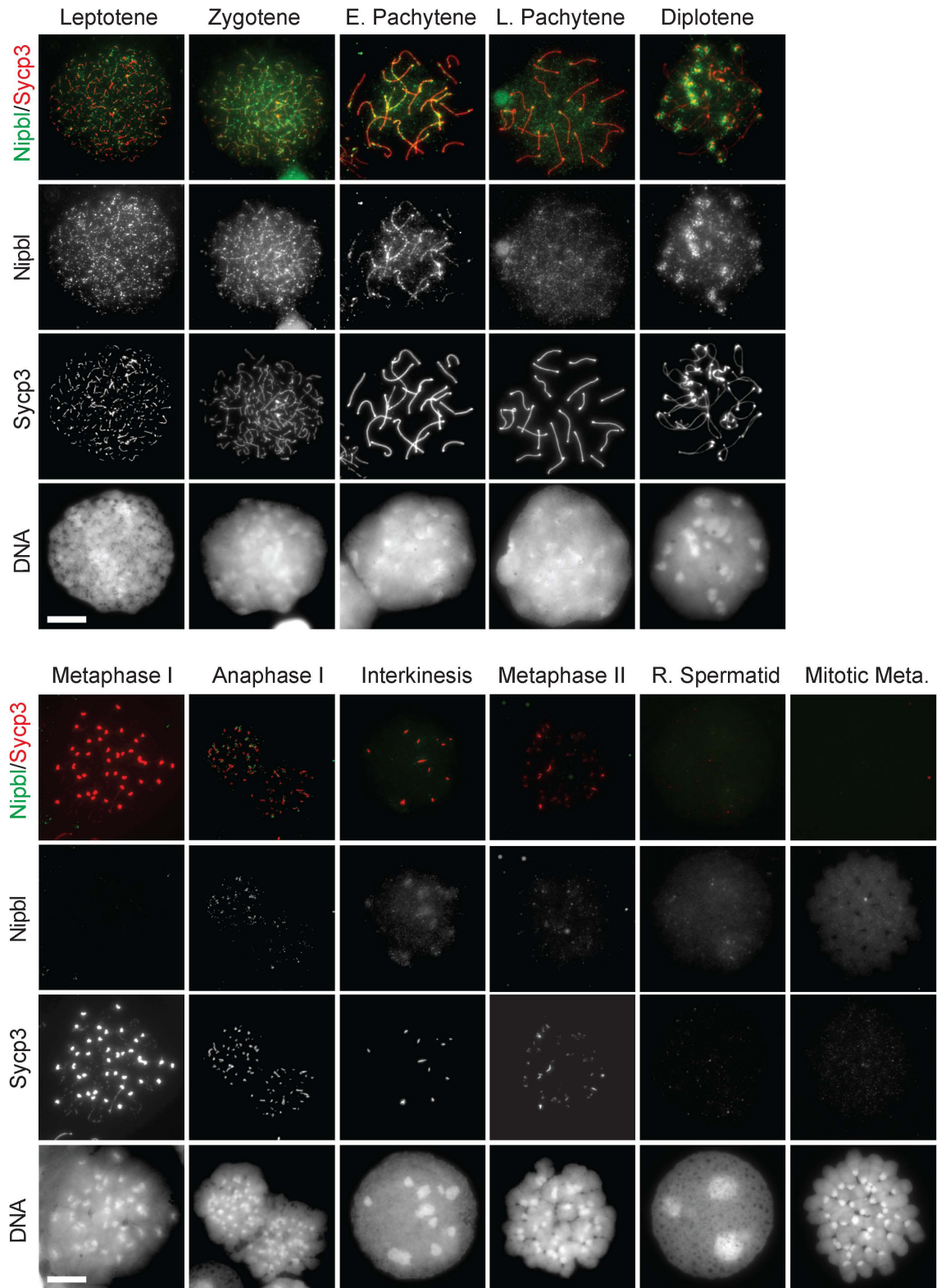


Figure I – 3 Immunofluorescence images of spermatocyte chromosomes stained with anti-Nipbl KT54 and anti-Sycp3 antibodies.

Chromosome spreads from testicular cells were subjected to immunofluorescent staining with rat monoclonal antibody against Nipbl A (KT54) and anti-Sycp3 mouse monoclonal antibody. Sycp3 marks the AE/LE of synaptonemal complex. DNA was counterstained with DAPI and merged images are shown. E., early L., late; R., round. Meta., Metaphase. Scale bar 5 μ m.

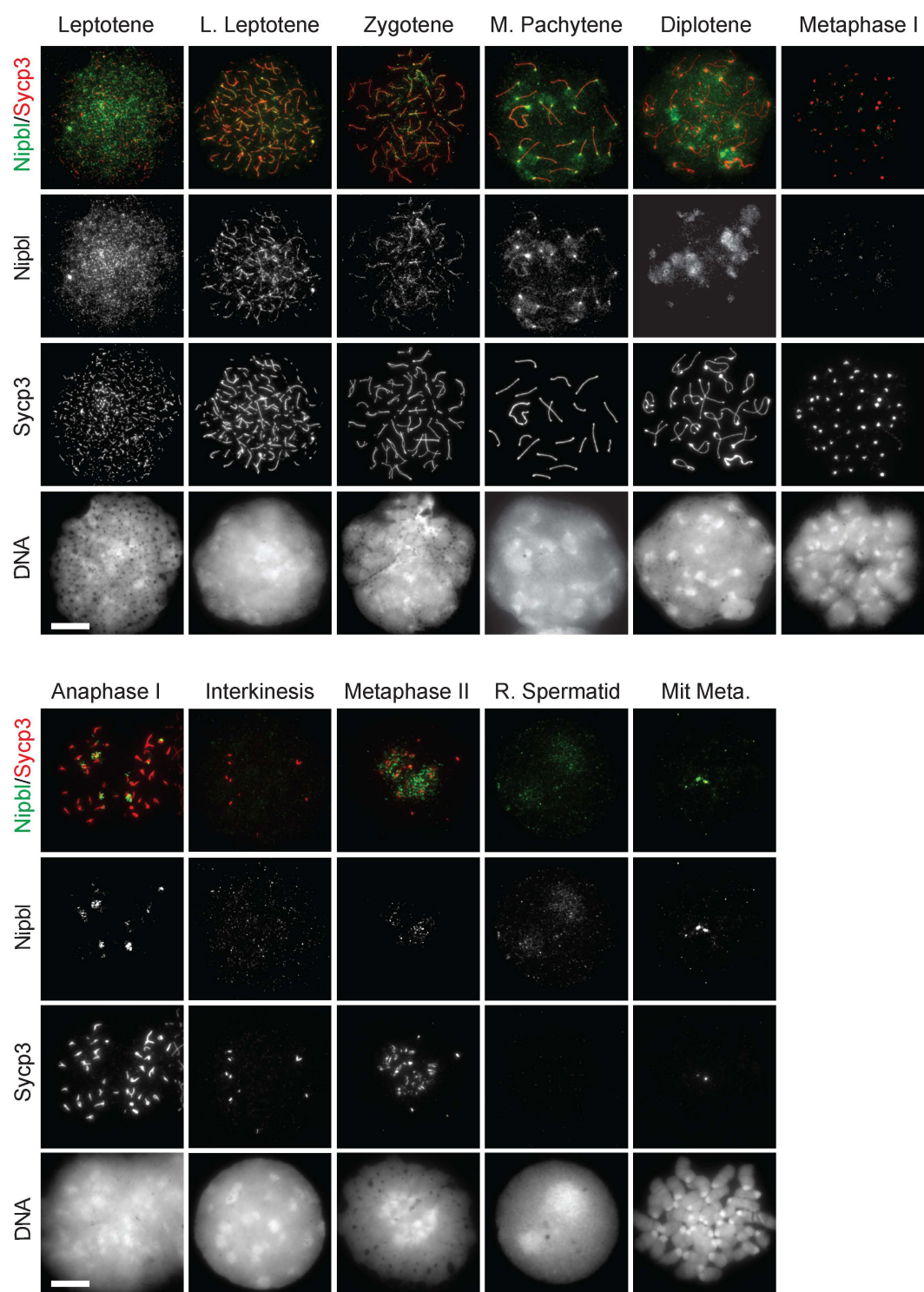


Figure I - 4 Immunofluorescence images of spermatocyte chromosomes stained with anti-Nipbl 114 and anti-Sycp3 antibodies.

Chromosome spreads from testicular cells were prepared and subjected to immunofluorescent staining with rabbit polyclonal antibody against both isoforms of Nipbl (114) and mouse monoclonal antibody against Sycp3. Sycp3 marks the AE/LE of synaptonemal complex. DNA was counterstained with DAPI and merged images are shown (Sycp3 is in red and Nipbl is in green). L., late., M., mid, Mit. Meta., mitotic metaphase. Scale bar 5 μ m

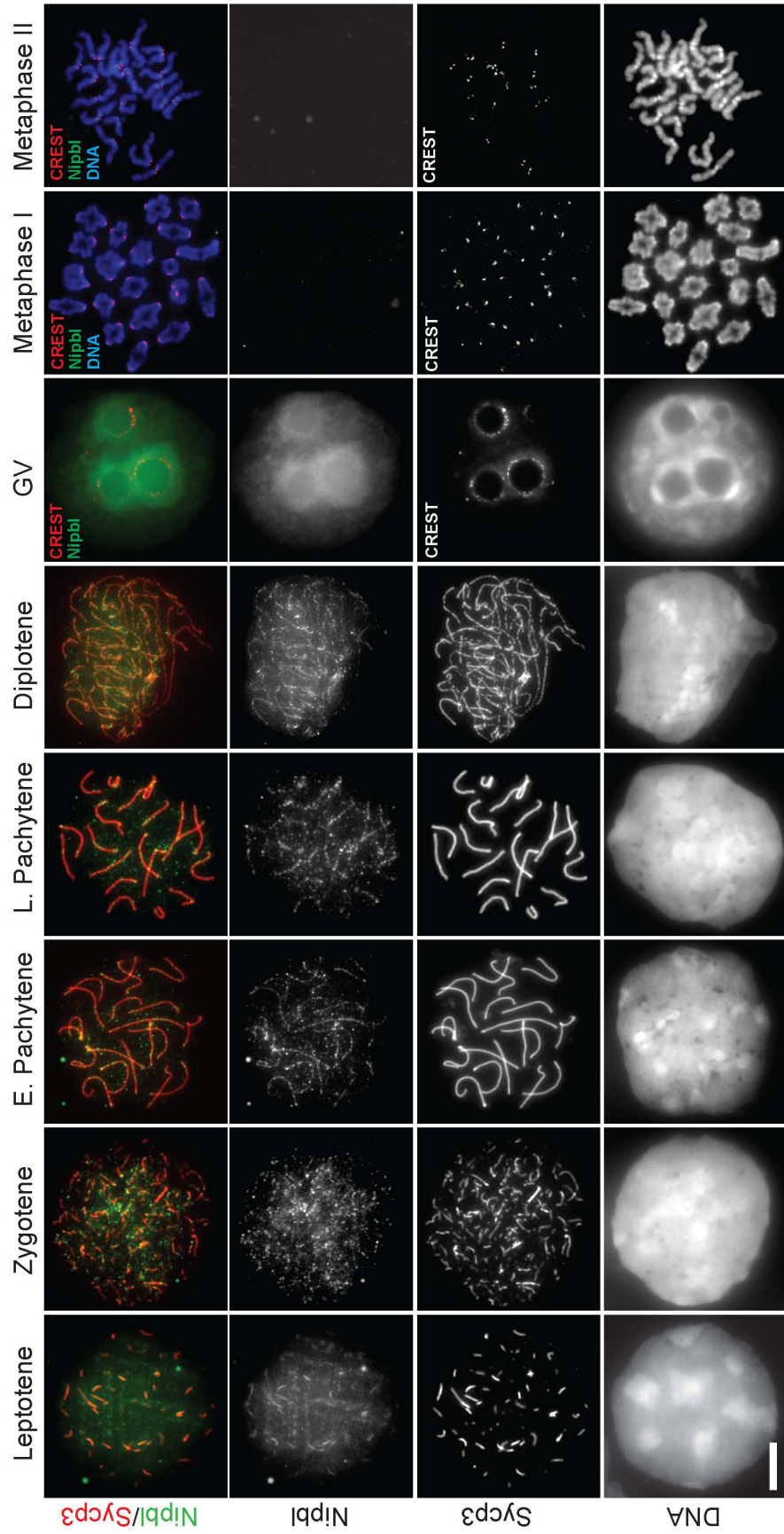


Figure 1 - 5 immunofluorescence images of oocytes chromosomes stained with anti-Nipbl KT55 and anti-Sycp3 antibodies.

Chromosomal spread assay during meiotic prophase stages in E15.5 wild-type fetal ovaries. Spreads were immunofluorescently labelled with rat monoclonal antibody against Nipbl B (KT55) and anti-Sycp3 mouse monoclonal antibody. Sycp3 marks the AE/LE of synaptonemal complex. CREST serum marks kinetochores. DNA was counterstained with DAPI and merged images are shown (Sycp3 is in red and Nipbl is in green). In colour merge images co-localisation of red and green signals appear yellow. E., early; L., late. Scale bar 5µm.

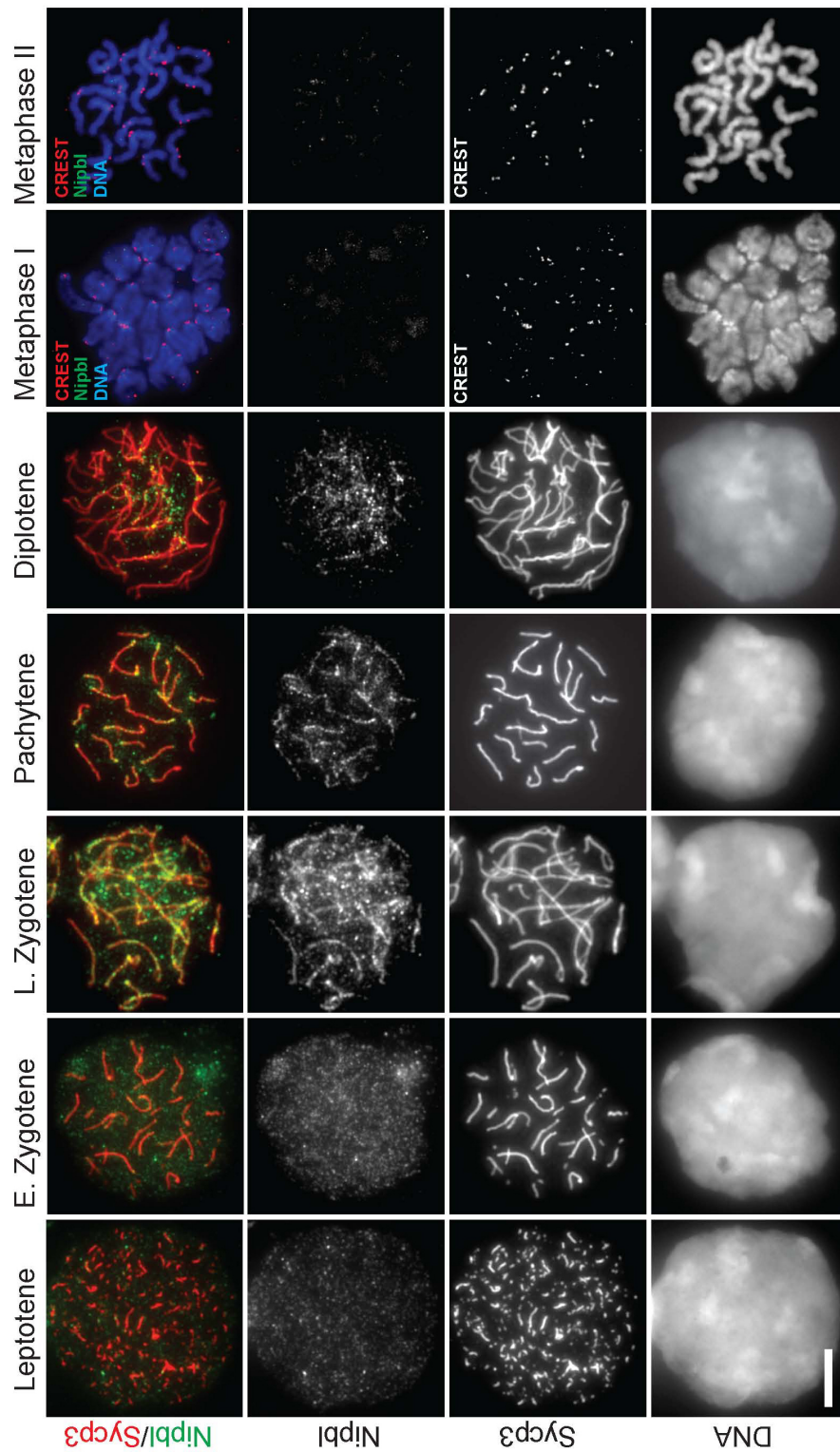


Figure 1 - 6 Immunofluorescence images of oocytes chromosomes stained with anti-Nipbl (114) and anti-Sycp3 antibodies.

Chromosomal spread assay during meiotic prophase stages in E15.5 wild-type fetal ovaries. Spreads were immunofluorescently labelled with rat monoclonal antibody against both Nipbl isoforms (114) and anti-Sycp3 mouse monoclonal antibody. Sycp3 marks the AE/LE of synaptonemal complex. CREST serum marks kinetochores. DNA was counterstained with DAPI and merged images are shown (Sycp3 in red and Nipbl in green). In colour merge images co-localisation of red and green signals appear yellow. E., early; L., late. GV, germinal vesicle. Scale bar 5µm.

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