Epigenetic regulation of germ cell and early embryonic development by Polycomb group proteins

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Abbreviations

5meC	5-methyl cytosine
Arg, R	arginine
BrdU	5-bromo-2-deoxyuridine
BrUTP	5-bromouridine-5-triphosphate
cAMP	3',5'-cyclic adenosine monophosphate
Cdk	cyclin dependent kinase
ChIP	chromatin immuno-precipitation
CPE	cytoplasmic polyadenylation element
CPEB	cytoplasmic polyadenylation element binding protein
Cys	cystein
DAPI	4,6-diamidino-2-phenylindole
dm	double mutant
Dnmt	DNA methyl transferase
Dpc	day post coitum
DSB	double-stranded break
dsRNA	double-stranded RNA
DUB	de-ubiquitintating emzyme
E	embryonic day
ES	embryonic stem
GO	gene ontology
GV	germinal vesicle oocyte
GVBD	Germinal vesicle breakdown
H2AK119ub1	histone H2A lysine 119 mono-ubiquitination
H3K27me3	histone H3 lysine 27 tri-methylation
HAT	histone acetyl transferase
HCP	high-CpG content
HDAC	histone deacetylase
HMTase	histone methyl transferase
Hox	homeobox
HP1	heterochromatin protein 1
Hpf	hours post fertilization
ICM	inner cell mass
ICR	imprinting control region
IF	immunofluorescent
IVF	*in vitro* fertilization
KMTase	lysine methyltransferase
KO	knockout
LCP	low CpG-content
Lys, K	lysine
mat	maternal
ME	meridional division followed by equatorial division
me1/me2/me3	mono-/di-/tri-methylation
MEF	mouse embryonic fibroblast
MET/MZT	maternal to embryonic transition; maternal to zygotic transition
M-I/M-II	metaphase-I/metaphase-II oocyte
miRNA	micro RNA

MSCI	Meiotic Sex Chromosome Inactivation
MSUC	Meiotic Silencing of Unsynapsed Chromatin
MZT	maternal to zygotic transition
ncRNA	non-coding RNA
NPC	neural progenitor cell
NSN	non-surrounding nucleolus oocyte
PB	polar body
P-body	Processing body
PcG	Polycomb group
PCH	pericentric heterochromatin
PCR	polymerase chain reaction
PN	pronucleus
PRC	Polycomb repressive complex
PRE	Polycomb response element
pre-RC	pre-replication complex
pri-miRNA	primary microRNA
Prm	protamine
PRMTase	arginine methyltransferases
PTM	post-translational modification
Rb	retinoblastoma
RC	replication coupled
RI	replication independent
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNAP	RNA polymerase
RT-PCR	reverse transcriptase PCR
SC	synaptonemal complex
Ser, S	serine
SET	Suppressor of variegation, Enhancer of Zeste, Trithorax
SN	surrounding nucleolus oocyte
TC	transcription coupled
TCR	transcription requiring complex
TE	trophectoderm
TG	trophoblast giant
Thr	threonine
TP	transition protein
TrxG	Trithorax group
TS	trophoblast stem
TSA	trichostatin A
TSS	transcription start site
XIC	X-inactivation center
Xist	X-inactivation specific transcript
ZGA	zygotic genome activation
ZP	zona pellucid
γH2AX	histone variant H2AX phosphorylated at serine residue 139

1. Summary

Mammalian development begins at fertilization, when two highly specialized gametes, sperm and oocyte fuse to form a zygote. It is well established that early development of the embryo is driven by factors that are inherited from the oocyte. A complex maternal program, consisting of mRNAs, proteins and chromatin modifications is prepared in the oocyte that bears the intimate potential to execute events that will ultimately lead to the formation of a totipotent embryo. The components of this maternal program and the players involved in establishing it are poorly characterized.

Polycomb group proteins (PcG) are evolutionarily conserved chromatin–modifying factors that maintain cellular identity during many rounds of cell division by transcriptionally repressing developmental regulator genes that are inappropriate for the given cell lineage. Lack of Polycomb function has been shown to result in de-repression of differentiation specific factors in embryonic stem (ES) cells and interfere with proper differentiation. Similarly *in vivo*, embryonic deficiency for some core PcG members in the mouse results in embryonic lethality around gastrulation. PcG proteins are known to function in at least two major complexes, termed Polycomb Repressive Complex 1 (PRC1) and 2 (PRC2). Silencing is thought to be in part a consequence of the ubiquitin ligase activity of the PRC1 complex towards histone H2A, inhibiting productive transcriptional elongation of genes. Previous data from our laboratory indicated that PcG proteins were present in the oocyte and transmitted to the embryo.

The aim of this PhD project is to address the role of the Polycomb silencing pathway, focusing on the PRC1 complex, during oogenesis and early embryonic development.

We show that embryos lacking the maternal contribution of Rnf2 (Ring1b) -the main catalytic subunit of PRC1- and its paralog Rnf1 (Ring1) fail to develop beyond the 2-cell stage. Expression profiling of fully grown oocytes revealed de-repression of numerous developmental regulator genes, most of which are established Polycomb targets in other cellular systems. We show however, that these differentiation-specific transcripts are only translated after fertilization, resulting in the inappropriate presence of differentiation factors during the otherwise totipotent stage of early embryogenesis. Additionally, maternal pro-nuclear transfer experiments between control and maternal *Rnf1/Rnf2* double mutant zygotes revealed that the developmental block is not only due to inappropriate cytoplasmic factors, but also to a defective chromatin setup inherited from the PRC1 deficient oocyte.

Our findings demonstrate that PRC1-mediated silencing during oocyte growth is an essential component in the preparation of the maternal to embryonic transition program required for proper initiation of embryonic development.

2. Introduction

At the onset of mammalian development a totipotent embryo is formed by the union of sperm and oocyte. Development of an organism involves carefully regulated differentiation processes during which the totipotent cells of the early embryo give rise to many distinct cell types of the adult body. During this phase, developmental potential of the differentiating cells is gradually decreasing and specificity towards a single terminal fate is acquired. The character of a differentiated cell is defined by its constituent proteins, which are the result of specific patterns of gene expression. Specific gene expression programs on one hand need to be stable enough to maintain cell identity but also need to be flexible to allow changes if change in developmental potential is required, for example during differentiation of embryonic cells. This balance of stability versus plasticity presents an inherent regulatory challenge for developing organisms.

Cell type specific gene expression programs need to be executed on the same genomic DNA template. Crucial determinants of gene expression patterns are DNA-binding transcription factors that choose genes for transcriptional activation or repression by recognizing the sequence of DNA bases in their promoter regions. However if transcription factors alone were responsible for the regulation of gene expression then the gene expression pattern of a differentiated nucleus would be completely reversible upon exposure to a different set of factors. As reproductive cloning of animals by transferring somatic nuclei to an enucleated oocyte is successful only with low efficiency, the “transcription factor only” model seems to be incorrect and suggests that other components like chromatin structure plays an important role in regulating gene expression.

Over the past few decades the rapidly evolving field of epigenetics explored the molecular mechanisms which shape or modify chromatin structure and thereby present an opportunity for regulating DNA-templated events such as transcription, replication and repair which then contribute to the maintenance or the establishment of new cell type specific gene expression programs.

2.1. Epigenetics and heritability

In 1942 a developmental biologist C.H. Waddington, who was interested in how gene expression patterns are modified during development, coined the word epigenetic which he described as “the causal interaction of genes and their products, which bring the phenotype into being” (Waddington, 1942).

To date a more narrow definition has become more widely accepted among molecular biologists, where epigenetics refers to mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the DNA sequence. Of note, there is still considerable disagreement about what falls under this definition. Strictly, to be epigenetic, a phenomenon has to be inherited through rounds of

cell division without relying on the initial trigger (Ptashne, 2007). Due to the inherent difficulty to distinguish between sequence-independent self-propagation of epigenetic states and a re-establishment after cell division mediated by sequence-dependent recruitment of enzymatic activities, most chromatin modifications are termed epigenetic without the knowledge of the molecular mechanisms that mediate propagation (Bird, 2007).
Many examples of mitotic heritability of epigenetic information have been documented where epigenetic marks are passed on to daughter cells to "remember" active or repressive transcriptional states. Recent years have even yielded the first clues about the molecular mechanism involved in propagation of certain epigenetic modifications (Margueron *et al.*, 2009).

Much less is known about meiotic or essentially transgenerational heritability of epigenetic information. A certain epigenetic state is inherited by the embryo from the maternal oocyte and the paternal spermatozoon which then after fertilization is in part reset to suit the needs of embryonic development. This change of epigenetic marks correlates with the establishment of totipotency in the early embryo and is essential for allowing new gene expression programs to be established that will drive cellular differentiation. However, evidence mostly based on DNA methylation studies, that epigenetic information is not completely erased and can be inherited from parent to offspring comes from several observations (Ashe and Whitelaw, 2007; Blewitt *et al.*, 2004; Chandler and Stam, 2004; Chong and Whitelaw, 2004).

One of the best understood examples of trans-generational inheritance of epigenetic information is the case of genomic imprinting. Genomic imprinting is a phenomenon in mammals where a gene is expressed only from one allele, either coming from the mother of from the father (Reik *et al.*, 1987). This differential expression is dependent on DNA methylation at imprinting control regions (ICRs), which are located within or outside the differentially expressed loci. Depending on their methylation status, ICRs either enhance or repress expression of neigbouring genes. Sex specific DNA methylation patterns are established on ICRs during male and female germ cell development and are brought to the embryo by the gametes. There are so far around 80 genes identified that undergo genomic imprinting. Most ICRs are methylated at the maternal allele and only three on the paternal one (Feil, 2009). Proper DNA methylation of ICRs in sperm and oocyte are necessary for the successful development of the embryo. Oocytes lacking maternal imprints give raise to embryos that die *in utero*. Males with impaired DNA methylation, including on paternal ICRs, are infertile, thus its effect on the embryo cannot be determined (Bourc'his *et al.*, 2001; Kaneda *et al.*, 2004). Nonetheless, embryos carrying two maternal genomes (parthenogenetic or gynogenetic) die *in utero*, showing that the paternal genome is necessary for the development (Surani and Barton, 1983). Further, bi-maternal embryos constructed by combining a haploid genome of a fully grown oocyte, which contains maternal imprints, and a haploid genome of an 'imprint free' non-growing oocyte in which two out of three paternally methylated ICRs were deleted (mimicking paternal repression of the ICR), developed into viable and fertile females (Kawahara *et al.*, 2007). These results indicate that

correct maternal imprints and at least two out of three correct paternal imprints are crucial for normal development.

Trans-generational epigenetic inheritance has also been demonstrated using the agouti viable yellow (A^{vy}) mouse model (Bultman *et al.*, 1992; Morgan *et al.*, 1999). The *Agouti (A)* locus is responsible for the production of yellow hair pigment. In wild type animals, the pigment is produced only during a short period of the hair growth resulting in a light brown (agouti) coat color (Bultman *et al.*, 1992). In the A^{vy} line, an IAP retrotransposon has integrated upstream of the agouti promoter and the LTR of the IAP element took over the control of A^{vy} expression. The IAP element can be silenced by DNA methylation, allowing normal expression of agouti locus under the endogenous promoter, therefore resulting in agouti fur color or it can be un-methylated, resulting in a constitutively active yellow gene and therefore a yellow coat color. A^{vy} mice display a wide range of coat colors, depending on the DNA methylation state of the IAP element inherited from the mother which is thought to be incompletely cleared in the embryo (Morgan *et al.*, 1999). However, this modification was shown to be entirely erased from the A^{vy} locus immediately post fertilization (Blewitt *et al.*, 2006). Recently, a number of epigenetic factors have been identified that influence the expression of A^{vy} and show trans-generational effects, supporting novel mechanisms other than DNA methylation to be involved in transmission across early embryonic development (Blewitt *et al.*, 2006; Chong *et al.*, 2007).

Further examples of maternal and even paternal transgenerational inheritance of epigenetic information is reviewed in Chong and Whitelaw, 2004 (Chong and Whitelaw, 2004).

Taken together, concrete functional evidence for the transgenerational inheritance of epigenetic information is very limited and apart from imprinting, is mostly dependent on observations of transgenes or genes under the control of inserted retrotransposons. Moreover, majority of these phenomena are related to the inheritance of DNA methylation. Importantly, we and others have shown that histones along with post translational modifications are passed through the female (Puschendorf *et al.*, 2008) and even the male germline (Brykczynska, in press*.;* Hammoud *et al.*, 2009) to the embryo, thus are attractive candidates for transgenerational epigenetic inheritance. (Discussed later, in chapters 2.7.1.4. and 2.7.3.1.)

2.2. Epigenetics and plasticity

The original view of epigenetic modification stability, even of the “most stable” modifications, such as histone methylation and DNA methylation has been changed over the past decades. Since the groundbreaking discovery of the first histone demethylase (Shi *et al.*, 2004) numerous others have been identified (Agger *et al.*, 2008). Although still the relatively most stable epigenetic modification known, DNA

methylation is also known to be removed at certain developmental stages and recently the elongator complex and enzymes involved in DNA repair processes have been proposed to be involved in this process (Bhutani *et al.*, 2010; Okada *et al.*, 2010; Popp *et al.*, 2010).

During mammalian development two waves of epigenetic (re-)programming are known to take place. One right after fertilization where some of the inherited gametic epigenetic marks are reset in order to execute the embryonic transcription program and the other during primordial germ cell development were even DNA methylation from imprints is erased followed by a sex specific re-establishment. Both of these epigenetic re-programming events are known to be essential for proper development (Dean *et al.*, 2003; Reik *et al.*, 2001).

Dynamics of epigenetic marks has also been demonstrated by studies examining genome-wide changes of different modifications during cellular differentiation (Mikkelsen *et al.*, 2007; Mohn *et al.*, 2008). The current view is that epigenetic mechanisms can confer robustness to steady state gene expression and thereby stabilize cell identity; however, upon external stimuli that induce differentiation, they can impact on changes in gene expression patterns and thereby direct further developmental potential.

2.3. Chromatin

Chromatin is the basic organizational form of DNA in the eukaryotic nucleus. The repeat unit of chromatin is the core nucleosome in which 147 base pairs of DNA are wrapped around the histone octamer that consists of two molecules each of the core histones H2A, H2B, H3 and H4 (Kornberg, 1974). Between these nucleosome cores is a variable length of DNA that is bound by the linker histone H1. The interaction between histone H1 molecules helps mediating the assembly of the nucleosomal array into a chromatin fiber, which then is further folded into a higher order structure. Eukaryotic genomes are ubiquitously packaged into chromatin; however, cells need to spatially and temporally regulate specific loci independently of bulk chromatin (O'Malley *et al.*, 1977; Weisbrod, 1982). In order to achieve the high level of control required to co-ordinate nuclear processes such as DNA replication, repair and transcription, cells have developed a variety of means to locally and specifically modulate chromatin structure defining accessibility of transcription factors and RNA polymerase to promoter elements (Li *et al.*, 2007). While nucleosomes have long been viewed as stable entities, today considerable evidence exists supporting their dynamic nature, capable of being altered in their composition and structure. This can involve the incorporation of histone variants, covalent modification of histones and non-covalent remodeling by ATP-dependent remodeling enzymes.

Whilst histones are remarkably conserved throughout evolution, several variant forms have been identified that are characterized by specific expression and localization patterns, allowing specialized

regulation of chromatin dynamics at certain loci compared to the bulk of chromatin that is occupied by canonical histones (Talbert and Henikoff, 2010).

Since they were discovered in the mid 1960's we know of over 60 residues on histones that are subject to post-translational modifications (PTMs), including acetylation and methylation of lysines (Lys) and arginines (Arg), phosphorylation of serines (Ser) and threonines (Thr), ubiquitination and sumoylation of lysines, as well as ribosylation (Kouzarides, 2007). Some modifications have been shown to be correlated with gene silencing; others seem to be correlated with gene expression.

At first, these chromatin modifications and the overall chromatin structure were thought to be stable and simply providing a structural scaffold. However, the discoveries that the yeast transcriptional co-activator Gcn5 bears histone acetylation activity (Brownell *et al.*, 1996), while the co-repressor Rpd3 mediates histone deacetlyation (Taunton *et al.*, 1996) for the first time directly linked transcriptional regulation to PTMs. A few years later Suv39h, a previously identified transcriptional regulator and suppressor of variegation in Drosophila (Tschiersch *et al.*, 1994) and its yeast homolog Clr4 were shown to specifically methylate lysine 9 at histone H3 (H3K9) (Rea *et al.*, 2000). This subsequently led to a change of paradigms towards a regulatory role of chromatin and PTMs of histones in DNA templated processes. Ultimately, the discovery of histone demethylases indicated that PTMs are much more dynamic than previously anticipated (Shi *et al.*, 2004).

The fact that most of the early PTMs found were concentrated within the tail extensions that protrude from the nucleosome core lead to two main theories regarding the regulatory mechanism of histone modifications. One theory suggests that they may affect electrostatic interactions between the histone tails and DNA to "loosen" chromatin structure. Later it was proposed that combinations of these modifications may create binding epitopes which recruit other proteins, leading to the "histone code" hypothesis (Strahl and Allis, 2000).

Although many of these PTMs are considered to be epigenetic and are thought to be inherited during mitotic cell divisions, so far the propagation mechanisms are unknown for most of these modifications.

ATP-dependent chromatin remodeling enzymes convey alterations to DNA accessibility by mediating a variety of reactions such as sliding nucleosomes along DNA (Whitehouse *et al.*, 1999), disrupting histone-DNA contacts to the extent of destabilizing the H2A/H2B dimer (Bruno *et al.*, 2003; Kassabov *et al.*, 2003), introducing a histone variant into nucleosomes (Mizuguchi *et al.,* 2004) and generating negative superhelical torsion in DNA and chromatin (Havas *et al.,* 2000).

2.4. Histone variants

In most organisms, there are multiple copies of the histone genes encoding for the major histone proteins. These genes are highly similar in sequence, code for the bulk of the cellular histones, are expressed in a tightly regulated manner during the cell cycle with the histones deposited onto DNA in a process that is strictly coupled to DNA replication.

While histones are among the slowest evolving proteins known, there are non-allelic variants of the major histones that can have significant differences in the primary sequence. The similarity between the major histone subtypes and the variants on amino acid level can range from almost no differences to extremely divergent changes. Some variants have distinct biophysical characteristics that are thought to alter the properties of nucleosomes, while others localize to specific regions of the genome. The variants are usually present as single-copy genes (also called orphan genes) that are not restricted in their expression to the S-phase but are expressed throughout the cell cycle and can also be incorporated outside of S-phase. Unlike the major subtypes, variant genes contain introns and the transcripts are often polyadenylated. These features are thought to be important in the post-transcriptional regulation of these proteins (Old and Woodland, 1984). Some variants exchange with the pre-existing histones during development and differentiation, and are therefore referred to as replacement histones (Bosch and Suau, 1995; Grove and Zweidler, 1984; Wunsch *et al.*, 1991). These observations have led to the suggestion that the histone variants have specialized functions regulating chromatin dynamics. Interestingly, this diversification of histone function is restricted to H2A and H3, with H2B and H4 being mostly invariant. Assembly factors and functions are not identified yet for all variants.

2.4.1. H2A replacements

Among core histones, H2A has the largest number of variants identified, among them H2AX, H2AZ, macroH2A and H2A-Bbd (H2A-bar-body-deficient) (Ausio and Abbott, 2002; Fernandez-Capetillo *et al.*, 2004; Redon *et al.*, 2002).

The H2A.Z variant has been linked to both transcriptional repression and activation, localizing to silent or active chromatin in various organisms (Draker and Cheung, 2009). First in yeast (Rusche *et al.*, 2003) and later in mammalian cells (Rangasamy *et al.*, 2004) H2AZ was also postulated to participate in the establishment or maintenance of the boundary between heterochromatin and euchromatin.

H2AX is a histone variant in mammals, while in other species, like in budding yeast it is the main histone H2A. In both cases H2AX has a unique extended C-terminus compared to the canonical H2A. The Ser139 in this C-terminal region is phosphorylated in response to DNA double-stranded breaks (DSBs), implicating the involvement of this histone in the early steps of response to DNA damage (Rogakou *et al.*, 1998).

MacroH2A and H2A-Bbd are vertebrate specific variants, MacroH2A localizing predominantly to the inactive X-chromosome (Costanzi and Pehrson, 1998), while H2A-Bbd localizes to the active X-chromosome and autosomes (Chadwick and Willard, 2001).

2.4.2. H3 variants

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Most studies concerning histone variants have focused on variants of H3. In mammals, there are two canonical forms of H3: H3.1 and H3.2 as well as three other H3 variants: H3.3, a testis specific H3 variant (H3t) and centromeric H3 variants (CENP-A).

The H3 variant CenH3 (in mouse/human termed CENP-A, in *Drosophila* Cid) exclusively localizes to centromeres and is essential for proper centromer functioning and chromosome segregation (Black and Bassett, 2008). CenH3s form a highly divergent family of histone H3 variants that are characterized by an H3-like histone fold domain and a variable N-terminus tail. (Dalal *et al.*, 2007).

In the mouse 13 canonical histone genes are present in the genome, encoding the two major H3 histones H3.1 and H3.2, which only differ in one amino acid located at position 96: Cys in H3.1 and Ser in H3.2. Cysteins are rarely used amino acids in nature and can form disulfide bonds under oxidative conditions which are known to be involved in dimerization of proteins. All H3 proteins contain a conserved Cys at position 110 (Luger *et al.*, 1997), but the additional Cys 96 in H3.1 was speculated to confer further chromatin compaction and gene silencing roles for this histone version through forming disulfide bridges with other H3s or proteins containing Cys. Deposition of H3.1 and H3.2 was also suggested to involve different proteins, which could lead to specific targeting of these variants to different genomic loci (Hake and Allis, 2006). Another notion that H3.1 and H3.2 could have different functions was shown by mass-spectrometry analysis of histone H3s which revealed that the PTMs occurring on the two canonical H3s are different: H3.2 is enriched in repressive marks (H3K27me2/3) while H3.1 contains PTMs of active (H3K14 acetylation) and inactive chromatin (H3K9me2) (Garcia *et al.*, 2008; Hake *et al.*, 2006; McKittrick *et al.*, 2004). The functional importance however for having two different replicative histones is unclear.

The H3.3 variant is present in all eukaryotes and is among the most conserved proteins (Malik and Henikoff, 2003). Five or four amino acids are different between H3.3 and H3.1 or H3.2, respectively (Graves *et al.*, 1985). The differences in amino acid sequence amongst H3.1/2 and H3.3, albeit surprisingly small, lead to different pathways of incorporation into the chromatin (Ahmad and Henikoff, 2002a; Ahmad and Henikoff, 2002b). In somatic cells, it is well established that the canonical variants H3.1 and H3.2 are synthesized only during S-phase and deposited into the chromatin through a Replication Coupled (RC) pathway (Polo and Almouzni, 2006).

In mammals two *H3.3* genes (*H3.3A* and *H3.3B*) encode for the same protein, but the transcripts only differ in their untranslated regions (Frank *et al.*, 2003). The fact that expression of *H3.3* genes is not linked to S-phase (Replication Independent (RI)) has been known for a long time (Wu *et al.*, 1982). This

observation led to the so-called neutral-replacement idea, in which the function of H3.3 is to replace H3 whenever nucleosome assembly takes place independently of S phase.

As a generalal rule, H3 is deposited as H3-H4 tetramers (Akey and Luger, 2003). The activities responsible for loading H3.1 or H3.3 have been characterized (Tagami *et al.*, 2004); the complexes responsible for H3.1 or H3.3 deposition include several common subunits (eg ASF-1), as well as unique histone chaperones. The canonical H3.1 is deposited by a complex containing ASF-1 and the entire CAF-1 complex, whereas the H3.3 deposition complex contains ASF-1, the smallest subunit of the CAF-1 complex (p48) and a specific histone chaperone HIRA (Ray-Gallet *et al.*, 2002; Tagami *et al.*, 2004). CAF-1 can interact with PCNA and promote subsequent deposition of H3.1-H4 heterodimers to sites of DNA synthesis, allowing for the assembly of new nucleosomes at the replication fork (Shibahara and Stillman, 1999). Amino acids 87-90 in H3.3 have been shown to be important for the RI deposition into chromatin, suggesting that this region serves as a chaperone recognition site where HIRA binds H3.3 and CAF-1 to H3.1. It is not known whether H3.2 binds to a different chaperone and whether the Ser at position 96 plays a role in this (Ahmad and Henikoff, 2002b; Tagami *et al.*, 2004).

An example supporting the neutral-replacement model of H3.3 comes from differentiated cells which have exited the cell cycle. In the absence of S-phase and RC histone gene expression, differentiating cells have abundant H3.3 transcripts, while canonical transcripts are not detected (Krimer *et al.*, 1993; Pantazis and Bonner, 1984). Another example in line with the neutral-replacement model describes H3.3 deposition during DNA repair at sites of heterochromatin in human cells after treatment with HDAC inhibitors (Zhang *et al.*, 2007).

The observation that H3.3 deposition is not homogenous throughout the genome, but is enriched at highly transcribed regions, has led to the model of H3.3 being a marker of active chromatin. As the RNAP complex passes through a transcribed gene, it displaces nucleosomes, creating a situation were nucleosomes need to be re-deposited in a RI manner (Li *et al.*, 2007; Schwabish and Struhl, 2004). Therefore H3.3 is deposited in a transcription coupled manner (TC). For example incorporation of H3.3 at large transcription units on the *Drosophila* polytene chromosomes has been described, linking H3.3 to transcriptional elongation (Schwartz and Ahmad, 2005). Studies of high resolution mapping of H3.3 distribution by ChIP analysis have also found this variant enriched at sites of active transcription (Mito *et al.*, 2005; Wirbelauer *et al.*, 2005). Finally, others found H3.3 enrichment at regulatory elements of active, but also inactive genes (Jin and Felsenfeld, 2006; Mito *et al.*, 2005; Nakayama *et al.*, 2007). These last two observations link H3.3 deposition yet to another phenomenon: H3.3 marking sites of rapid histone turnover at regulatory elements. Whether H3.3 would have any role in facilitating accessibility of regulatory factors to bind to their elements or this is a mere consequence of high nucleosome turnover at these regions is not clear. On a side note, the TC and chromatin remodeling associated roles for H3.3 discussed so far do not interfere per se with the neutral-replacement model, as these processes are not limited to the S-phase of the cell cycle.

Correlating with its appearance at transcriptionally active site, mass spectrometry studies showed that H3.3 harbors PTMs associated with active chromatin (hyperacetylation and dimethylation of K36 and K79) (Hake *et al.*, 2006; McKittrick *et al.*, 2004). Remarkably, a study by *Loyola et al.* showed that H3.3 histones already carry a distinct set of PTMs before they are deposited into the nucleosome, impacting its final PTM composition in chromatin (Loyola *et al.*, 2006).

A study in *Xenopus* has put forward the potential role of H3.3 in epigenetic memory. By transferring a somatic cell nucleus into a *Xenopus* oocyte, authors demonstrated the inheritance of an active chromatin state of a gene, whose activity correlates with the presence of H3.3 harboring H3K4 methylation. This epigenetic memory persisted throughout numerous rounds of cell division in the absence of transcription, supporting the model that H3.3 is transmitted through cell generations, rather than always being re-established by re-occurring transcription (Ng and Gurdon, 2008). Unfortunately, this study did not address whether this memory was simply due to K4 methylation on any H3 or K4 methylation specifically on H3.3, although the importance of K4 methylation specifically of H3.3 has since been demonstrated in a different system (see below) (Hodl and Basler, 2009).

Besides its neutral-replacement and epigenetic roles discussed above, several recent studies pointed out that the function of H3.3 in RI chromatin remodeling processes is unique to the germline (Ooi and Henikoff, 2007). For example, an interesting study highlighted the importance of H3.3 in *Drosophila* germline (Hodl and Basler, 2009). Deletion of the two fly *H3.3* genes did not affect somatic tissues of the animal, but caused sterility. The major fraction of active H3K4 methylation is harbored by the H3.3 variant. Surprisingly, in contrast to the germ line, somatic cells did not seem to mind loss of H3.3 or loss of bulk K4 methylation levels. Fertility however, could not be rescued by H3.2, even when engineered to be expressed outside of S-phase, or by an H3.3 mutant containing an Ala instead of a Lys at position 4. These results imply that K4 methylation specifically on H3.3 is essential for proper germ line function in the fly.

The laboratory of Peter De Boer with collaborative help from us has demonstrated a role for H3.3 during Meiotic Sex Chromosome Inactivation taking place in the male germ line. This will be discussed in the Results section (van der Heijden *et al.*, 2007).

The function of H3.3 in male pronucleus formation in the zygote will be mentioned later in the context of early embryonic development.

Recently, a novel function for H3.3 was identified at telomeres of mouse ES cells. Localization of H3.3 at interphase telomeres and enrichment of Ser31-phosphorylated H3.3 (H3.3S31P) at metaphase telomeres was shown in pluripotent mouse ES cells. Upon differentiation, telomeric H3.3S31P signal decreased, accompanied by an increase of heterochromatic repressive marks and decreased micrococcal nuclease sensitivity at the telomeres. RNAi-depletion of H3.3 induced a telomere-dysfunction phenotype, providing evidence for a role of H3.3 in the regulation of telomere chromatin integrity in ES cells (Wong *et al.*, 2009). Surprisingly, two studies have recently shown that this H3.3 localization at telomeres is HIRA

independent, but is instead dependent on the SWI2/SNF2 chromatin remodeling protein ARTX (alpha thalassemia / mental retardation syndrome X-linked) (Goldberg *et al.*, 2010).

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2.5. Epigenetic modifications

Epigenetic modifications serve as an extension of the genetic information encoded by the genome. These modifications mark genomic regions and act as heritable and stable instructions for defining chromatin organization and structure that dictate transcriptional states. In mammals, DNA methylation and the modification of histones account for the major epigenetic alterations (Berger, 2002).

2.5.1. DNA methylation

Methylation is the only physiological post-synthetic modification of DNA able to modify DNA function and consists in the introduction of methyl groups on cytosines mainly at the CpG dinucleotides of the mammalian genome. DNA methyltransferases (Dnmts) like Dnmt1 and Dnmt3a/Dnmt3b are differentially responsible for establishing and maintaining methyl-CpG patterns. This epigenetic modification introduces a fifth base into DNA, the 5-methyl-cytosine (5mC). It is well-known that 5mCs are distributed in a non-random fashion in genomic DNA. The distribution is characterized by the presence of methylated cytosines on the bulk of DNA (98% of the vertebrate genome) while the unmethylated ones are mainly located within particular regions termed CpG islands (1-2% of the genome) (Suzuki and Bird, 2008). CpG islands are found in the promoter regions of genes and the methylation of these CpG islands correlates with silencing of the gene (Bird, 2002). While methylation may directly interfere with the binding of some transcription factors, indirect repression by proteins that specifically recognize methylated CpGs is currently believed to be responsible for the majority of methylation dependent silencing. A family of proteins which all share a common methyl-CpG-binding domain have been identified as capable of reading the methylation pattern.

DNA can be demethylated passively via blocking maintenance during DNA replication and thereby avoiding methylation of the newly synthesized DNA strand. Alternatively it has been proposed that DNA methylation can be removed actively via specific enzymes. While in plants, active demethylation occurs widely and is carried out by 5meC glycosylases such as Demeter and Demeter-like proteins (Gehring *et al.*, 2009a; Gehring *et al.*, 2009b; Hsieh *et al.*, 2009) existence active demethylation in mammals is highly controversial. There are two stages during mammalian development where the occurrence of DNA demethylation is highly suspected: first in the pre-implantation embryo and later during primordial germ cell (PGC) development (Reik, 2007). Very recently a number of studies identified factors involved in DNA

demethylation events. A study by Popp et al. claims to have identified the cytidine deaminases AID as the enzyme responsible for DNA demethylation during PGC development (Popp *et al.*, 2010). AID-dependent DNA de-methylation has also been demonstrated in the context of somatic cell nucleus re-programming to a pluripotent state (Bhutani *et al.*, 2010). Another group showed involvement of the elongator complex in active paternal genome de-methylation in the zygote (Okada *et al.*, 2010). DNA demethylation in the context of early embryonic development will be discussed in more detail later.

2.5.2. Histone acetylation

Lysines are the major source of the net positive charge of histone octamers and therefore crucial for binding the negatively charged phosphate backbone of DNA. For transcription, replication or DNA repair it is important that histone-DNA interactions can be modified in order to facilitate passage of polymerases. All four core histones bear lysine residues which are subject to acetylation and deacetylation by specific histone acetyltransferases (HAT) and deacetylases (HDAC) (Kouzarides, 2007). Generally, acetylated lysines on histones H2B, H3 and H4 are highly correlated with actively transcribed regions (Pokholok *et al.*, 2005; Schubeler *et al.*, 2004; Wang *et al.*, 2008). This is thought to be due to neutralization of the positive charge of lysines upon acetylation, which lowers the electrostatic interactions with the negatively charged phosphate backbone of DNA and consequently weakens the DNA-histone interaction. Indeed, acetylation increases DNA accessibility, destabilizes nucleosomes and leads to an increase of non-histone protein binding to DNA *in vitro* (Lee et al., 1993; Vettese-Dadey et al., 1996; Wolffe and Hayes, 1999). Many proteins however, in chromatin-associated complexes contain highly conserved Bromodomains which specifically bind to acetylated lysines, indicating that there might be more specific regulatory potential to acetylation than previously anticipated (Taverna *et al.*, 2007).

2.5.3. Histone methylation

There are two types of histone methylation, targeting either arginine (R) or lysine (K) residues. Adding to the complexity is the fact that each K residue can accept one, two or even three methyl groups (abbreviated me1, me2, and me3), and an R can be either mono- or di-methylated (Sims *et al.*, 2003). It is possible that methylation induces alterations in chromatin structure, either condensing or relaxing it. However, a methyl group is relatively small and in contrast to acetylation, does not neutralize the charge of a K or R residue, so it is unlikely that methylation per se will significantly alter chromatin structure. It is more likely that it creates binding sites for regulatory proteins. Unlike DNA methylation, histone methylation can represent active as well as repressive states depending on the modified residue (Barski *et al.*, 2007; Pokholok *et al.*, 2005; Saunders *et al.*, 2006; Schubeler *et al.*, 2004).

Arginine methylation is performed by protein arginine methyltransferases (PRMTs) and is antagonized by PADI4 (Klose *et al.*, 2006a; Zhang and Reinberg, 2001). Lysine methylation is carried out by specific lysine methyltransferases (KMTases or HMTases), that all but one (Dot1) contain the catalytically active "SET" domain. This domain is named after the three evolutionary conserved founding members Suv39h, Enhancer of Zeste and Trithorax. All three play major roles in chromatin formation and gene expression among the species.

Numerous proteins have been discovered that bind to different methylated lysine marks, thereby reading the modification pattern and translating it into a biological meaning. Several protein domains have been found to specifically recognize methylated lysines, such as Tudor domains, chromodomains, PHD-finger domains and WD40 domains. Each domain has characteristic affinities for different lysines and methylation states which further depend on other domains of the respective protein and its interaction partners (Margueron *et al.*, 2009; Martin and Zhang, 2005; Taverna *et al.*, 2007).
For a long time, histone methylation – unlike all other histone modifications – was considered a permanent mark. The discovery of enzymes capable of demethylating histones however has proven histone methylation a dynamic modification as well (Bannister and Kouzarides, 2005). Lysine methylation can be removed by two distinct classes of histone demethylases (HDMs): the LSD1 enzyme and the JmjC protein family (Klose *et al.*, 2006a).

Chromatin immunoprecipitation experiments have revealed that active genes are methylated at lysine 4 of histone H3 (H3K4), H3K36 and H3K79 (Barski *et al.*, 2007; Pokholok *et al.*, 2005; Saunders *et al.*, 2006; Schubeler *et al.*, 2004). These modifications are thus thought to have a role in transcription. This is supported by data from yeast indicating that Set1 and Set2, which methylate H3K4 and H3K36, directly interact with factors bound to the RNA polymerase II (RNAPII) complex (Krogan *et al.*, 2003a; Krogan *et al.*, 2003b). Genetic evidence also predicts a recruitment of Dot1 (a H3K79 HMTase) to chromatin via elongating RNAPII (van Leeuwen *et al.*, 2002). H3K4me peaks around the transcription start site and is gradually diminished further 3'. H3K36me and H3K79me display a broader distribution within the gene body, starting just downstream of the H3K4me peak (Bell *et al.*, 2007; Wirbelauer *et al.*, 2005). Consistent with a role for H3K36me in transcription, data from yeast show that H3K36me prevents cryptic initiation via recruiting a histone deacetlyase to the body of genes, which presumably leads to a less accessible chromatin structure (Carrozza *et al.*, 2005). H3K4 methylation has been implicated in transcriptional activation pathways since many chromatin remodeling and co-activator complexes bear a module which specifically recognizes H3K4me2/3. For example a PHD-domain in the NURF chromatin remodeling complex specifically recognizes H3K4me3 and might facilitate transcriptional activation via opening the chromatin structure around H3K4me2/3 modified promoters (Wysocka *et al.*, 2006).
More recent data from mammalian systems indicates that in contrast to invertebrates H3K4me2/3 are not exclusively marking actively transcribed regions (Bernstein *et al.*, 2006; Guenther *et al.*, 2007; Mikkelsen *et al.*, 2007; Roh *et al.*, 2006; Weber *et al.*, 2007). Interestingly, these loci are CpG-rich sequences and

many bear low but detectable levels of RNAPII and acetylated histone H3 (Guenther *et al.*, 2007; Roh *et al.*, 2006). Moreover, virtually all CpG-rich promoters reside in chromatin carrying H3K4 di-/tri-methylation independent of transcriptional activity. This is in sharp contrast to CpG-poor promoters. These promoters are only H3K4 methylated when transcribed, which is reminiscent of the situation in invertebrates such as fly and yeast (Pokholok *et al.*, 2005; Schubeler *et al.*, 2004).

Inactive loci display a different set of methylation marks mainly consisting of methylation of H3K9, H4K20, and H3K27. These will be discussed in the next part on the basis of which silencing pathway they participate in.

2.6. Silencing mechanisms

2.6.1. Constitutive heterochromatin formation

Constitutive heterochromatin has been historically defined as chromosomal regions that remain condensed throughout the cell cycle. The proper formation of heterochromatin is very important for gene regulation as well as maintaining genome stability. A classical example of constitutive heterochromatin is formed on satellite repeats around the centromeres, termed pericentric heterochromatin (PCH).
RNA interference (RNAi) and histone methylation have been shown to play central roles in constitutive heterochromatin formation in fission yeast, where long double stranded RNA molecules, transcribed from underlying tandem satellite repeats, are thought to be processed by the RNAi machinery into small interfering RNAs that subsequently target other HTMases to repeats (Grewal and Elgin, 2007).
Although mammals do not possess the RNA-dependent RNA polymerase activity to produce dsRNA, evidence is accumulating that an RNAi(-like) pathway is functioning at mammalian heterochromatin too. (Chen *et al.*, 2008; Fukagawa *et al.*, 2004; Kanellopoulou *et al.*, 2005)
Constitutive heterochromatin is enriched in tri-methylated H3K9 (H3K9me3), which in mammals is mediated by the Suv39h1 and Suv39h2 enzymes (Peters *et al.*, 2001). H3K9me3 creates a binding site for the heterochromatin protein 1 (HP1) (Bannister *et al.*, 2001; Lachner *et al.*, 2001). Although H3K9me3 is required for heterochromatin targeting of HP1 (Lachner *et al.*, 2001), it does not seem to be sufficient. In mammalian cells, HP1 localization to heterochromatic foci requires a structural RNA of unknown identity (Maison and Almouzni, 2004) In mammals, HP1 interacts with the DNA methyl transferases Dnmt1 and Dnmt3a/b (Fuks *et al.*, 2003), directing DNA methylation to satellite repeats (Lehnertz *et al.*, 2003). Moreover, additional repressive histone methylation at H4K20 is mediated by the recruitment of the Suv4-20h HMTase (Schotta *et al.*, 2004). Taken together, mammalian heterochromatin is marked by a number of characteristic chromatin modifications establishing a transcriptionally repressed state. Loss of *Suv39h*-mediated chromatin modification leads to an increase in satellite transcription from PCH (Lehnertz *et al.*,

2003). Importantly, in *Suv39h*-deficient mice chromosome segregation is perturbed, indicating that proper marking of PCH is essential to ensure correct chromosome segregation and genome stability (Peters *et al.*, 2001).

2.6.2. Non-coding RNA and domain wide gene silencing

Perhaps the best studied phenomenon of non-coding RNA (ncRNA) mediated silencing is X-chromosome inactivation in female mammals required for dosage compensation. X-chromosome inactivation begins with the synthesis of the *Xist* (X-inactivation specific transcript) non-coding RNA from the XIC (X-inactivation center) locus. The expression and spreading of *Xist* RNA along the X chromosome triggers a cascade of events leading to the inactivated state and ultimately to the formation of facultative heterochromatin.

Facultative heterochromatin, in contrast to constitutive heterochromatin does not stay condensed throughout the cell cycle and development, but can change its chromatin state in response to developmental signals. H3K4 hypomethylation, H3K9me2, H3K27me3 (set by PRC2 and then bound by PRC1, see later) (de Napoles *et al.*, 2004), H4K20me1 hypermethylation, macroH2A histone variant incorporation and DNA methylation are characteristic marks of the inactivated X-chromosome (Okamoto *et al.*, 2004; Peters and Schubeler, 2005).

Another well known example of silencing involving ncRNAs - along with DNA methylation and histone modifications - is genomic imprinting. Most mammalian imprinted genes are found in clusters that also contain large imprinted ncRNAs. In most cases, expression of the ncRNA correlates with repression of the protein-coding genes in the opposite strand (*in cis*). So far, two of the three tested imprinted long ncRNAs (*Airn* in the *Igf2r* locus and *Kcnqot1* in the *Kcnq1* locus) have been shown to be required for the imprinted expression of the whole cluster (Koerner *et al.*, 2009).

2.6.3. H3K9 mediated gene silencing

One example of gene silencing is conferred by the methylation of H3K9. Several distinct SET domain containing HMTases are known to methylate mono-, di-, and tri-methyl H3K9: *G9a* and *Glp1* confer mono- and di-methylation, *Eset* mediates di- and tri-methylation while the *Suv39h* HMTs direct tri-methylation. It is well known that the H3K9me2 and me3 marks serve as binding sites for HP1. Binding of HP1 is however alleviated during mitosis by phosphorylation at the adjacent Ser10. HP1 in turn recruits Dnmts, which methylate DNA and drive the formation of heterochromatin, either at genes or repetitive sequences (Fischle *et al.*, 2003; Fuks, 2005; Snowden *et al.*, 2002).

2.6.4. Polycomb-mediated silencing

At the core of development lies the specialization of cells that make up an organism. Upon specialization different cells acquire different programs of gene expression. During the growth of an organism, these specialized states need to be maintained and the particular configurations of gene expression transmitted to daughter cells in the cell lineage. This requirement, referred to as 'transcriptional memory' or 'cellular memory' is fulfilled in part by the antagonistic functions of Polycomb group (PcG) and Trithorax group (TrxG) proteins (Dellino *et al.*, 2004; Klymenko and Muller, 2004; Ringrose and Paro, 2001). Both groups of proteins are involved in maintaining the spatial patterns of *homeotic box* (*Hox*) gene expression, which are established early in embryonic development (Deschamps *et al.*, 1999). In general, PcG proteins are transcriptional repressors that maintain the repressed state and TrxG proteins are transcriptional activators that counteract the repressed state. PcG and TrxG proteins function in large multiprotein complexes which harbor different intrinsic histone methyltransferase or ubiquitin ligase activities, which suggests that PcG and TrxG proteins participate in cellular memory through methylation or ubiquitination of core histones (Ringrose and Paro, 2004).

2.6.4.1. Polycomb repressive complex 1 and 2

PcG proteins can be separated biochemically and functionally into at least two major multiprotein complexes termed Polycomb repressive complexes (PRCs). In *Drosophila* Polycomb Repressive Complex 1 (PRC1) is composed of the chromodomain containing protein Polycomb (Pc), Polyhomeotic (Ph), Posterior Sex Combs (Psc) and the dRing protein which contains a Ring finger-motif, a characteristic of ubiquitin ligases. In mouse, depending on cell type, several redundant homologues exist for each core component: Pc- Cbx2, 4, 6, 7, 8; Ph- Rae28, Mph2; Psc- Bmi1, Mel18, Mblr and dRing- Rnf1 (Ring1a), Rnf2 (Ring1b). The Polycomb repressive complex 2 (PRC2) is comprised of the SET domain containing HMT Enhancer-of-zeste (E(Z)) (mammalian homologs Ezh2 and Ezh1), different isoforms of the WD-repeat protein Extra sex combs (Esc) (mammalian Eed (embryonic ectoderm development)), the Zn-finger protein suppressor-of-zeste-12 (Su(Z)12) (mammalian Suz12) and another WD-repeat protein Nurf55 (mammalian RpAp48/46) (Lund and van Lohuizen, 2004) (**Figure 1**).

The signature of the PRC2 complex is tri-methylation of lysine 27 on histone H3 (H3K27me3) (**Figure 1**), carried out by the cathalytic subunit E(Z). In *Drosophila*, loss of *E(Z)* or *ESC* results in loss of mono-, di- and trimethylation of H3K27, suggesting that a complex containing these subunits are the only source for K27 HMTase activity. In mammals, loss of *E(Z)* homolog *Ezh2* reduces only di- and tri-methylation, indicating a different complex responsible for mono-methylation. The mono-methylating complex was shown to contain

the other mammalian E(Z) homolog Ezh1 (Shen *et al.*, 2008) and it still requires Eed, as deletion of this subunit eliminates all three layers of K27 methylation (Montgomery *et al.*, 2005).

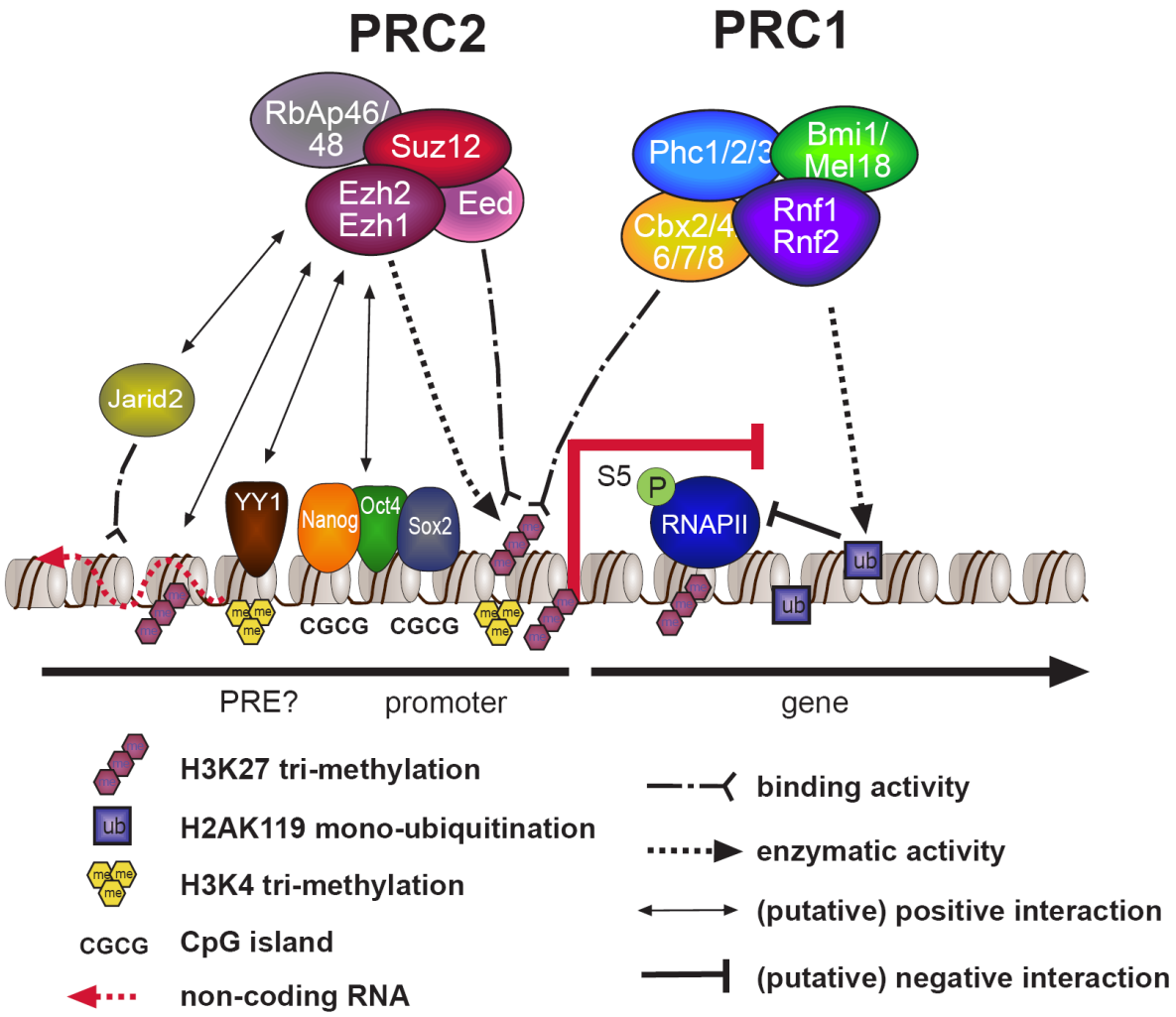

Figure 1. Main mammalian Polycomb complexes and a model for Polycomb-mediated targeting and silencing.

Recent studies have identified distinct functions for the PRC2 complex containing Ezh1 in contrast to PRC2 with Ezh2 (Margueron *et al.*, 2008). While Ezh2 is highly expressed in during embryonic development and in proliferating cells, Ezh1 is mostly present in non-proliferating adult tissues (Bracken *et al.*, 2003). Ezh2

containing PRC2 (Ezh2-PRC2) was shown to have robust H3K27me3 HMTase activity, while the HMTase activity of Ezh1-PRC2 was weak. Additionally, the study by Margueron et al. claims that the Ezh1-PRC2 complex can compact polynucleosomes, unlike Ezh2-PRC2. Ezh1-PRC2 was shown to be able to repress transcription from chromatinized templates *in vitro*, while Ezh2-PRC2 could not (Margueron *et al.*, 2008). They propose that Ezh2-PRC2 mediates repression through a catalytic mechanism, whereas Ezh1-PRC2 functions in a non-catalytic fashion. However, it is more conceivable that Ezh1-PRC2, albeit with a weaker HMTase activity, functions together with Ezh2-PRC2 in tri-methylating H3K27 at target genes (Shen *et al.*, 2008). Ezh1 occupies a subset of Ezh2 target genes in ES cells, and residual H3K27me3 at these genes in *Ezh2-/-* cells only disappears upon knockdown of *Ezh1* (Shen *et al.*, 2008).

The composition of the PRC2 complex can be further complicated by the presence of different Eed isoforms. Alternative transcription start sites can yield 4 different isoforms of Eed. The PRC2 complex which contains the two shortest isoforms of Eed methylates H3K27, while the complex containing the longest Eed isoform confers histone methylation at H1K26 (Kuzmichev *et al.*, 2004). The potential role of H1K26 methylation in Polycomb-mediated silencing is not known.

An additional variation of PRC2 is achieved by binding of the PcG protein Polycomb-like (PCL in *Drosophila*, PHF1 in mammals), which alters the properties of PRC2. Intriguingly, PRC2-PCL is found specifically at PcG target genes (Tie *et al.*, 2003) and in the absence of PLC function H3K27me3, but not H3K27me2 is lost (Nekrasov *et al.*, 2007; Sarma *et al.*, 2008). This may be a very important component in targeting PRC2-mediated silencing, as H3K27me2 is a wide-spread mark, not correlating with the more restricted domains of H3K27me3 at silenced Polycomb target genes.

Numerous studies have accumulated genetic and biochemical evidence that PRC1 proteins are at the heart of Polycomb-mediated silencing (Francis and Kingston, 2001; Levine *et al.*, 2004; Muller and Verrijzer, 2009; Schuettengruber *et al.*, 2007; Schwartz and Pirrotta, 2007). PRC1-mediated ubiquitination of histone H2A at lysine 119 (H2AK119Ub) (**Figure 1**) is one of the most abundant ubiquitinated nuclear proteins and is required for Polycomb-mediated silencing (Jason *et al.*, 2002). The RING finger containing protein Rnf2 (Ring1b) was identified as the main catalytic subunit of PRC1, whose enzymatic activity was greatly enhanced by Bmi1 (Buchwald *et al.*, 2006; Cao *et al.*, 2005; Wang *et al.*, 2004a). Rnf2 does seem to be the major H2A ubiquitin ligase *in vivo*, as loss of *Rnf2* dramatically decreases global H2AK119Ub levels and de-represses Polycomb target genes in cells (Stock *et al.*, 2007; van der Stoop *et al.*, 2008). However, Rnf1 (Ring1a), a paralog of Rnf2 in mammals can substitute for Rnf2 *in vitro* (Buchwald *et al.*, 2006). Consistently, in the absence of Rnf2, Rnf1 can still mediate H2AK119Ub at the inactive X chromosome in fibroblasts and during ES cell differentiation (de Napoles *et al.*, 2004; Leeb and Wutz, 2007), arguing for functional redundant roles of these proteins in certain biological contexts. An additional observation supporting functional redundancy comes from a study of *Rnf1/2* double knockout (KO) ES cells (Endoh *et al.*, 2008). In *Rnf1/2* double KO ES cells over twice as many genes were de-repressed then in *Rnf2* single KO cells. The overlap between the two groups of de-repressed genes was significant, but the level of de-

repression was always higher in *Rnf1/2* double mutant cells. This suggests that Rnf1 can even compensate to a certain extent at genes, as full de-repression is only achieved when both proteins are lost (Endoh *et al.*, 2008).

As for PRC2, there is great diversity among PRC1 complexes as well. This diversity is in part due to the existence of multiple paralogs of each PcG gene, creating various possible combinations of core subunits. Additionally, binding to the core complex are a variety of non-core Polycomb proteins, including Rybp, Mblr, NSPc1, Smcl2 and L(3)Mbt.

A recent study in *Drosophila* identified the dRAF complex, containing dRing and Psc with an additional lysine demethylase Kdm2 (Lagarou *et al.*, 2008). Interestingly, Kdm2 has H3K36me2 de-methylase activity, but it also stimulates the ubiquitin ligase activity of dRing and Psc. More strikingly, reconstituted complexes containing dRing, Psc and the two other PRC1 core subunits Pc and Ph did not increase ubiquitin ligase activity of dRing and Psc alone, while addition of Kdm2 instead of the original PRC1 subunits increased enzymatic activity. Moreover depletion of Kdm2 revealed that this component, along with dRing and Psc is also responsible for global H2AUb in *Drosophila* cells (Lagarou *et al.*, 2008). This study therefore suggests that in flies the primary source for H2AUb is dRAF rather than PRC1. Whether an analogous situation exists in mammals, remains to be addressed, although the previously identified Rnf1 and Rnf2 containing complex Bcor shares similarities with the fly dRAF (Gearhart *et al.*, 2006; Sanchez *et al.*, 2007).

Another model organism, *Caenorhabditis elegans* provides further evidence of evolutionary conservation of the components of PRC1 that are the most central to ubiquitination. Based on homology it is clear that PRC2 members are present in the worm, however PRC1 was thought to be absent, as obvious homologs were not identified (Bender *et al.*, 2004). Recently however, two proteins with distant homology to Rnf2 and Bmi1 have been shown to catalyze H2AUb in the worm (Karakuzu *et al.*, 2009).

Taken together, the emerging picture is that PRC1 and PRC2 come in a variety of flavors. Thus, different target genes, different tissues or different developmental stages may involve differentially composed Polycomb complexes that confer distinct functionalities.

2.6.4.2. Targeting of Polycomb complexes

An important question of the Polycomb-field is how the complexes are recruited to their target genes, as PcG proteins do not possess DNA sequence specific binding properties.

In *Drosophila* it has been known for a long time that PcG proteins are targeted to specific regulatory elements called Polycomb response elements (PREs). PREs are not easily recognizable, as they are not a binding site for a single transcription factor, but rather a collection of different combinations of transcription

binding sites, defined as an “element” (Ringrose and Paro, 2007). The recruitment of PcG proteins to these elements is thought to be mediated by trans-acting factors that recognize PREs and recruit PcG proteins by direct or indirect interactions. One such factor Pho (mammalian Yy1) -in complex with Sfmbt forms the Pho-repressive complex - has been shown to co-occupy most PREs with PRC1 and PRC2 components in *Drosophila* embryos (Klymenko *et al.*, 2006; Kwong *et al.*, 2008; Oktaba *et al.*, 2008). Other DNA binding proteins such as Zeste, GAGA factor (GAF), Pipsqueak and Dorsal switch protein1 (DSP-1), have also been implicated in PcG recruitment, although contributions of these factors to Polycomb silencing are not fully understood (Muller and Kassis, 2006; Ringrose and Paro, 2007).

Despite mapping thousands of Polycomb binding sites in mammalian cells, no PRE motif has been identified to date (Boyer *et al.*, 2006; Bracken *et al.*, 2006; Lee *et al.*, 2006) (**Figure 1**). Partially explaining this is the fact that in mammals PcG proteins bind to a much broader domain at target genes, in contrast to *Drosophila*, where PcG proteins localize in a well recognizable peak (Boyer *et al.*, 2006; Ku *et al.*, 2008; Lee *et al.*, 2006). It seems that in mammals an even more complex system is responsible for targeting PcG proteins, involving many more combinations of recruiting factors. A good candidate for a recruiter is the Yy1 protein, a homolog of the fly PHO (Thomas and Seto, 1999) (**Figure 1**). Indeed, knockdown of *Yy1* disables Ezh2 binding and removes H3K27me3 from target genes in mouse myoblasts (Caretti *et al.*, 2004). However, only limited regions are co-occupied by Yy1 and PRC2 in ES cells (Squazzo *et al.*, 2006), suggesting that Yy1 is not a general factor at all PcG binding sites. It has been proposed that in ES cells the core transcription factors Oct4, Nanog and Sox2 might be involved in PcG recruitment, as these factors co-occupy a subset of PcG target genes in human ES cells (Boyer *et al.*, 2006; Lee *et al.*, 2006) (**Figure 1**). A functional link has been proposed by showing reduced PRC1 levels in ES cells on *Oct4* knockdown (Endoh *et al.*, 2008). As loss of *Oct4* induces differentiation, changes in PRC1 occupancy may be a mere secondary effect. Another study carried out genome-wide mapping of PRC1 and PRC2 subunits at high resolution in ES cells. As a result they found a very significant overlap among PRC2 and CpG islands or CG rich regions, suggesting that CpG-binding proteins also contribute to PcG recruitment (Ku *et al.*, 2008).

Long non-coding RNAs (ncRNAs) have also been implicated in PcG targeting (**Figure 1**). Three well studied cases involve production of ncRNAs: *Hox* gene silencing, X chromosome inactivation and imprinting (Mercer *et al.*, 2009). Interestingly, all three scenarios also involve accumulation of H3K27me3, raising the possible link between ncRNAs and PcG proteins. A recent study identified ncRNAs transcribed from human *Hox* loci, for example *Hotair* from the *HoxC* locus. Interestingly *Hotair* is required to repress expression form the *HoxD* locus in *trans* through recruiting PRC2, as loss of *Hotair* RNA results in eviction of H3K27me3 from the *HoxD* locus (Rinn *et al.*, 2007). How *Hotair* exactly recruits PRC2 and whether the interaction is direct remains to be addressed.

X inactivation and imprinting involve ncRNAs that act *in cis*. The key ncRNA in X inactivation is *Xist*, which contains a repetitive element called repA. This repA motif has been shown to bind PRC2, implying a recruitment option to the inactive X chromosome (Zhao *et al.*, 2008a). The paternally imprinted *Kcnq1* locus

Gene repression by PcG proteins has been proposed to involve changes in chromatin remodeling and/or by interfering with the transcriptional machinery. Reconstructed or purified fly and human PRC1 can bind and compact nucleosomal arrays *in vitro*, thereby making them refractory to SWI/SNF-class remodelers, which could otherwise move nucleosomes out of the way and allow binding of e.g. transcription factors (Francis *et al.*, 2004; Francis *et al.*, 2001; King *et al.*, 2002; Shao *et al.*, 1999). PRC1 and SWI/SNF remodeler binding seems to be competitive, as remodelers can remove bound PcG proteins from endogenous target genes (Kia *et al.*, 2008). However, there is no strong evidence to support PcG induced chromatin compaction *in vivo* (Schwartz and Pirrotta, 2007).

Another option for exerting repression is by blocking transcription. PRC1 does not seem to block transcription initiation or steps before that, as several studies have shown that transcription factors or the transcription machinery and PRC1 proteins can bind at target genes at the same time in both fly and mammalian cells (Breiling *et al.*, 2001; Dellino *et al.*, 2004; Papp and Muller, 2006; Saurin *et al.*, 2001; Stock *et al.*, 2007).

Several studies have proposed that PRC1 blocks transcription elongation. Many genes in mouse ES cells are termed bivalent because they possess the active H3K4me3 mark and the repressive H3K27me3 mark at the same time (Azuara *et al.*, 2006; Bernstein *et al.*, 2006) (**Figure 1**). These bivalent genes are expressed at very low levels. Searching for RNAPII on promoters of these PcG target genes revealed that they are bound by a special poised form of RNAPII, which is phosphorylated at Ser 5 (Stock *et al.*, 2007) (**Figure 1**). Depleting H2AK119Ub by deletion of Rnf2 and Rnf1 in ES cells releases the poised state of RNAPII and leads to gene de-repression (Stock *et al.*, 2007). These results suggest that PRC1 mediated H2AK119Ub achieves repression by holding RNAPII in check. Recent work in fly also finds paused RNAPII at two classical Polycomb target genes, *Ultrabithorax* and *AbdominalB*, although a direct role for PcG proteins in generating or maintaining this paused RNAPII was not addressed (Chopra *et al.*, 2009). Of note, the previously discussed chromatin compaction function might also contribute to stalling the polymerase on Polycomb target genes.

Overall, mechanisms of PcG-mediated silencing still hold many unknowns and will be an exciting field for new discoveries.

2.6.4.4. PRC1-PRC2 dependence or independence in targeting and silencing?

The “classical” sequence of Polycomb complex recruitment described above stems from the affinity of the chromodomains of Cbx proteins for H3K27me3 (Fischle *et al.*, 2003; Min *et al.*, 2003) and studies in *Drosophila* (Cao *et al.*, 2002; Wang *et al.*, 2004b) and mammalian cells (Boyer *et al.*, 2006; Cao *et al.*, 2005), where disruption of PRC2 leads to the loss of PRC1 from chromatin targets. Moreover,

is also known to transcribe a long ncRNA, *Kcnq1ot1* that is essential for imprinted silencing. PRC2 has also been shown to be required for imprinted silencing at this locus. Physical interaction between *Kcnq1ot1* and PRC2 has been demonstrated as well as the requirement of *Kcnq1ot1* for PRC2 targeting (Pandey *et al.*, 2008; Terranova *et al.*, 2008). NcRNAs are wide-spread in the mammalian genome, therefore the possibility is there for a more general Polycomb-targeting via this mechanism.

Very recently a number of studies demonstrated the involvement of the Jumonji family member Jarid2 in recruiting PRC2 to target genes (Li *et al.*; Pasini *et al.*; Peng *et al.*, 2009; Shen *et al.*, 2009) (**Figure 1**). Jarid2 was sufficient to target PcG proteins to a heterologous promoter and inhibiting of *Jarid2* expression resulted in altered PcG protein binding and H3K27me3 levels at target genes. The DNA-binding ARID domain of Jarid2 was shown to be required for Polycomb-targeting. Previous studies have shown that the AIRD domain of Jarid2 can bind to diverse DNA sequences (Kim *et al.*, 2003b). It was suggested that the broader specificity of Jarid2 binding to DNA could be narrowed by other transcription factors to achieve specific PRC2 targeting to genes (Pasini *et al.*).

Although the listed examples are promising, further work is clearly needed to fully unravel PcG targeting mechanisms in mammalian cells.

2.6.4.3. Mechanism of Polycomb-mediated silencing

The classical model for PcG mediated silencing involves recruitment of PRC2 to target genes, setting H3K27me3 and thereby creating an anchorage point for Cbx proteins (**Figure 1**). These latter proteins bind to H3K27me3 via their chromodomains and recruit other PRC1 complex members that establish silencing (Cao *et al.*, 2002; Wang *et al.*, 2004b). It has been proposed that histone H2A ubiquitination plays an important role in PRC mediated silencing. The PRC1 component Rnf2 protein was identified as an E3 ubiquitin ligase, monoubiquitinating lysine 119 on histone H2A (H2AK119Ub) (Wang *et al.*, 2004a).

Depletion of *dRing*, the *Drosophila* homolog of *Rnf1/2* by RNAi in cell culture resulted in loss of ubiquitinated H2A and caused de-repression of the *Ubx* homeotic box gene, a well known target of PRC mediated silencing. Moreover, a single amino acid substitution in the catalytic RING finger domain that abolishes ubiquitin ligase activity *in vitro*, behaves as a loss of function allele for *dRing in vivo* (Wang *et al.*, 2004a). These findings indicated that ubiquitination of H2AK119 plays a central role in the PRC mediated silencing pathway. Since then, numerous other studies have supplied evidence highlighting the important role of Rnf2-, Rnf1- or Bmi1-dependent H2AK119Ub in repression (de Napoles *et al.*, 2004; Endoh *et al.*, 2008; Kallin *et al.*, 2009; Leeb *et al.*, 2010; Stock *et al.*, 2007; van der Stoop *et al.*, 2008). Although key to Polycomb-mediated silencing, the precise molecular mechanism of repression mediated by PRC1 is only poorly understood.

overlapping genomic distribution of PRC1, PRC2 members and H3K27me3 suggests concerted actions of these complexes (Boyer *et al.*, 2006; Ku *et al.*, 2008).
Two recent studies have further addressed the role of H3K27me3 in PRC1 targeting, importantly, by altering the modification level but not the PRC2 complex itself. Either by knocking down a H3K27 de-methylase (Utx) (Lee *et al.*, 2007) or by constructing a viral HMTase capable of mediating H3K27me3 (Mujtaba *et al.*, 2008), authors showed increased H3K27me3 levels at targeted *Hox* genes. In both cases increasing H3K27me3 levels corresponded with increasing PRC1 levels, thus providing further evidence of inter-dependency of the two complexes.

An increasing number of observations however, demonstrate PRC2 independent recruitment of PRC1, contradicting the generality of the classical model of PcG recruitment. In flies, PRC1 specifically peaks at PREs, while H3K27me3 comprises a broader domain (Schwartz *et al.*, 2006). Physical interaction of the recruitment factor Pho and PRC1 has been described in *Drosophila*, alleviating the need for PRC2-mediated targeting (Mohd-Sarip *et al.*, 2006).

In mammals both random and imprinted X inactivation initiate normally in *Eed*-deficient embryos that have no detectable level of H3K27me3 (Kalantry and Magnuson, 2006). *Xist* expression at an autosomal locus recruits Rnf2 and establishes long-term silencing in *Eed* knockout ES cells (Schoeftner *et al.*, 2006). The PRC1 complex is recruited to paternal pericentric heterochromatin in zygotes generated by *Ezh2* mutant gametes that (paternally) lack detectable H3K27me3 (Puschendorf *et al.*, 2008). In *Ezh2* mutant embryos, Rnf2 is recruited to the silenced allele of the *Kcnq1* locus, although in theory PRC2 function may still be partially rescued by redundancy of Ezh1 (Terranova *et al.*, 2008). During differentiation of *Suz12* deficient ES cells, two PRC1 members (Bmi1 and Cbx8) are recruited to most of the target genes they occupy in wild type ES cells (Pasini *et al.*, 2007).

A recent study analyzed PRC1 (*Rnf2*)/PRC2 (*Eed*) double mutant ES cells (Leeb *et al.*, 2010). They identified a group of genes that are redundantly repressed by both complexes (only de-repressed when both are gone) and a distinct group of genes where loss of either complex is already sufficient for de-repression (for example *Hox* genes). However, their analysis is not satisfying, lacking a clear overview of genes and levels of de-repression in single (*Rnf2* or *Eed*) and double (*Rnf2/Eed*) mutant ES cells. Their data indicate that in most cases loss of either PRC from a target gene already results in a certain level of de-repression, while the loss of the second PRC adds to this de-repression.

Genome-wide ChIP analysis of PRC1 (Rnf2) and PRC2 (Ezh2, Suz12) occupancy in mouse ES cells showed many genomic sites which accumulate PRC2, but not PRC1 (Ku *et al.*, 2008). This however, may simply be due to the different efficiencies of antibodies used for PRC1 and PRC2 components.

In summary, only few cases confirm that PRC1 can be targeted to chromatin in a PRC2-independent manner. In general, PRC1 and PRC2 seem to act through cooperatively to silence target genes.

2.6.4.5. Role in pluripotency, differentiation and development

Polycomb during development

Mice deficient for any core PRC2 member are not viable and die during early post-implantation stages displaying severe developmental and proliferative defects (Faust *et al.*, 1995; O'Carroll *et al.*, 2001; Pasini *et al.*, 2004). This demonstrates that Polycomb function is essential for proper embryonic development. Deletion of several PRC1 members leads to homeotic transformations (Akasaka *et al.*, 1996; Core *et al.*, 1997; del Mar Lorente *et al.*, 2000; van der Lugt *et al.*, 1994). The most severe phenotype, resulting in embryonic lethality, is observed for *Rnf2* deficiency (Voncken *et al.*, 2003). Deletion of *Rnf2* results in gastrulation arrest and cell cycle inhibition. In contrast, mice deficient for the homologous *Rnf1* are viable and fertile with only minor homeotic transformations (del Mar Lorente *et al.*, 2000). With respect to the differences in the severity of these phenotypes (del Mar Lorente *et al.*, 2000; Voncken *et al.*, 2003) it is interesting that global H2A ubiquitination levels are drastically reduced in *Rnf2* but not in *Rnf1* mutant ES cells (de Napoles *et al.*, 2004). Mutations in other PRC1 members yield relatively mild phenotypes, possibly due to the functional redundancy of the different mouse PRC1 homologs.

Polycomb in stemmness and differentiation

Pluripotent embryonic stem cells have the unique ability to self-renew and to differentiate into all cell types of the embryo and adult organism. During differentiation, gene expression patterns are gradually specified and restricted along with the developmental potential of the cell, ultimately resulting in commitment to a specific fate. Developmental stage specific transcription factors are known to orchestrate gene expression patterns, which become heritable through acquiring epigenetic modifications, providing cellular memory of lineage restriction. Polycomb proteins have emerged as important regulators of stemness and differentiation.

This role of Polycomb is best illustrated by various PcG-knockout ES cells. Although different knockouts have slightly different phenotypes, there are common trends. Core components of PRC2 (Ezh2, Eed and Suz12) and the main catalytic subunit of PRC1 (Rnf2) are not required for stem cell proliferation and maintenance, although they do stabilize self-renewal (Chamberlain *et al.*, 2008; Leeb and Wutz, 2007; Pasini *et al.*, 2007; Shen *et al.*, 2008; van der Stoop *et al.*, 2008). PcG mutant ES cells are prone to spontaneous differentiation. Expression of pluripotency factors such as Oct4 are maintained in mutant ES cells, but differentiation specific genes are activated. Upon *in vitro* induced differentiation, mutant cells are unable to give rise to the full range of cell types in *in vitro* differentiation assays, accompanied by failure to

repress pluripotency genes as well as failure to activate appropriate differentiation markers (Chamberlain *et al.*, 2008; Leeb and Wutz, 2007; Pasini *et al.*, 2007; Shen *et al.*, 2008; van der Stoop *et al.*, 2008). Although slightly more severe, this phenotype resembles that of ES cells double deficient for a PRC1 (*Rnf2*) and a PRC2 (*Eed*) complex member (Leeb *et al.*, 2010). The only exception known so far are ES cells double deficient for *Rnf1* and *Rnf2*, which seize proliferation upon PRC1 loss (Endoh *et al.*, 2008). We can draw two interesting conclusions from these studies: first, in ES cells *Rnf1* is capable to compensate in part for the loss of *Rnf2* and to maintain stem cell self-renewal; second, there seems to be a discrepancy between the need for PRC1 and PRC2 for ES cell maintenance. This result points to a surprising importance-hierarchy among PRC1 and PRC2 for silencing. However, this could be simply due to different culture conditions, as pointed out in *Leeb et al. 2010*.

Interestingly many reports demonstrate that PRC1 proteins have an essential role in maintenance of more differentiated cell types as multipotent adult stem cells. For example, *Bmi1* is required for maintenance of self-renewing hematopoietic stem cells (Park *et al.*, 2003), neuronal stem cell proliferation (Molofsky *et al.*, 2003) and mammary stem cell maintenance (Liu *et al.*, 2006). *Bmi1* homozygous null mice can survive to adulthood, but show progressive postnatal growth retardation and the loss of hematopoietic and neural stem cells (Bruggeman *et al.*, 2005). In addition, Ph1 *(*Rae28*)*, a PRC1 member that interacts directly with Bmi1 is also required for sustaining activity of hematopoietic stem cells (Kim *et al.*, 2004; Valk-Lingbeek *et al.*, 2004). While inactivation of Rnf2 in embryonic neural stem cells affects self-renewal and results in precocious neuronal but not glial differentiation (Roman-Trufero *et al.*, 2009), deletion of *Rnf2* in adult stem cells or progenitor cells of the hematopoietic system results in proliferation, rather than differentiation defects (Cales *et al.*, 2008).

Genome-wide mapping studies of Polycomb protein occupancy in mouse and human ES cells have shown that PcG complexes are predominantly bound at genes that encode key developmental regulator factors that control diverse developmental pathways (Boyer *et al.*, 2006; Lee *et al.*, 2006). Most of these factors are expressed at very low levels or not at all in ES cells. However, upon differentiation a discrete set of these genes become activated, pointing out an intimate involvement of Polycomb proteins in the dynamic regulation of stem cell identity and cell fate determination.

Further studies have shown that many PcG targets in ES cells are broadly covered with H3K27me3, but surprisingly also harbor H3K4me3, an active mark, and RNAPII Ser5-phospho at the transcription start site (Bernstein *et al.*, 2006; Mikkelsen *et al.*, 2007; Pan *et al.*, 2007; Stock *et al.*, 2007; Zhao *et al.*, 2007) (**Figure 1**). Such regions marked by dual histone modifications were termed bivalent domains. By mapping histone modifications and determining gene expression during the course of differentiation, the current hypothesis is that bivalent domains poise genes for subsequent activation (accompanied by the loss of the H3K27me3 mark) or permanent repression (accompanied by the loss of the H3K4me3 mark) (Mikkelsen *et al.*, 2007). Permanent repression of genes in some cases also involves additional silencing mechanisms, like H3K9me or DNAme that may contribute to locking in the silenced state (Feldman *et al.*, 2006; Mohn *et*

al., 2008). Bivalent domains are not unique to ES cells as initially thought but are also found in differentiated cells, like NPCs, MEFs and T cells (Mikkelsen *et al.*, 2007; Mohn *et al.*, 2008; Roh *et al.*, 2006). However, the number of genes marked by bivalency in differentiated cells is much lower compared to ES cells, suggesting that the resolution of these domains is closely related to the commitment of cells. With progressive commitment, lineage choices become increasingly limited by the resolution of 'poised' promoter states into either “on” or “off” states (Pietersen and van Lohuizen, 2008).

Interestingly, bivalent domains in ES cells were found to correlate with the presence of CpG-rich promoters (High-CpG content promoters; HCP) (Mikkelsen *et al.*, 2007; Mohn *et al.*, 2008) (**Figure 1**). In general, genes with HCP and bivalent marks were found to mainly include key developmental transcription factors, morphogens, and cell surface markers, while genes bearing HCP and H3K4me3 marks generally have ‘housekeeping’ functions, including replication and basic metabolism. On the other hand, genes with low-CpG content promoters (LCP) are associated with tissue-specific genes (Mikkelsen *et al.*, 2007). To illustrate the Polycomb-bivalency-CpG richness connection in numbers: a study by van der Stoop el al. 2008 showed that Rnf2 in ES cells almost exclusively associated (93% of Rnf2 targets) with and regulated (94% of Rnf2 targets) transcription of genes with HCPs. Moreover, 99% of Rnf2 targets carried bivalent chromatin marks (van der Stoop *et al.*, 2008).

Taken together, apart from the known role of heritable silencing mediated by Polycomb proteins, a new, more plastic role has emerged for Polycomb-mediated regulation, providing means for a more flexible regulation of developmental transcription factors during differentiation.

Coordinated transcription factor networks have emerged as master regulatory mechanisms of stem cell pluripotency and differentiation. Pluripotency factors Oct4, Sox2 and Nanog function in combinatorial complexes to activate the expression of genes promoting stemness and repress differentiation inducing factors (Boyer *et al.*, 2005). They are also known to form a core regulatory feedback circuit, in which all three factors regulate their own, as well as each other’s expression to sustain precise levels of pluripotency factors required for ES cell maintenance (Catena *et al.*, 2004; Kuroda *et al.*, 2005; Okumura-Nakanishi *et al.*, 2005; Rodda *et al.*, 2005). Initiation of differentiation requires interruption of this positive feedback loop by differentiation factors, for e.g. Gata6. Given the crucial role of PcG proteins in stem cells, it was obvious that Polycomb-pluripotency network relationships had to be examined. First reports demonstrated that PRC2 members co-occupy a significant subset of genes targeted by the (repressive function of the) core pluripotency network in human ES cells (Boyer *et al.*, 2006; Lee *et al.*, 2006). Additionally, it has now been shown that there is a functional link between these regulatory mechanisms (Endoh *et al.*, 2008) (PRC1 and PRC2 occupancy is reduced at target genes upon loss of Oct4, while Oct4 binding does not change significantly upon loss of PRC1. Furthermore, molecular links between PRC1 and Oct4 have been shown, all pointing towards Polycomb silencing functioning downstream of the core pluripotency circuitry (Endoh *et al.*, 2008).

2.6.4.6. Polycomb proteins in cell cycle regulation and DNA damage response, a highly dynamic role?

Beyond the role in stable maintenance of repressive chromatin during development, Polycomb proteins have long been implicated in playing a role in cell proliferation. For example *Drosophila* E(Z) mutants display a small disc phenotype with no discernible mitotic figures (Phillips and Shearn, 1990). Mutations in PH, PC and PSC display segregation defects caused by the formation of anaphase bridges during syncytial embryonic mitoses (O'Dor *et al.*, 2006). Examples in mammalian systems include cell cycle arrest generated by Rnf2 knockdown in U2OS cells (Wang *et al.*, 2004a), and proliferation halt of ES cells where both *Rnf1* and *Rnf2* were deleted (Endoh *et al.*, 2008). Unfortunately, in both studies the cell cycle block was not characterized.

An essential requirement for regulating the cell cycle is flexibility of the system; even more flexible than the previously discussed plasticity that developmental progression requires. Surprisingly, studies on mobilities of PRC1 proteins in *Drosophila* and mammalian cells using photobleach recovery showed exactly such a dynamic behavior, challenging the previous view of Polycomb protein always being in static association with chromatin. In U2OS cells two Bmi1 populations were identified based on their mobilities: a highly mobile one and one that is immobile (Hernandez-Munoz *et al.*, 2005). In ES cells the rates of exchange for over 85% of Cbx proteins were orders of magnitude faster than the mean exchange rates of core histones. The mobility of PcG proteins decreased and the immobile population grew during differentiation of ES cells and also during *Drosophila* development (Ficz *et al.*, 2005; Meshorer *et al.*, 2006; Ren *et al.*, 2008). The significance of these two populations remains to be determined, although it is intriguing to speculate that the immobile population may be the one relatively stably repressing developmental gene promoters, while the mobile portion may be involved in completely different functions, such as cell cycle regulation.

Although several Polycomb mutants show cell cycle defects it remains unclear how Polycomb complexes control cell cycle progression. In theory there are different possibilities to achieve this: 1) by direct silencing of cell cycle genes, 2) by indirect control of cell cycle genes through de-repression of Polycomb-targets, 3) by having a role in proper chromatin setup that is required for cell cycle progression or even 4) by directly modifying cell cycle factors and thereby altering their properties. Below I have listed examples supporting each possibility.

In a bioinformatic search of PREs in *Drosophila*, authors pointed out a number of potential Polycomb targets involved in cell cycle control (Ringrose *et al.*, 2003). Functional evidence for this prediction first came from identifying Cyclin A as a bona fide PcG target in the fly (Martinez *et al.*, 2006), followed by the first study reporting genome-wide binding profiles of PcG proteins in developing *Drosophila*, which also identified cell cycle genes directly regulated by Polycomb (Oktaba *et al.*, 2008).

In mammals, the tumor suppressor *Ink4a/Arf* locus has been identified as a target of PRC. The *Ink4a/Arf* locus encodes two proteins, p16 and p19 by use of alternative reading frames. p16 and p19 are important players in the retinoblastoma (Rb) and p53 pathways, respectively and their activation results in growth arrest, senescence or apoptosis. *Bmi1* has been shown to negatively regulate the *Ink4a/Arf* locus, thereby repressing the p16 and p19 mediated senescence pathways, promoting stem cell self-renewal and maintaining of multipotency (Molofsky *et al.*, 2005). Disruption of the *Ink4a/Arf* locus in *Bmi1-/-* stem cells partially restores self-renewal capacity, indicating that inappropriate activation of the locus negatively influences stem cell fate (Jacobs *et al.*, 1999a; Lowe and Sherr, 2003; Sharpless and DePinho, 1999). Since then, the *Ink4a/Arf* locus was shown to be also targeted by a number of other PcG proteins, including Cbx4, Cbx7, Cbx8, Mel18, Rnf2, Ezh2 and Suz12 (Bracken *et al.*, 2007; Dietrich *et al.*, 2007; Gil *et al.*, 2004; Jacobs *et al.*, 1999a; Voncken *et al.*, 2003). Cbx7 was shown to extend the life span of human and mouse cells by bypassing replicative senescence through down regulation of the *Ink4a/Arf* locus (Gil *et al.*, 2004). Similarly, ectopic expression of *Cbx8* leads to repression of the *Ink4a/Arf* locus and immortalization of mouse embryonic fibroblasts (Dietrich *et al.*, 2007). Another cell cycle inhibitor, Cdkn1c (p57, Kip2) has also been shown to be a direct target of PRC2 (Yang *et al.*, 2009) and the paternally imprinted allele to be expressed upon the loss of *Eed* (Mager *et al.*, 2003), *Ezh2* or *Rnf2* (Terranova *et al.*, 2008).

Taken together, Polycomb proteins can directly regulate the expression of positive or negative (like checkpoint components) regulators of the cell cycle.

Well established Polycomb targets like *Hox* genes have previously been shown to be involved in cell proliferation control (Core *et al.*, 1997; Sauvageau *et al.*, 1994; Sordino *et al.*, 1995). In mice for example, *Hoxa1* mutants have cell growth defects (Dolle *et al.*, 1993) and *Hoxd3* has been suggested to regulate proliferation roles of precursor cells (Condie and Capecchi, 1993). In humans, *Hoxb4* expression is involved in hematopoetic stem cell proliferation (Antonchuk *et al.*, 2002). Finally, misregulation of some *Hox* genes leads to alterations in lymphocyte proliferation (Perkins *et al.*, 1990; Sauvageau *et al.*, 1994). Thus cell cycle defects in PcG mutants may be an indirect consequence of changes in *Hox* gene activities.

An observation suggesting a chromatin-based role in cell cycle regulation comes indirectly from the study of de-ubiquitintating emzymes (DUBs) (Discussed in more detail in chapter 2.6.4.9.). Disturbing the dynamics of H2AUb leads to chromosome condensation and mitotic defects in HeLa cells (Joo *et al.*, 2007), although direct PRC1 involvement in setting this Ub marks has not been demonstrated.

Finally, an interesting study reports that PRC1 can directly ubiquitinate the cell cycle regulator Geminin and therefore target it for ubiquitin-proteosome-mediated degradation (Ohtsubo *et al.*, 2008). Geminin is a central component in controlling DNA replication licensing by binding to and antagonizing the replication initiation factor Cdt1 (Melixetian and Helin, 2004; Pitulescu *et al.*, 2005). Loss of *Geminin* permits re-licensing of replication initiation complexes and leads to endoreplication in higher eukaryotes (Melixetian and Helin, 2004; Tachibana *et al.*, 2005; Zhu and Dutta, 2006a), while overexpression of *Geminin* causes

cells to accumulate in G1-phase, possibly because of induction of a licensing checkpoint (Shreeram *et al.*, 2002). In PRC1 member *Phc1* knockout embryos hematopoetic stem cell activities were eliminated, presumably due to the accumulation of Geminin and subsequent cell cycle arrest (Ohtsubo *et al.*, 2008).

A very recently published report set out to dissect the mitotic defect of Polycomb mutant Drosophila embryos (Beck *et al.*, 2010). Time-lapse imaging of PH mutant embryos showed that the absence of PcG proteins was actually accelerating S-phase by abrogating the DNA damage checkpoint, which then reflected in aberrant mitosis. Additionally Ring1b in the context of PRC1 has been shown to mediate ubiquitination of H2A in response to DNA damage, probably to facilitate chromatin relaxation or to mediate transcription pausing during repair (Bergink *et al.*, 2006).

2.6.4.7. Propagation of Polycomb-mediated marks

A feature of chromatin based epigenetics is that chromatin structure can be modified either to allow or to inhibit transcription, and that these regulatory features of chromatin structure are propagated throughout the cell cycle. Recent studies on Polycomb proteins have begun to unravel the mechanisms by which Polycomb-memory is inherited through DNA replication.

The H3K27me3 mark is a good candidate for transmission of regulatory information, as parental histones with this mark are segregated into daughter chromatin during replication. PRC2 was show to be able to bind H3K27me3 *in vitro*, moreover when targeted to a reporter gene, it was able to establish H3K27me3, recruit PRC1 and initiate gene silencing (Hansen *et al.*, 2008). Importantly, all these features were maintained at the reporter gene even after the targeted version of PRC2 was no longer expressed. This suggests that stable epigenetic silencing was established by transient PRC2 recruitment. Recently, the WD40 domain of Eed was shown to selectively bind to H3K27me3 by virtue of an aromatic cage and this interaction stimulated HMTase activity of the PRC2 complex by an allosteric mechanism (Margueron *et al.*, 2009). These studies support a model where replication segregates H3K27me3 marked nucleosomes into daughter chromosomes where it interacts with PRC2, which in turn directs methylation of newly deposited histones, maintaining high levels of histone methylation at Polycomb target sites.

Replicating chromatinised templates in a cell free system revealed that PRC1 complexes stay associated with chromatin throughout S-phase, although no further data on mechanisms for propagation is available (Francis *et al.*, 2009).

Chromosome condensation and mitosis may present further problems for transmission of epigenetic information. While chromatin is stably H3K27me3-modified during mitosis (Puschendorf *et al.*, 2008), ubiquitination of H2A needs to be removed at the onset of mitosis to allow cell cycle progression (Joo *et al.*, 2007). How is then the repressive memory mediated by H2AUb remembered? One possibility is that

the PRC1 complex stays anchored to condensed mitotic chromatin and re-ubiquitinates its target sites in anaphase (Joo *et al.*, 2007). Indeed, PRC1 members are known to stay chromatin-bound during M-phase (Buchenau *et al.*, 1998; Messmer *et al.*, 1992; Puschendorf *et al.*, 2008), however re-ubiquitination by these proteins has not been directly shown.

2.6.4.8. Other complexes containing PRC1 members

Some PRC1 members, such as Rnf1 and Rnf2 have been found to interact with other repressive complexes other than PRC1. Immuno-affinity purification of E26F interacting proteins identified Rnf1 and Rnf2 together with L3mbtl2, Yaf2, Dp1, Mga, Max, Eu-HMTase1/Glp1 and G9a (Ogawa *et al.*, 2002). The two last proteins are H3K9 HMTases, which may contribute to silencing of E2F6 target genes. Similarly, Rnf1 and Rnf2 associate with the Bcl6 co-repressor (Bcor), Nspc1, LSD1/Aof2 and Fbxl10/Jhdm1B (Gearhart *et al.*, 2006; Sanchez *et al.*, 2007). Bcor target genes are ubiquitinated *in vivo*, suggesting that Rnf1/2 are functional in this complex and may contribute to silencing of Bcor target genes. Fbxl10 is a H3K36 de-methylase, a homolog of the *Drosoplila* KDM2, but it has not been addressed whether it also functions to stimulate ubiquitin ligase activity of the complex as it does in *Drosophila*. Nevertheless, it is an interesting possibility that the histone de-methylase activities within this complex (LSD1/Aof2 and Fbxl10/Jhdm1B) may provide additional regulatory function through these chromatin-modifying mechanisms.

Therefore, it is of note that knockouts of PRC1 members may not only model Polycomb deficiency, but show a complex phenotype of deficiencies arising from loss of function of several complexes.

2.6.4.9. Reversing Polycomb-mediated marks

In contrast to histone acetylation or phosphorylation, for a long time histone methylation was considered a permanent and irreversible mark, locking epigenetic states, especially histone tri-methylation, as the thermodynamic stability of the N-CH_3 bond is very high. It was thought that the only way to remove histone methylation was by exchanging histones or by cleaving histone tails. The groundbreaking discovery of the first histone demethylase LSD1 changed this view (Shi *et al.*, 2004). LSD1 was shown to demethylate histones through a flavin adenine dinucleotide (FAD)-dependent amine oxidase reaction, a reaction in theory only capable of demethylating mono- and di-methylated states. Indeed, LSD1 has been shown to demethylate H3K4me1/2 in a complex with the repressive CoREST (Shi *et al.*, 2004) and to demethylate H3K9me1/2 in complex with the androgen receptor (Metzger *et al.*, 2005). It was only until the identification of the Jumonji (JmjC) catalytic domain containing histone demethylase family when the possibility of

reversal of the tri-methyl mark became apparent (Cloos *et al.*, 2006; Klose *et al.*, 2006b; Tsukada *et al.*, 2006; Whetstine *et al.*, 2006; Yamane *et al.*, 2006). To date a number of JmjC domain protein family members have been assigned demethylase activities. Of our interest are the two enzymes that have been shown to demethylate H3K27me2/3: Utx and Jmjd3 (Agger *et al.*, 2007; De Santa *et al.*, 2007; Hong *et al.*, 2007; Lan *et al.*, 2007; Xiang *et al.*, 2007). *In vitro*, these enzymes catalyze transition of H3K27m2/3 to H3K27me1 on bulk histones, H3K27me3 being the preferred substrate. However, their enzymatic reaction on nucleosomal substrates is very weak, indicating the need for additional binding partners for activity in the context of chromatin (Lan *et al.*, 2007). Indeed, ectopic expression of *Utx* alone seems to have little effect on K3K27 methylation levels in most cell types (Cloos *et al.*, 2008).

H3K27 demethylases have been shown to be required for normal development, as morpholino-mediated knockdown of the two zebrafish Utx homologs results in posterior abnormalities in the embryo (Lan *et al.*, 2007) and knockdown of a Jmjd3 homolog in *C.elegans* impaired normal gonad development (Agger *et al.*, 2007). Utx has also been shown to regulate *Hox* gene expression. Upon differentiation of NT2/D1 cells with retinoic acid, Utx is recruited to the promoters of the anterior genes of *HoxA* and *HoxB* loci. Utx recruitment correlates with the removal of H3K27me3, decreased occupancy of PRC2 members and gene activation. Furthermore, knockdown of Utx expression disables H3K27 demethylation and *Hoxb1* activation upon differentiation (Agger *et al.*, 2007).

Another study demonstrates transient Jmjd3 binding to *HoxA7* and *HoxA11* promoters during bone marrow cell differentiation (De Santa *et al.*, 2007). Jmjd3 is also rapidly induced in macrophages in response to an inflammatory stimulus. For example, at the promoter of the inflammatory response gene *Bmp2*, Jmjd3 levels increase, while H3K27me3 decreases and transcription is initiated in response to inflammation (De Santa *et al.*, 2007).

Taken together, these results demonstrate that H3K27 de-methylases are functioning during differentiation at known Polycomb target genes like *Hox* clusters and *Bmp2*, to counteract Polycomb silencing at genes that need to be activated.

In contrast to histone methylation, it has been clear for decades that monoubiquitination of histone H2A is a dynamic mark, as global levels vary over the cell cycle (Goldknopf *et al.*, 1980; Matsui *et al.*, 1979; Mueller *et al.*, 1985; Wunsch and Lough, 1987). To date five H2A de-ubiquitinating (DUBs) enzymes have been identified: 2A-DUB belonging to the JAMM/MPN+ family and USP3, USP-16 (Ubp-M), USP-21 and USP-22 from the Ubiquitin Specific Protease family (USP) (Joo *et al.*, 2007; Nakagawa *et al.*, 2008; Nicassio *et al.*, 2007; Zhang *et al.*, 2008; Zhao *et al.*, 2008b; Zhu *et al.*, 2007). USP-3 and USP-22 are also capable of de-ubiquitinating histone H2B. Although Rnf1/2 is known to be responsible for most H2AUb in the mammalian genome, it is likely that not all these DUBs would function to antagonize Rnf1/2.

Only studies on the USP-16 enzyme suggest that a DUB functions in counteracting Polycomb repression. Depleting USP-16 in *Xenopus* embryos lead to de-regulation of *HoxD10* expression and defects in posterior

development, (Sparmann and van Lohuizen, 2006) a phenotype consistent with Polycomb-antagonistic function. Furthermore, USP-16 knockdown in HeLa cells resulted in increased H2AK119Ub levels at the promoter and transcriptional repression of the *HoxD10* gene, a known Polycomb target (Joo *et al.*, 2007). However the function of USP-16 seems to be multi-layered, as it not only regulates gene expression, but also cell cycle progression (Joo *et al.*, 2007). As previously mentioned it is known for a long time that de-ubiquitination occurs at the G2/M transition and it is re-established in early anaphase (Goldknopf *et al.*, 1980; Matsui *et al.*, 1979). It has been shown that USP-16 is the DUB responsible for this de-ubiquitination. Depletion of USP-16 in HeLa cells causes a G2/M delay, in part due to the H2AUb mark inhibiting Aurora B binding to chromatin, therefore inhibiting H3 serine 10 phosphorylation (H3S10P), a hallmark of condensing chromatin (Joo *et al.*, 2007).

USP-21, USP-22 and 2A-DUB have also been implicated in transcriptional control; however Polycomb-links have not been investigated (Nakagawa *et al.*, 2008; Zhang *et al.*, 2008; Zhao *et al.*, 2008b; Zhu *et al.*, 2007).

H2A ubiquitination/de-ubiquitination is emerging as an important regulator of gene activity, cell cycle control and even DNA damage response. Study of DUBs can provide new insights on H2AUb function. However, to what extent this is Polycomb-relevant, remains to be addressed.

2.7. Development - the epigenetic cycle

At the onset of life, two gametes, sperm and oocyte come together to form a zygote. Both gametes are highly specialized types of cells, carrying a specialized set of epigenetic modifications. However, they are specialized for executing a program upon fusion that will produce a totipotent embryo. This program involves extensive chromatin remodeling, erasure of certain epigenetic modifications, followed by re-establishment of others (Santos and Dean, 2004). This epigenetic re-programming event is thought to account for totipotency, correct initiation of embryonic gene expression, and early lineage development in the embryo. As the embryo develops and differentiates, epigenetic modifications direct and stabilize gene expression patterns that define a more and more restricted cell fate. However, certain epiblast cells of the post-implantation embryo that already contain epigenetic modifications characteristic of differentiating cells, undergo major re-programming again to reset the epigenetic slate and allow formation of primordial germ cells (PGCs), the precursors of the future gametes. PGCs undergoing the differentiation process of gametogenesis acquire sex-specific epigenetic modifications, and will ultimately specialize into the tiny one-cell transmission programs of life (Hajkova *et al.*, 2002; Morgan *et al.*, 2005; Surani, 2001).

2.7.1. The male germ line

2.7.1.1. Spermatogenesis

Spermatogenesis is a highly regulated sequence of differentiation and maturation events that result in the formation of spermatozoa from precursor cells (Clermont, 1972). In humans the entire process takes approximately 85 days, while in mice around 35 days (de Rooij and Grootegoed, 1998). There are three major phases that together make up spermatogenesis: mitotic proliferation of spermatogonial stem cells, reduction of chromosomal number by meiosis and the morphological transformation of the haploid germ cell into a spermatozoon termed spermiogenesis (de Kretser *et al.*, 1998).

In the prenatal testis only gonocytes that have entered mitotic arrest are present (Huckins and Clermont, 1968). Shortly after birth the gonocytes resume proliferation and become spermatogonia. These stem cells divide mitotically to constantly replenish the pool of cells that will eventually enter meiosis. Spermatogonia undergo differentiation to produce primary spermatocytes that enter meiosis.

During meiosis reciprocal exchange of genetic information takes place between parental homologs, after which two subsequent divisions occur. The first division yields secondary spermatocytes that then further divide into haploid round spermatids (Clermont, 1972; de Kretser *et al.*, 1998). Meiosis in the male mouse takes about 12 days, large part of which is spent in the prophase of the first division. Prophase 1 is further broken up into four stages: leptotene, zygotene, pachytene and diplotene. During leptotene, chromosomes start to condense and DNA double stranded breaks (DSBs) start to occur. DSBs are essential for recombination between homologs that takes place later in meiosis (Cobb *et al.*, 1997; Roeder, 1997). During zygotene, sister chromatids start to pair and form synaptonemal complexes (SCs). The SC is a proteinaceous scaffold, consisting of two axial elements forming between sister chromatids and a central element that joins the axial elements as synapsis takes place (Yang and Wang, 2009). Meiotic DNA is arranged in loops that attach at their base to the axial elements. During pachytene synapsis is completed and homologous recombination takes place. In diplotene chromosomes start to separate and SCs disappear (Baarends and Grootegoed, 2003) at the end of prophase1.

In the final post-meiotic part of spermatogenesis haploid round spermatids undergo extensive cytoplasmic metamorphosis and dramatic chromatin remodeling. This involves transcriptional silencing and condensing the chromatin into a volume of about 5% of that of a somatic cell nucleus, which is assisted by DSBs and histone-to-protamine exchange (Doenecke *et al.*, 1997; Govin *et al.*, 2004; Marushige and Marushige, 1975).

2.7.1.2. Epigenetics of the male germ line

Epigenetic changes are known to accompany and to be critical for different steps of spermatogenesis. In this chapter I will discuss literature describing changes in DNA methylation and histone modifications during spermatogenesis.

Before and during early steps of spermatogenesis transposable elements are silenced to prevent genomic instability. DNA methylation is essential for transposable element silencing and the involvement of Dnmt3l (Dnmt3-like) has been demonstrated in this process (Bourc'his and Bestor, 2004; Deininger *et al.*, 2003; Hata *et al.*, 2006; Webster *et al.*, 2005). *Dnmt3l* is sequence-related to *Dnmt3a* and *Dnmt3b* but lacks enzymatic activity. It is expressed in germ cells specifically at the time when the de novo methylation occurs (Bourc'his and Bestor, 2004). In a complex with either Dnmt3a or Dnmt3b, it stimulates their activity (Suetake *et al.*, 2004).

Paternally imprinted genes are DNA methylated and silenced during germ cell development. To date three paternally imprinted regions have been identified: *H19-Igf2*, *Rasgrf* and *Dlk1-Gtl2* (Davis *et al.*, 1999; Li *et al.*, 2004). All four Dnmts have been implicated in paternal imprinting (Li *et al.*, 1993; Li *et al.*, 1992; Sasaki *et al.*, 2000).

During meiosis several aspects of chromosome condensation, pairing and recombination are dependent on epigenetic modifications. *Dnmt3l* mutants fail to form appropriate heterochromatin and therefore chromosomes fail to pair at zygotene stage (Bourc'his and Bestor, 2004; Webster *et al.*, 2005). The H3K9me3 HMTase Suv39h enzymes have also been shown to be essential for meiosis. In mice there are two paralogs *Suv39h1* and *Suv39h2*, *Suv39h2* being testis specific in the adult. Single knockout of either homolog does not affect viability and fertility; however, mice mutant for both enzymes displayed impaired spermatogenesis. Spermatocytes underwent apoptosis at the pachytene stage as a consequence of incomplete homolog pairing and synapsis (Peters *et al.*, 2001). Mutant mice for the H3K9me1/2 HMTase *G9a* are also sterile due to spermatocytes unable to proceed beyond the pachytene stage due to deficient SC formation (Tachibana *et al.*, 2007). Therefore H3K9 methylation seems to be crucial for male meiosis. Prdm9 (also known as Meisetz), a germ line specific H3K4 tri-methylase, is also crucial for synapsis and recombination of homologous chromosomes during meiotic prophase (Hayashi *et al.*, 2005). In *Prdm9*-deficient spermatocytes, a number of genes, including those that are specifically expressed in meiotic germ cells, were repressed. These results suggest that Prdm9 mediated H3K4 methylation is involved in the activation of genes important for synapsis and recombination (Hayashi *et al.*, 2005).
During meiosis, homologous recombination occurs preferentially at defined sites, known as hotspots. Crossovers generated during meiotic recombination are important for proper alignment of homologous chromosomes during metaphase of the first meiotic division and therefore for proper segregation of chromosomes. Moreover, crossovers are the central source for genetic variation among offspring.

Recently, a number of studies independently identified Prdm9 as a key factor responsible for specifying and initiating the activity of recombination hotspots in mammals (Baudat *et al.*, 2010; Myers *et al.*, 2010; Neale, 2010; Parvanov *et al.*, 2010).

2.7.1.3. Meiotic Sex Chromosome Inactivation (MSCI)

MSCI is the process of transcriptional silencing of the X and Y chromosomes during the pachytene stage of meiosis. Shortly after the zygotene-pachytene transition, when meiotic synapsis between autosomes is complete, the heterologous sex chromosomes that only homologously synapse at their pseudoautosomal regions, are rapidly silenced and compartmentalized into a peripherial nuclear subdomain termed the sex-body (McKee and Handel, 1993; Solari, 1974; Turner, 2007). Phosphorylation of the histone variant H2AX at serine residue 139 (γH2AX) is essential for MSCI. In general γH2AX plays a crucial role in DNA DSB repair (Celeste *et al.*, 2002; Rogakou *et al.*, 1999). DSBs generated for homologous recombination on autosomes during leptotene and zygotene stages also acquire this modification (Mahadevaiah *et al.*, 2001). However, at the time MSCI is initiated H2AX is rapidly and extensively phosphorylated at the sex chromosomes independent of DNA breaks (Turner *et al.*, 2005). H2AX-null mice display complete meiotic arrest associated with MSCI failure (Fernandez-Capetillo *et al.*, 2003). (H2AX phosphorylation is dependent on the DNA repair protein ATR (Ataxia telangiectasia and Rad3 related) and ATR recruitment is dependent on the tumor suppressor BRCA1 (Breast cancer 1) (Turner *et al.*, 2004). MSCI is defective in BRCA1 mutant mice, because H2AX phosphorylation does not take place on the XY chromosomes, but at ectopic sites throughout the nucleus, as a result of failure to properly localize ATR (Turner *et al.*, 2004; Xu *et al.*, 2003). Thus the linear recruitment and function of BRCA1-ATR-γH2AX act to initiate MSCI. Other post-translational modifications such as H2AUb (Baarends *et al.*, 1999; Baarends *et al.*, 2005), H3K9me2 as well as de-acetylation of histones H3 and H4 (Khalil *et al.*, 2004) may serve in the maintenance of MSCI, as they are established shortly after MSCI initiation, but then remain associated with XY chromosomes throughout meiosis and into spermiogenesis, long after γH2AX is gone. Over the past years it has become apparent that MSCI is a consequence of synaptic failure and is therefore part of a more general meiotic silencing mechanism termed Meiotic Silencing of Unsynapsed Chromatin (MSUC). Inducing unsynapsis at autosomal regions using a mouse line with an X-16 reciprocal translocation showed that indeed, unsynapsed chromatin is inducing binding of BRCA1, ATR, γH2AX and silencing (Turner *et al.*, 2005). Even female mice with only one X undergo MSCI at unsynapsed chromatin (Speed, 1986). Furthermore providing the normally unsynapsed chromatin with a synaptic partner, like in XYY male mice, lead to the YY chromosomes escaping MSCI (Turner *et al.*, 2006).

Despite previous debates, it is now clear that, at least in part, silencing of XY chromosomes persists throughout spermatogenesis, a process known as post-meiotic sex chromosome repression. Examining X

chromosome transcriptional activity using microarrays revealed that approximately 87% of X-linked genes are repressed in the post-meiotic period (Namekawa *et al.*, 2006). This correlates with previous observations that modifications characteristic of heterochromatin stay associated with sex chromosomes after meiosis (Khalil *et al.*, 2004). It has also been suggested that this repressed stated is carried over to the next generation, providing the basis for non-random paternal X chromosome inactivation in the early embryo (Huynh and Lee, 2003). This model however is highly debated, as other studies argue that paternal X inactivation only takes place in the early embryo in a *Xist*-dependent manner (Okamoto *et al.*, 2005) or as recently proposed, in *Xist*-independent manner (Kalantry *et al.*, 2009).

Inactivation of the XY chromosomes during meiosis is intriguing and many questions remain regarding this phenomenon. It is clear that MSCI is essential for male fertility, but why sex chromosomes have to be silenced during meiosis is still poorly understood.

2.7.1.4. Histone-to-protamine exchange and the unique chromatin of the spermatozoon

The high degree of condensation of spermatid chromatin during spermiogenesis is achieved by replacing histones by protamines (Marushige and Marushige, 1975). The histone-to-protamine exchange involves replacement of histones with transition proteins (TP1 and TP2) that are subsequently replaced by protamines. This exchange process is associated with hyperacetylation of of histone H4, which was proposed to promote a looser nucleosomal structure facilitating exchange (Hazzouri *et al.*, 2000; Sonnack *et al.*, 2002).

Mice mutant for either TP1 or TP2 are fertile with only subtle abnormalities in chromatin packaging during spermiogenesis (Yu *et al.*, 2000; Zhao *et al.*, 2001). However, mice deficient for both TPs exhibited severe spermatogenesis defects. Defective chromatin condensation and DSBs were noted, which resulted in male sterility (Zhao *et al.*, 2004).

Protamines are small basic proteins rich in arginine and cysteine and are specific to spermatids (Wouters-Tyrou *et al.*, 1998). Mouse and human genomes encode for two different protamine (Prm) molecules, Prm1 and Prm2. Both are encoded by relatively short genes comprising two exons. *Prm2* encodes a precursor protein that binds to DNA and undergoes proteolytic processing during the last stages of spermatid elongation. The mature forms of Prm1 and Prm2 bind to 10 and 15 base pairs of DNA, respectively. This binding neutralizes the negative charge of the DNA backbone and enables the DNA molecules to pack closely together. In a final step, which is happening after the spermatozoa leave the testis and proceed through epididymis, a network of bisulfate bonds is formed between the adjacent protamine molecules leading to a very compact arrangement (Balhorn, 2007). Disruption of one copy of either gene, i.e. haploinsufficiency for either protamine gene disrupts nuclear formation, processing of

Prm2 and sperm function (Cho *et al.*, 2003). In humans even the ratio of Prm1:Prm2 is important, as perturbation of the normally 1:1 ratio is associated with male infertility (Balhorn *et al.*, 1988; de Yebra *et al.*, 1993). Furthermore, the JmjC-domain containing H3K9me1/2 de-methylase Jhdm2A was shown to at least in part regulate activation of *TP1* and *Prm1* genes (Okada *et al.*, 2007). Jhdm2A binds directly to *TP1* and *Prm1* genes, and is responsible for the reduction of H3K9 methylation at their promoters. In the absence of Jhdm2A the promoters remain H3K9 methylated and silenced, causing defects in spermatid elongation, abnormal nuclear morphology and infertility (Okada *et al.*, 2007).

Expression of both TPs and protamines is tightly regulated during spermatogenesis. Global transcription ceases along with the chromatin condensation. Therefore, transcription and translation of protamines are uncoupled. Protamine transcription starts in transcriptionally active round spermatids and the transcripts are stored in the cytoplasm as messenger ribonucleoprotein particles. They are activated for translation in elongated spermatids only one week later (Braun, 2000).

The histone-to-protamine exchange process is not complete, 1% of nucleosomes in mouse and up to 10% in humans are retained (Tanphaichitr *et al.*, 1978; Wykes and Krawetz, 2003). The role of these retained nucleosomes is not understood, but it was speculated that they may be mere remnants of incomplete histone-to-protamine exchange or that they may have a biologically significant role in transgenerational epigenetic inheritance. Recent studies, including one from our lab, have demonstrated that retained histones in mouse and human sperm are not simply randomly distributed remains of inefficient protamine replacement, but are associated with specific promoters (Arpanahi *et al.*, 2009; Brykczynska, in press.*;* Hammoud *et al.*, 2009). Moreover these histones can carry PTMs such as the active H3K4me2 or the Polycomb mediated repressive H3K27me3 that occupy functionally defined groups of genes. H3K4me2-marked promoters control genes with functions in spermatogenesis and cellular homeostasis, suggesting that this mark reflects germline transcription. By contrast, multiple developmental regulators, which are Polycomb targets in pluripotent somatic cells, are marked by H3K27me3 in human sperm. Similarly to somatic cells, the presence of this mark correlates with gene repression during spermatogenesis and in the early embryo. These data suggest a model in which H3K27me3, transmitted by sperm, assures repression of developmental regulators at the totipotent stage of the pre-implantation development (Brykczynska, in press.). Functional testing of this model is a current project in our laboratory.

2.7.2. The female germ line

2.7.2.1. Oogenesis

Oogenesis begins with the formation of primordial germ cells in the post-implantation embryo that migrate into the genital ridge. Once established in the prospective ovary, primordial germ cells lose their motility and go through several rounds of mitosis to create a large supply of oogonia. In the mouse, oogonia enter meiosis (hereafter referred to as oocytes) at approximately 13.5 dpc, pass through leptotene, zygotene and pachytene stages of meiotic prophase 1 before arresting at diplotene (referred to as dictyate arrest) (Pepling, 2006). This is in contrast to male germ cell development, where gonocytes halt in mitosis. Oocytes remain in this cell cycle stage until end of folliculogenesis. Coincident with the initiation of meiosis, oocytes become enclosed in a single layer of squamous pregranulosa cells resting on the basement membrane. This nest of cells together with the resting oocyte in the center is called the primordial follicle; the oocyte itself is classified as the primordial oocyte. Primordial oocytes remain resting until recruitment into the growing population starting a few days after birth. The dictyate arrest is characterized by 4C DNA content (four times the haploid amount), paired homologous chromosomes in a fully extended conformation and active transcription (Bukovsky *et al.*, 2005). The mechanism by which primordial follicles are maintained or leave the resting pool and start the growing phase is not well understood. Follicle activation is initiated with granulosa cell proliferation and their change in shape to a cuboidal form (Braw-Tal, 2002; Hirshfield, 1991). In the mouse ovary, the first wave of oocyte growth is thought to be initiated synchronously shortly after birth. As oocytes progress through primary, secondary and different antral stages, granulose cells proliferate, eventually reaching 6-7 layers around the oocyte (Fortune, 2003). In growing follicles granulose cells become metabolically coupled with the oocytes through gap junctions that are of vital importance to oogenesis. Follicle activation also triggers the production and secretion of the oocyte specific Zona pellucida (ZP). The ZP is a glycoprotein membrane formed in the perivitelline space of the follicle that consists of three glycoproteins: ZP1, ZP2 and ZP3. The zona provides a protective coat around the oocyte and has a key role in fertilization (Litscher *et al.*, 2009; Philpott *et al.*, 1987; Ringuette *et al.*, 1988). Numerous studies have been carried out to identify factors responsible for folliculogenesis, but the notion that the oocyte is the main driving force has been gaining acceptance (Eppig, 2001; Fair, 2003). During the growth phase the oocyte increases its volume more than 100-fold (Gougeon, 1996). This growth is not a simple process of increasing mass, but involves qualitative and quantitative changes in key molecules for metabolism, structure and information that are fundamental for acquiring meiotic competence (i.e. the ability to resume meiosis and progress to and arrest at metaphase-II) (Sorensen and Wassarman, 1976; Wickramasinghe and Albertini, 1992; Wickramasinghe *et al.*, 1991) and developmental competence (i.e. the ability to be fertilized and develop to term) (Eppig and Schroeder, 1989).

The most striking change is the burst of transcription in the oocyte following follicle activation, which is sustained until the end of folliculogenesis. Increased transcription is also associated with increased protein synthesis, in part to meet the needs of the rapidly growing oocyte. However, a great portion of mRNAs made at this stage are stored and will only be recruited for translation during meiotic maturation or after fertilization (Bachvarova, 1985; Bachvarova *et al.*, 1985). Transcription is rapidly shut down when the oocytes reach the fully grown Germinal vesicle (GV) stage (Bouniol-Baly *et al.*, 1999; Liu and Aoki, 2002; Miyara *et al.*, 2003).

2.7.2.2. Epigenetics and chromatin in the female germ line

Chromatin in growing mouse oocytes is initially decondensed in a configuration termed Non-surrounded-nucleolus (NSN). During the last stage of oocyte growth major chromatin remodeling takes place, condensing the chromatin around the nucleolus and resulting in oocytes with Surrounded-nucleouls configuration (SN) (Bouniol-Baly *et al.*, 1999; De La Fuente and Eppig, 2001). Although transcription does correlate with chromatin configuration: NSN oocytes being transcriptionally highly active and SN oocytes virtually transcriptionally silent (Bouniol-Baly *et al.*, 1999; Miyara *et al.*, 2003), further studies have shown that chromatin remodeling is not required for transcriptional shut-down. For example, oocytes of mice mutant for the chromatin remodeler Nucleoplasmin 2 (Npm2) fail to condense into the SN configuration, but still achieve transcriptional silencing (De La Fuente *et al.*, 2004a).

In addition, the transition into SN configuration also correlates with timely progression of meiotic maturation (Debey *et al.*, 1993; Wickramasinghe *et al.*, 1991) and when fertilized and further cultured, SN oocytes readily developed to bastocyst stage, while embryonic development of NSN oocytes was comprised (Zuccotti *et al.*, 1998). These results demonstrate that changes in large-scale chromatin structure are essential to confer growing oocytes with meiotic and developmental competence.

Other chromatin-based processes, like epigenetic modifications have also been described to play important roles in the regulation of oocyte chromatin structure. Histone acetylation (H3K9, H3K18; H4K5 and H4K12) was shown to actually increase during oocyte growth, fully grown oocytes having most modifications, despite that acetylation is associated with relaxation of chromatin (De La Fuente, 2006; Kageyama *et al.*, 2007; Kim *et al.*, 2003a). Surprisingly, treatment of SN oocytes with an HDAC inhibitor, trichostatin A (TSA) resulted in decondensation of primarily euchromatic regions of chromatin (De La Fuente *et al.*, 2004a). Furthermore, this decondensation did not restore transcriptional activity. In addition, overexpression of Hdac6 in GV oocytes induced premature and even over condensation of chromatin (Verdel *et al.*, 2003). Two conclusions can be drawn from these studies: first, the appropriate balance of acetylation-deacetylation has to be orchestred to achieve proper chromatin condensation and second, that acetylation and as previously pointed out, chromatin compaction is not associated with transcriptional activity.

HDACs also regulate critical aspects of chromatin remodeling during meiosis, as a wave of genome-wide deacetylation was shown to coincide with resumption of meiotic maturation (De La Fuente *et al.*, 2004a; Kim *et al.*, 2003a; Sarmento *et al.*, 2004). Specifically, deacetylation of the H4K5 residue was essential to allow the chromatin remodeler ATRX to bind to centromeric heterochromatin in condensed chromosomes (De La Fuente *et al.*, 2004b). Inhibition of HDACs by TSA resulted in hyperacetylated chromosomes with no ATRX binding, which in turn lead to abnormal chromosome alignments at metaphase-II (M-II) (De La Fuente *et al.*, 2004b).

2.7.2.3. Transcription during oocyte growth

As mentioned before, oocytes have the unique capacity to build up a program that will sustain further development such as meiotic maturation and early embryogenesis. Essential part of this program is production of maternal transcripts during oocyte growth (**Figure 2**). Over the past years considerable effort was made to characterize this maternal transcriptome. A number of studies performed expression profiling of oocytes and different stages of pre-implantation embryogenesis. By drawing out expression profiles for individual genes across different stages of development, sets of maternal transcripts with distinctive degradation patterns during pre-implantation development were identified.

In one of the first reports published these maternal transcripts were associated with Gene Ontology (GO) terms such as “circadian rhythm”, “M-phase of mitotic cell cycle”, “DNA replication”, ”DNA repair”, “Golgi-apparatus/intracellular protein transport”, “intracellular signaling cascade”, among others (Hamatani *et al.*, 2004). This shows that that the oocyte is already equipped with the transcripts that will produce the machinery to conduct essential processes in the embryo. Moreover, another simultaneously published study found maternal transcripts with predicted function in regulating polarity during pre-implantation embryogenesis (Wang *et al.*, 2004c).

Further insight on transcription during oocyte growth came from a report in which they performed global gene expression profiling of different stages of oocyte development, comparing transcriptomes of primordial, primary, secondary, small antral and large antral follicle oocytes (Pan *et al.*, 2005). Hierarchical clustering analysis revealed that primordial oocytes have the most distinct transcription profile compared to any other oocyte stage. This major transition from primordial to primary oocyte is also reflected by the fact that ~50% of genes detected at these stages show an increase or decrease in transcription level (Pan *et al.*, 2005). This transition likely reflects the dramatic reorganization in follicle structure and initiation of growth and development. Another apparent transition occurs between secondary and small antral follicle oocytes, which corresponds to the acquisition of meiotic competence (Sorensen and Wassarman, 1976; Wickramasinghe and Albertini, 1992; Wickramasinghe *et al.*, 1991). Biological themes such as “protein synthesis”, “DNA repair” and “DNA damage response” are associated

with genes expressed throughout oocyte development. The primordial-to-primary oocyte transition includes changes in genes associated with “secreted proteins” involved in many key signaling pathways, “cell cycle” and “chromatin assembly or disassembly”, while the transition from secondary to small antral oocyte is specific for “microtubule-based processes”. These categories represent functions needed for the specialized development of oocytes as well as functions only needed in later development, again confirming the notion that the oocyte is a stock house for maternal transcripts essential for later development (Pan *et al.*, 2005).

2.7.2.4. Meiotic maturation

Follicular response to a surge of LH initiates ovulation, by stimulating granulose cells to shift from estrogen to progesterone production. Granulose cells also start to produce hyaluronic acid which leads to the mucification and expansion of the cumulus granulose cells and the disruption of gap junction contacts. The observation that oocytes removed from the ovary spontaneously resume meiosis lead to the conclusion that the follicle provides an inhibitory signal for cell cycle progression (Pincus and Enzmann, 1935; Tsafriri, 1979). Indeed, 3’,5’-cyclic adenosine monophosphate (cAMP) provided to the oocytes from the surrounding cells via gap junctions was identified as the “meiotic arrestor”. Loss of intercellular contacts upon ovulation or isolation lowers cAMP levels in oocytes which initiates exit from the dictyate arrest (Dekel and Beers, 1978; Dekel and Beers, 1980). Resumption of meiosis commences with breakdown of the nuclear envelope, also known as Germinal vesicle breakdown (GVBD) and chromatin condensation. Oocytes progress through a prolonged (6-10 hours) metaphase-I (M-I), extrude the first polar body (PB) and without chromosome decondensation or replication proceed to M-II, where they become arrested again awaiting fertilization (**Figure 2**). The two M-phases of meiotic maturation are regulated differently to assure proper separation of maternal chromatin and appropriate maturity of the oocyte to support further development following fertilization. The cell cycle components involved in these processes are well described and excellently reviewed in *Kubiak et al 2008* (Kubiak *et al.*, 2008).

2.7.2.5. Translational regulation in oocytes

The stockpile of maternal messages that insures rapid development during later stages in the absence of transcription comes with a catch: the translation of these mRNAs must be prevented during their synthesis and storage during oogenesis, and then translation must be activated at specific time points of egg maturation or early embryonic development. The oocyte therefore needs a fine-tuned translation regulation

system. Mechanisms of translational regulation in oocytes have been extensively studied in *Drosophila* and *Xenopus*, but these basic mechanisms seem to be well conserved in mammals as well.

The best described regulatory mechanism is regulation through the 3' poly(A) tail. The translational potential of maternal mRNAs is determined by the length of the poly(A) tail. Longer poly(A) tails (80-500 nucleotides) are associated with translational recruitment, while short tails (40-60 nucleotides) are linked to transcriptional repression (Piccioni *et al.*, 2005).

The 3' poly(A) tail is believed to mediate circularization of the mRNA mediated by interactions between 3' and 5' binding proteins. The poly(A)-binding protein (PABP) interacts with eIF4G, a member of the pre-initiation complex on the 5' leading to the stabilization of the whole eIF4F pre-initiation complex. Poly(A) tail shortening thus leads to the destabilization of the pre-initiation complex (de Moor *et al.*, 2005).

Accurate regulation of translation through poly(A) tail length requires the coordinated action of the poly(A) polymerase and of the deadenylation complex. These proteins are recruited to the target mRNA by sequence specific factors that bind elements in the 3'UTR, providing a mechanism by which poly-adenylation can be regulated.

Polyadenylation requires two elements: the hexanucleotide AAUAAA (Hex) (Sheets *et al.*, 1994), which is bound by CPSF (cleavage and polyadenylation specificity factor) (Dickson *et al.*, 1999) and the nearby Cytoplasmic polyadenylation element (CPE), which recruits CPEB (CPE-binding protein) (Hake and Richter, 1994; McGrew *et al.*, 1989). Activated (phosphorylated) CPEB binds CPSF, which in turn recruits the poly(A) polymerase GLD-2 (Germ line deficient 2) and decreases the affinity for the deadenylation complex (Kim and Richter, 2006; Mendez *et al.*, 2000; Rouhana *et al.*, 2005).

Translation of mRNAs can be blocked by what's called "masking". This involves binding of proteins to the 3'UTR of messages that inhibit translation by blocking lengthening of the poly(A) tail (Tadros and Lipshitz, 2005). This inhibition requires unphosphorylated CPEB, which recruits an eIF4E-binding factor named Maskin, precluding the interaction of eIF4G with eIF4F and therefore the 40S ribosomal subunit recruitment (Stebbins-Boaz *et al.*, 1999). Alternatively, CPEB can bind 4E-T, which in turn recruits the oocyte specific eIF4E isoform (eIF4loo in mouse), suggesting an oocyte specific version for CPEB-mediated mRNA repression (Evsikov *et al.*, 2006; Minshall *et al.*, 2007).

Finally, another *trans*-acting factor required by some repressed mRNAs is Pumilio, which binds to PBEs (Pumilio binding element) in the 3'UTR and interacts with CPEB to participate in translational repression.

Recently, two interesting studies in *Xenopus* defined a combinatorial code based on the elements present in the 3'UTRs of messages, the position of elements along the 3'UTR and the distance between the binding sites to predict differential translational behavior of mRNAs during meiotic maturation. Furthermore, they identify positive and negative feedback loops that generate waves of polyadenylation and deadenylation,

creating a translational regulatory circuit that drives meiotic progression (Belloc and Mendez, 2008; Pique *et al.*, 2008).

As pointed out before, these mechanisms were primarily identified and studied in *Xenopus* and *Drosophila*. An example, to demonstrate that poly(A)-mediated mechanisms also exist in the mouse, comes from expression profiling of GV and M-II mouse oocytes. When expression profiles were compared, a large number of genes showed up-regulation between the two stages, despite the well known fact that there is no transcription during meiotic maturation (Bachvarova, 1985; Bachvarova *et al.*, 1985). This "increase" in transcription was due to the technique used for RNA amplification, which involves annealing between the poly(A) tail and an oligo-(dT) primer. The elongation in the poly(A) tail of mRNAs was responsible for increased efficiency of annealing and therefore the detection of such transcripts (Wang *et al.*, 2004c).

Indeed, another study also comparing expression profiles of mouse GV and M-II oocytes but using amplifying primers that are unbiased towards the poly(A) tail length, basically found only message loss during maturation; 98.5% of transcripts changing between the two stages being down-regulated. GO analysis showed that transcripts involved in processes that are associated with meiotic arrest at the GV-stage and the progression of oocyte maturation, such as oxidative phosphorylation, energy production and protein synthesis and metabolism were dramatically down-regulated. In contrast, transcripts encoding participants in signaling pathways essential for maintaining the unique arrested M-II stage, such as protein kinase pathways, were the most prominent among the stable transcripts (Su *et al.*, 2007).

Furthermore, the importance of poly(A) tail length-mediated regulation in mammals is also highlighted by polysome analysis performed in mouse oocytes and zygotes. This study provides direct analysis of transcripts that are utilized at these stages, demonstrating that a large portion of mRNAs are differentially associated with polysomes at each stage (Potireddy *et al.*, 2006).

A very recent study by the lab of Petr Svoboda, proposes means by which maternal transcripts are stored in the oocyte until recruitment (Flemr *et al.*, 2010). They identify a novel type of RNA granule in oocytes, which is related to Processing bodies (P-bodies). P-bodies are cytoplasmic ribonucleoprotein particles that are the sites of translational repression and mRNA decay in somatic cells. P-bodies were also detected in the cytoplasm of growing, meiotically incompetent oocytes, however they disappear by the end of oocyte growth. Instead, sub-cortical accumulation of granules is observed that contain maternal messages and RNA binding proteins, some of which are shared with P-bodies, such as Ddx6, CPEB, Ybx2 Msy2 and the exon junction complex. These granules disperse during oocyte maturation, consistent with the recruitment of maternal mRNAs that occur at this time. In contrast to P-bodies, a component of the decapping complex Dcp1a is not associated with these sub-cortical oocyte granules. Dcp1a levels however increase during meiotic maturation, which correlates with the first wave of maternal message destabilization (Flemr *et al.*, 2010).

Another interesting type of transcript regulation in oocytes that involves small regulatory RNAs has emerged in the past few years.

MicroRNAs (miRNAs) have been identified in oocytes by several groups (Murchison *et al.*, 2007; Tang *et al.*, 2007; Watanabe *et al.*, 2006). MiRNAs are single-stranded RNA molecules of about 21-23 nucleotides in length with 3' two-nucleotide overhangs and are complementary to sites in the 3'UTR of their target messages (Murchison and Hannon, 2004). MiRNAs are first transcribed as primary transcripts (pri-miRNA) and processed to 70 nucleotide stem-loop structures known as pre-miRNAs by the RNase III nuclease Drosha. These pre-miRNAs are further processed to mature miRNAs by the endonuclease Dicer which also initiates the formation of the Argonaut (ago)-containing RNA-induced silencing complex (RISC). The small RNA directs the RISC complex based on sequence homology to target mRNAs, which are subsequently translationally silenced or cleaved.

Do miRNAs influence transcript regulation in oocytes? Conditional deletion of *Dicer* in growing oocytes results in loss of most maternal miRNAs. Moreover, maternal *Dicer* mutants are sterile. The mutant oocytes mature and undergo GVBD but are defective in meiotic spindle organization (Murchison *et al.*, 2007; Tang *et al.*, 2007). Some *Dicer* mutant oocytes are capable to support fertilization but do not progress through the first cell cycle (Tang *et al.*, 2007). Microarray analysis showed that in the absence of Dicer transcript levels of more than one-third of genes expressed in oocytes increased. Interestingly, the up-regulated set includes a number of genes involved in microtubule associated processes which might explain the observed chromosome segregation defect. However, the 3'UTRs of transcripts up-regulated in *Dicer*-deficient oocytes were not enriched for predicted miRNA binding sites.

This finding prompted other labs to further investigate the importance of miRNA-mediated regulation in oocytes. A recent study examined oocytes lacking *Dgcr8* (Suh *et al.*, 2010). Dgcr8 is an interacting partner of Drosha, the RNase that cleaves pri-miRNAs to release hairpin-shaped pre-miRNAs that are subsequently cut by the cytoplasmic RNase Dicer to generate mature miRNAs. The Drosha-Dgcr8 complex, unlike Dicer, is only required for miRNA biogenesis, but not the RNAi pathway. Maternal *Dgcr8*-deficiency was shown to result in a similar loss of miRNAs as in *Dicer* knockout oocytes, yet the transcriptome of *Dgcr8*-/- oocytes was more similar to wild-type. Moreover, *Dgcr8* is not a maternal effect gene, as viable offspring can be produced from mutant oocytes (Suh *et al.*, 2010). Another study tested the ability of endogenous miRNAs to mediate cleavage or translational repression of reporter mRNAs in oocytes (Ma *et al.*, 2010). They found that miRNAs only poorly repress translation of reporter mRNAs and their mRNA degradation activity was even more inefficient. Together, these findings point towards the idea that not miRNAs, but rather siRNAs may control transcript regulation in mouse oocytes.

Two very recent, simultaneous studies showed that uniquely, mammalian oocytes produce endogenous small interfering RNAs (siRNAs) and regulate transcripts through RNA interference (RNAi) (Tam *et al.*, 2008; Watanabe *et al.*, 2008).

In contrast to the miRNA pathway, RNAi is induced by long dsRNA. The two pathways are closely related and some of the components, such as Dicer are shared. Before these studies, endogenous siRNA mediated gene regulation has only been observed in organisms possessing RNA-dependent RNA polymerase activity, a feature which is absent in mammals (Ambros, 2004; Pak and Fire, 2007; Vaucheret, 2006).

In mouse oocytes, inverted repeat structures, bidirectional transcription and antisense transcripts from various loci are sources of dsRNA; such loci were shown to be either retrotransposons or pseudogenes.

Antisense pseudogene (nonfunctional homolog) transripts were able to hybridize to mRNAs of homologous protein coding genes. Moreover, examining *Dicer* knockout oocytes revealed genes with abundant, pseudogene-derived siRNAs showed significant increase in expression. Thus the siRNA pathway is functional in mouse oocytes and is involved in retrotransposon and protein-coding transcript regulation (Tam *et al.*, 2008; Watanabe *et al.*, 2008).

Taken together, it seems that the previously hypothesized miRNA pathway only has a weak impact on the maternal transcriptome, and the transcriptional misregulation in *Dicer* knockout oocytes can mainly be attributed to disruption of the siRNA pathway.

2.7.3. Fertilization and early embryonic development

Fully matured oocytes (M-II) are arrested in their cell cycle and show low metabolic activity (Acevedo and Smith, 2005). Triggered by external stimuli, oocyte activation initiates a number of events, which brings the oocyte out of its “dormant” state. In mammals, fertilization by a single sperm is responsible for activation. Sperm entry initiates Ca^{2+} spiking, a signal that is both necessary and sufficient to induce oocyte activation (Jones, 1998). Ca^{2+} oscillations lead to activation of a signaling cascade that triggers resumption of cell cycle progression (Jones, 2005). Meiosis II is completed by another asymmetric division that produces the secondary PB, rendering the maternal genome haploid. The paternal genome undergoes major remodeling events and eventually, like the maternal genome, forms a nucleosomal-configured pronucleus (PN). Initially both PN are small in size and are far apart from each other. As zygotic development proceeds, the PN undergo decondensation (the paternal one always being bigger) and move towards each other to the center of the embryo. Based on morphology, zygotic substages have been defined (Adenot *et al.*, 1997; Santos *et al.*, 2002). PN0 refers to the zygote immediately after fertilization characterized by maternal chromosome segregation and paternal sperm decondensation, PN1 pronuclei are small and reside at the periphery of the embryo, PN2 pronuclei have an increased size and have started to migrate toward the center of the embryo, PN3 pronuclei have migrated toward the center, large PN4 pronuclei are close to each other and PN5 refers to large central pronuclei. A striking feature of this phenomenon is that maternal and paternal genomes will replicate in two physically distinct compartments. At the end of zygotic development, the pronuclear membranes break down and parental

genetic material fuses during the first mitotic division. However maternal and paternal genomes will only become fully intermingled by the 8-cell stage (Mayer *et al.*, 2000b; Puschendorf *et al.*, 2008) (**Figure 2**).

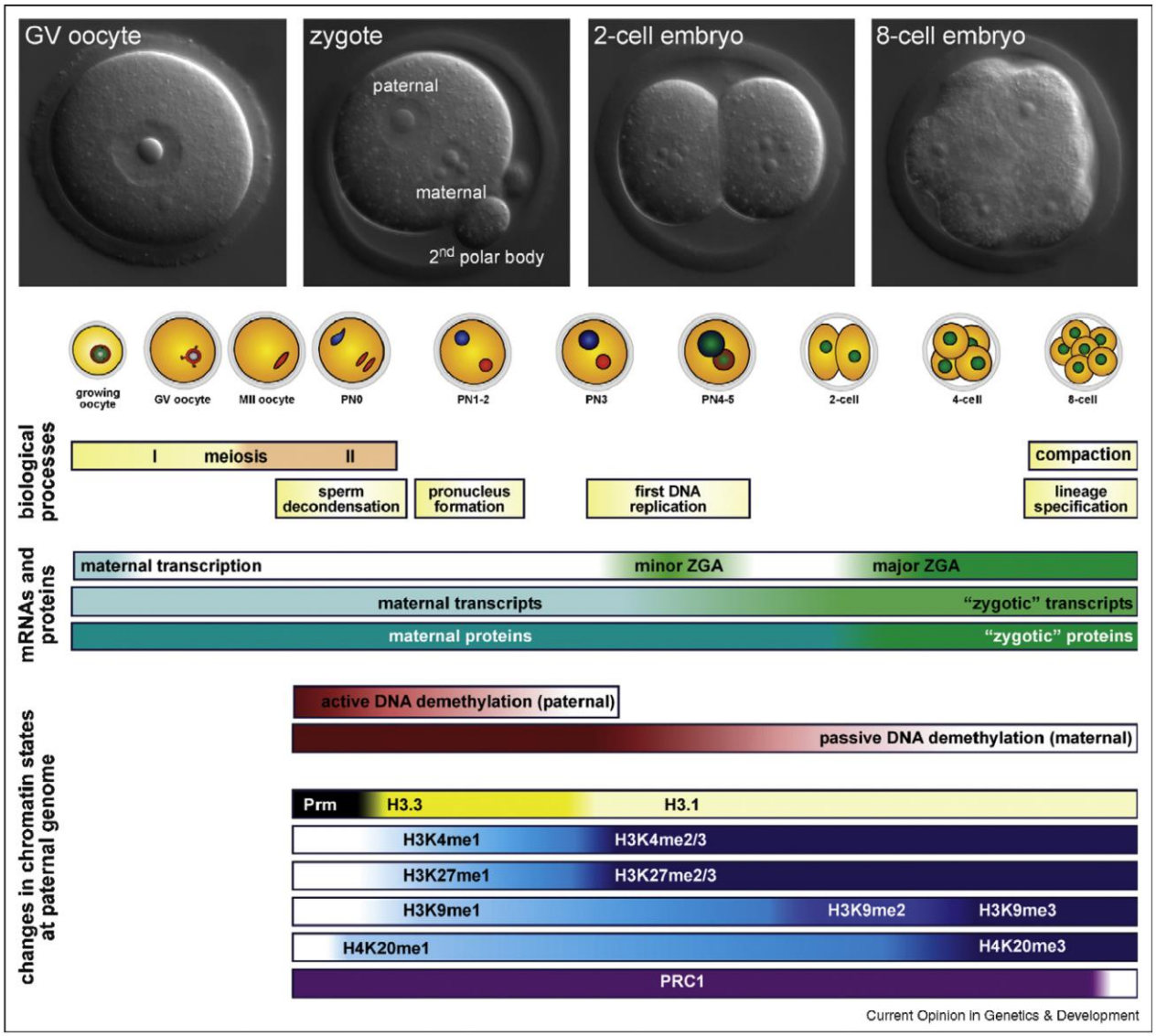

Figure 2. Epigenetic events during mouse pre-implantation development.
(Adapted from (Albert et al. 2009.))

2.7.3.1. Epigenetics of early development

At the time of fertilization, there is a marked difference in the way the genomes of the sperm and oocyte are organized, due to differential epigenetic programming during spermatogenesis and oogenesis (Sasaki and Matsui, 2008). The oocyte DNA comes in a nucleosomal configuration with a full arsenal of PTMs on histones, while the sperm DNA is tightly packed in protamines with only 1-10% of histones remaining. Upon fertilization, the sperm nucleus undergoes extensive remodeling in the oocyte cytoplasm (**Figure 2**). This chromatin reorganization can be divided into different phases, first dispersion, followed by rapid recondensation is observed as the nuclear envelope is removed and various cytoplasmic factors alter the highly compacted sperm nucleus. The final phase encompasses drastic expansion of the paternal genome (along with the maternal PN) (Adenot *et al.*, 1991; Nonchev and Tsanev, 1990). The first two phases are concomitant with the replacement of protamines with histones of maternal origin.

As this histone incorporation takes place in the absence of DNA replication, it is not surprising that the replication independent (RI) H3 variant H3.3 is specifically incorporated into paternal chromatin (Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005) (**Figure 2**). A similar pattern of asymmetric localization is observed for the H3.3 specific chaperone HIRA. Canonical H3 histones will only be assembled onto paternal chromatin during the first S-phase (van der Heijden *et al.*, 2005).

It has also been documented that the early paternal PN is enriched for acetylated histones in contrast to the maternal PN, which shows gradual accumulation of acetylation only later (Adenot *et al.*, 1997). The decondensing paternal genome already contains certain acetylated H4 histones (H4K8 and H4K12), indicating that some of these marks are inherited from sperm (van der Heijden *et al.*, 2006). Moreover, it has also been shown that histones are deposited onto paternal DNA in a pre-acetylated form on different residues (H4K5, H4K12, H4K16, H3K9, H3K14 and H3K18) (Sobel *et al.*, 1995; van der Heijden *et al.*, 2006). Histone acetylation is associated with a relaxed, transcriptionally active chromatin state (Eberharter and Becker, 2002), which may also account for the more expanded configuration of the paternal PN and the fact that the paternal PN supports a relatively higher transcriptional activity than the maternal PN (Aoki *et al.*, 1997). Supporting this hypothesis, it has been shown that treatment of mouse zygotes with HDAC inhibitors up-regulated the activity of an ectopic promoter to the levels observed in the male PN (Wiekowski *et al.*, 1993).

Differential acetylation levels persist between parental genomes until resolution during the mid zygote stage. At subsequent embryonic stages, histone acetylation remains high during interphase but is absent from cleavage chromosomes (Kim *et al.*, 2003a; Sarmento *et al.*, 2004).

Regarding other histone modifications, newly incorporated histones into the male genome are generally hypomethylated, whereas maternal chromatin displays abundant methylation, adding to the epigenetic asymmetry between the two genomes (**Figure 2**). Di- and tri-methylation of H3K4, H3K9, H3K27 and

H4K20 are initially only detected (by immunofluorescent staining) in the maternal PN. The earliest methylation mark that appears on paternal chromatin is H4K20me1 which is detected strikingly fast after gamete fusion, in the periphery of the expanding sperm nucleus (van der Heijden *et al.*, 2005). Following mono-methylation of H3K4, H3K9 and H3K27 appear after paternal PN formation (PN1/2), resulting in equal levels at both parental genomes. Generally, the acquisition of histone methylation seems to follow a strict spatially and temporally coordinated program with mono-methylation marks appearing first, followed by di- and tri-methylation.

Interestingly, the Polycomb proteins Ezh2 and Eed that confer the di- and tri-methylation of H3K27 are present very early in the paternal pronucleus (at PN1/2) but nonetheless H3K27me3 only appears around PN3/4 concurrent with DNA replication (Erhardt *et al.*, 2003; Santos *et al.*, 2005). The H3K27 mono-methylase has not been identified in mammals; however, since H3K27me1 is already present at PN1 in paternal chromatin, lack of substrate for PRC2 cannot be the limiting factor. Absence of other essential PRC2 complex members or post-translational modifications on PRC2 itself may explain the catalytic inactivity (Cha *et al.*, 2005).

Whereas H3K4 and H3K27 tri-methylation states are acquired on the paternal genome until the end of the first cell cycle (Lepikhov and Walter, 2004; Santos *et al.*, 2005; Sarmento *et al.*, 2004), acquisition of H3K9me2 and H3K9me3 is further delayed until the 4- to 8-cell stage (Lepikhov and Walter, 2004; Liu *et al.*, 2004; Merico *et al.*, 2007; Santos *et al.*, 2005; Yeo *et al.*, 2005) (**Figure 2**). H3K9me2 on the maternal genome, which is inherited from the oocyte, declines from the 1- to the 2-cell stage, indicating that no active H3K9me2 HMTase is present in the zygotic cytoplasm to maintain H3K9me2 during DNA replication (Liu *et al.*, 2004; Yeo *et al.*, 2005). In contrast, nuclear transfer of the unmethylated paternal PN into enucleated GV or M-II oocytes allows *de novo* H3K9 methylation to occur suggesting that the oocyte cytoplasm but not the embryonic cytoplasm contains H3K9 HMTase activity. Moreover, inhibition of protein synthesis with cyclohexamid in the zygote induces H3K9me2 at the paternal genome, suggesting that the H3K9me2 HMTase is active before fertilization but is deactivated by a newly synthesized protein in the early embryo (Liu *et al.*, 2004). Alternatively, a histone demetylase may be involved in antagonizing H3K9 HMTase activity specifically in the embryo (Agger *et al.*, 2008). Indeed, the KDM4 subgroup of demethylases known to demetylate H3K9 are expressed in the embryo (Katoh and Katoh, 2007). It remains to be resolved whether the stepwise appearance of the various histone lysine methylation marks simply reflects the consecutive mode of action of the mono-, di- and tri-HMTs or whether is it used as a means to distinguish the parental genomes.

A striking parental asymmetry in epigenetic silencing pathways at pericentric heterochromatin (PCH) has previously been reported by our lab (**Figure 2**). Namely, paternal PCH in the zygote lacks the canonical Suv39h heterochromatin silencing pathway, while maternal PCH is enriched for H3K9me3 and Suv39h-components, such as HP1 and H4K20me3. Instead, paternal PCH is targeted by the PRC1 complex, which is responsible for transcriptional repression of major satellite repeats specifically from the male

genome. Embryos maternally deficient for a Suv39h components accumulate PRC1 proteins at maternal PCH lacking H3K9me3, thereby revealing hierarchy between repressive pathways. The PRC1-mediated silencing pathway seems to function as a default back-up mechanism for PCH repression in the absence of the canonical Suv39h pathway. Moreover, parental epigenetic asymmetries are observed along cleavage chromosomes and are only resolved by the 8-cell stage, presumably marking the end of maternal to embryonic transition (Mayer *et al.*, 2000b; Puschendorf *et al.*, 2008).

The most studied epigenetic asymmetry, however, in the mouse embryo is the differential patterns of maternal and paternal DNA methylation (**Figure 2**). Within a few hours after fertilization the paternal genome is globally and rapidly demethylated by an active mechanism, since it occurs in the absence of DNA replication or transcription (Mayer *et al.*, 2000a; Santos *et al.*, 2002). The exact mechanisms involved in active demethylation are poorly understood. Recently the cytidine deaminase AID has been implicated in active DNA demethylation events during mammalian PGC development (Popp *et al.*, 2010), and in the context of somatic cell nuclear re-programming to a pluripotent state (Bhutani *et al.*, 2010). Other enzymes involved in DNA repair, such as PARP-1, have also been suspected to play a role in paternal genome de-methylation in the zygote (Mark Wossidlo, personal communication). Another group showed that the elongator complex is required for active paternal genome demethylation in the 1-cell embryo, but the exact role of the elongator complex or the molecular mechanism involved are not known (Okada *et al.*, 2010).

An important question is whether this paternal DNA demethylation is really global? A detailed study of a few genes that are highly methylated in sperm shows that they rapidly loose their methylation in zygotes (Oswald et al., 2000), except for paternally imprinted genes (Olek and Walter, 1997). But the overall hypomethylated state of promoters in sperm (Farthing *et al.*, 2008) argue against the importance of global demethylation for gene regulation. So then where is the genome-wide DNA methylation lost from? Bisulfite sequencing identified certain retrotransposons, such as Long Interspersed Nucleotide Elements (Line1) as targets of demethylation (Lane *et al.*, 2003), while others, like the intracisternal A particle (IAP) retrotransposon were excluded from active DNA demethylation (Lane et al., 2003).

Like the imprinted loci of the paternal genome, the female PN is protected from demethylation. Although the maternal factor Stella (Pgc7) localizes to both PN, it has been shown to specifically protect maternal PN as well as paternal imprinted loci from demethylation. The protective mechanism remains to be uncovered, as well as the mechanism which attributes specificity to it (Nakamura *et al.*, 2007).

The maternal genome on the other hand gradually looses DNA methylation, with lowest levels of DNA methylation at the morula stage. This is a passive demethylation event, as unmethylated DNA accumulates step-by-step, increasing after each round of DNA replication (Rougier *et al.*, 1998; Santos and Dean, 2004). This process seems to be primarily caused by the inability of the embryo to maintain current methylation patterns.

Indeed, the oocyte specific form of Dnmt1 maintenance DNA methyl-transferase (Dnmt1o), for a long time the only identified Dnmt to be present in early embryos, was shown to be retained in the cytoplasm of

developing embryos by a so far unknown mechanism (Cardoso and Leonhardt, 1999). Dnmt1o transiently localizes to the nucleus at the 8-cell stage, where it is thought to ensure the methylation status of imprinted loci before being shuttled back into the cytoplasm (Howell *et al.*, 2001). Offspring from females lacking Dnmt1o show around 50% reduction in the number of normally methylated alleles of imprinted genes (Howell *et al.*, 2001). These data argue, that Dnmt1o maintains the methylation status of imprinted genes only during one round of embryonic DNA replication at the 8-cell stage. How are then imprint-specific methylation patterns maintained during the rest of pre-implantation development? A more recent study by Hirasawa et al. reports the presence, albeit at low levels, of the somatic form of Dnmt1 in oocytes and early embryos. Conditional deletion of maternal and embryonic *Dnmt1* results in complete demethylation of imprinted loci by the blastocyst stage, whereas *Dnmt3a* or *Dnmt3b* are dispensable for this methylation (Hirasawa *et al.*, 2008).

Taken together, DNA methylation dynamics in the zygote might facilitate paternal epigenetic remodeling by erasing previously acquired spermatogenesis specific DNA methylation marks. Alternatively, paternal DNA demethylation may be required to allow for the generalized de-repression of paternal alleles to accommodate the minor transcriptional burst at the zygote stage. Paternal specific DNA demethylation is in line with the general more active paternal chromatin state defined by histone hyperacetylation and lack of repressive histone methylation marks.

2.7.3.2. Maternal to embryonic transition (MET)

Fertilization brings together the haploid genomes of two highly differentiated cells, the sperm and oocyte. One of the first functions of the embryo is to re-program the specialized parental genomes into a totipotent state. Totipotency is a rare and transient property, characterized by the ability of a single embryonic cell to give rise to all differentiated cell types that build up the adult body of an organism. It is displayed only by very early embryos in mammals, spans over few cell cycles and is already lost at the blastocyst stage (or even earlier). This extensive re-programming in early embryos is also known as the maternal to embryonic transition (MET). MET comprises mainly of two acts: the destabilization of maternal transcripts and proteins and the activation of an embryo specific expression program on the initially silent parental genomes.

Early development relies on maternal transcripts and proteins which are loaded into the egg during oocyte growth. Maternal RNAs have been implicated in oocyte specific processes and also in basic biosynthetic processes that fuel embryonic development. Embryo specific mRNAs are stored in an inactive, masked form and recruited for translation in a stage specific manner during embryogenesis (Stebbins-Boaz and Richter, 1997). Their recruitment for translation is thought to be mediated by similar mechanisms as described above for message regulation during meiotic maturation, although considerably fewer studies are available, especially for mammalian embryonic translational regulation. In general in mouse and *Xenopus*, regulating the length of the poly(A) tail is crucial, and it is also achieved through specific binding elements in

the 3'UTR, such as CPEs (Paillard *et al.*, 2000; Simon *et al.*, 1992). The mechanisms by which proteins that bind to these elements and accomplish poladenylation are not known, but it is speculated that they resemble events that take place during oocyte maturation (Paillard *et al.*, 2000; Wu *et al.*, 1997). There is a connection between the Ca^{2+} increase and translation initiation of maternal mRNAs, but the molecular pathways involved are not understood. In mouse, inhibiting Ca^{2+} release after fertilization prevents many activation associated changes in protein synthesis (Xu *et al.*, 1994). In contrast, translation can artificially be stimulated by Ca^{2+} increase in the unfertilized egg (Ducibella *et al.*, 2002).

Maternal message recruitment was demonstrated to be essential for zygotic genome activation (ZGA) and embryo development. Treating 1-cell mouse embryos with 3'-deoxyadenosine, which functions as a chain terminator of poly(A) tail elongation of maternal mRNA, but not 3'-deoxyguanosine, decreased transcriptional activity in 1-cell embryos (Aoki *et al.*, 2003). Accordingly, inhibiting protein synthesis with cycloheximide also leads to inhibition of embryonic transcription (Wang *et al.*, 2001; Wang and Latham, 2000).

Further studies provided examples as to which maternal messages are targets of recruitment: following fertilization, spindlin and cyclin A2 mRNAs undergo poly(A) tail elongation and translation (Fuchimoto et al., 2001; Oh et al., 2000). Moreover, cyclin A2 translation was shown to be essential for zygotic genome activation and development beyond the 2-cell stage (Foygel *et al.*, 2008; Hara *et al.*, 2005).

Insights into what kind of messages are recruited for translation in the zygote on a global scale comes from the previously mentioned study, in which they performed polysome profiling of mouse zygotes. They identified actively translated mRNAs at this stage involved in metabolic processes, transcription, translation, cell cycle regulation (Potireddy *et al.*, 2006).

Concurrent with the translation of some maternal mRNAs is the degradation of others. While seemingly at cross-purposes, both processes are essential for maternal regulation of embryonic development. Degradation can be coupled to translation to remove mRNAs that have already been translated and whose products are no longer needed and/or degradation may also simply occur without translation, to remove oocyte specific messages that are not needed or may even inhibit embryonic development.

There are at least two types of degradation activities in the early embryo. The first, "maternal", degradation activity is exclusively maternally encoded and functions right after fertilization. The second, "zygotic" activity requires embryonic transcription. For example in *Drosophila*, due to the possibility to separate egg activation from fertilization, the two activities can easily be observed (Bashirullah *et al.*, 1999; Tadros *et al.*, 2003). In mouse, a large portion of maternal mRNAs are degraded by the 2-cell stage (Piko and Clegg, 1982). Large scale transcriptome analysis at different stages of mouse pre-implantation development have also identified different kinetics of maternal message degradation, one prominent wave just after fertilization and one coinciding with the timing of zygotic genome activation at the 2-cell stage. Of note, a third set of maternal transcripts show a relatively stable profile, indicating that some maternal information may still contribute to embryonic development after zygotic genome activation (Hamatani *et al.*, 2004).

Just as polyadenylation is important for translation of messages, removal of the poly(A) tail is a key step in degradation. Specific sequences in the 3'UTR have been shown to target messages for deadenylation. Again, not much is known in mammals, but in *Xenopus* two *cis*-elements have been identified that direct deadenylation after fertilization: an A/U rich element (ARE) with repeats of AUUUA and the Embryonic deadenlylation element (EDEN) (De Renzis *et al.*, 2007; Paillard *et al.*, 1998; Voeltz and Steitz, 1998). These sequences function as binding sites for RNA binding proteins that lead directly or indirectly to deadenylation of the transcript.

A recent study performing large-scale computational analysis of 3'UTRs of expressed genes from fully grown mouse oocytes and 2-cell embryos has uncovered valuable information regarding features associated with the stability of maternal transcripts during early mammalian development (Evsikov and Marin de Evsikova, 2009). Comparison of the transcriptomes of the two stages revealed two types of behaviour of maternal mRNAs: a stable and a transient group. Interestingly, the two sets of transcripts differed in the size of their 3'UTRs, the stable transcripts possessing significantly longer 3'UTRs than the transient ones. Furthermore, differential nucleotide content of 3'UTRs was also observed between the two groups, caused primarily by a higher portion of cytosines in the UTRs of transient and uracils in the UTRs of stable transcripts. Finally, they also concluded that CPEs and PBEs are predominant in the UTRs of stable transcripts (Evsikov and Marin de Evsikova, 2009). This study may be the first step towards defining a code, presumably more complex than in *Xenopus*, based on 3'UTR features that may define translational regulation of mammalian messages following fertilization.

The second accomplishment of MET is the activation of a new gene expression program on the newly formed embryonic genome. ZGA in the mouse has been reported to occur in two waves, a minor one during zygotic development and a major on at the 2-cell stage (Bouniol *et al.*, 1995; Flach *et al.*, 1982; Schultz, 1993) (**Figure 2**).

Various analyses based on, for example, reporter gene expression, BrUTP incorporation into nascent RNA and transgene expression in the paternal genome have demonstrated that the 1-cell embryo has transcriptional activity (Bouniol *et al.*, 1995; Matsumoto *et al.*, 1994; Ram and Schultz, 1993). Based on quantification of BrUTP incorporation, this wave of transcription reaches only approximately 40% of the transcription levels at the 2-cell stage and is primarily supported by the male PN (Aoki *et al.*, 1997).

Surprisingly, genome-wide expression profiling studies searching for products of *de novo* transcription in the zygote, meaning transcripts at the 1-cell stage that are sensitive to an RNAPII inhibitor α-amanitin, failed to detect any (Zeng and Schultz, 2005). It therefore remains elusive what BrUTP incorporation represents at this stage.

The chromatin structure in somatic cells is inherently repressive, as a consequence of DNA packaging into chromatin, which obstruct the access of transcription factors to DNA. Therefore, enhancers of *cis*-acting elements are required to relieve the transcriptionally repressive state of chromatin structure. In 2-

cell embryos, enhancers are necessary to achieve maximal transcription of reporter genes. However, in the 1-cell embryo reporter genes are actively transcribed even in the absence of enhancers (Wiekowski *et al.*, 1991). These results suggest that on top of ZGA, a transcriptionally repressed chromatin state is established by the 2-cell stage, perhaps to confer specificity to genome activation.

The chromatin-based nature of this repressive state is also supported by the observation that induction of a more active chromatin state by hyperacetylation at the 2-cell stage relieved the repression of enhancer-less reporter genes and increased global BrUTP incorporation (Aoki *et al.*, 1997; Wiekowski *et al.*, 1993).

Around 20% of genes detected at the 2-cell stage have been shown to be α-amanitin sensitive, also supporting the notion that ZGA is not as global as previously anticipated, but rather specific (Zeng and Schultz, 2005). Among the genes transcribed during major ZGA are genes involved in basic cellular function, ion transport, ribonucleotid metabolism, ribosome biogenesis, protein synthesis and transcription (Hamatani *et al.*, 2004; Zeng *et al.*, 2004; Zeng and Schultz, 2005).

A dramatic change in promoter utilization also takes place during early embryogenesis, such that TATA-less promoters are more efficiently utilized in early embryos compared to oocytes. Such change in promoter utilization could specifically enhance expression of genes needed in the embryo. For example, housekeeping genes are actually under-represented in the fully grown oocyte transcriptome (Evsikov *et al.*, 2006), but their high expression is needed to support the increased energy and metabolic demands of the rapidly developing pre-implantation embryo. Many housekeeping genes are regulated by TATA-less promoters (Nothias *et al.*, 1995), supporting the idea that promoter utilization change could contribute significantly to the re-programming of gene expression that occurs during the maternal-to-zygotic transition (Davis and Schultz, 2000).

Although in general the molecular mechanisms regulating ZGA are poorly understood, it is established that in general translation of maternal messages is a prerequisite, as discussed before. Only a few specific maternal transcripts that are known to be involved in ZGA have been identified. Some of these will be discussed in the next chapter.

2.7.3.3. Maternal effect genes

Maternal transcripts serve important roles during oocyte maturation and early embryonic development including embryonic genome activation. A handful of "maternal effect" genes have been identified over the past years which refers to the dependence of early embryonic development on maternal products and their deletion results in female sterility (Acevedo and Smith, 2005; Bettegowda *et al.*, 2008). Such genes are: *Mater* (maternal antigen that embryos require) (Tong *et al.*, 2000), *Hsf1* (heat shock factor 1) (Christians *et al.*, 2000), *Dnmt1o* (Howell *et al.*, 2001), *Npm2* (nucleoplasmin 2) (Burns *et al.*, 2003; De La

Fuente *et al.*, 2004a), *Stella* (Payer *et al.*, 2003), *Zar1* (zygotic arrest 1) (Wu *et al.*, 2003), *Pms2* (Gurtu *et al.*, 2002), *Dnmt3a* (Kaneda *et al.*, 2004), *mHR6A* (RAD6-related) (Roest *et al.*, 2004), *Basonuclin* (Ma *et al.*, 2006), *Tif1α* (Torres-Padilla and Zernicka-Goetz, 2006), *Brg1* (Bultman *et al.*, 2006), *Dicer (Tang et al., 2007)*, and *CTCF* (Wan *et al.*, 2008). In a strict sense, loss of maternal effectors only effects embryonic development, but not oogenesis, although for most identified genes the full extent of their effects on oocyte development are simply unknown. Majority of maternal effect genes have been identified using traditional knockout technology, however, this approach can only uncover maternal effect genes with relativly minor functions in other essential processes, as maternal effect genes that have important general functions preclude the analysis of homozygous null females. More recent studies have started to use oocyte specific promoter driven cre-recombinases to delete floxed alleles of target genes only in oocytes without affecting maternal viability or transgenic RNAi approaches. Loss of maternal effect genes in oocytes can lead to 1-cell arrest (like *Npm2*, *Zar1*, *Hsf1* and *Dicer*), 2-cell arrest (*Zar1*, *Mater*, *mHR6A* and *Brg1*) or comprised pre-implantation development (*Stella*, *Pms2* and *CTCF*). In most cases the exact phenotypes and the underlying molecular mechanisms are poorly described or not known at all.

Zar1 was identified as a gene exclusively expressed in the growing oocyte and accordingly, *Zar1-/-* mice are viable and grossly normal but *Zar1-/-* females are sterile. Oocytes from *Zar1-/-* mice progress normally through meiotic maturation, but early embryonic development arrests at the 1- or 2-cell stage though the exact mechanisms remain elusive (Wu *et al.*, 2003).

Similarly, oogenesis is unaffected in females mutant for *Mater*, a factor that has been identified as an oocyte antigen in a mouse model of autoimmune premature ovarian failure. Mater mutant 2-cell embryos were able to synthesize the transcription-related complex (TRC) a marker of ZGA in mice (Conover *et al.*, 1991), although only at 40% reduced levels (Tong *et al.*, 2000).

Another maternal effect gene, whose molecular role in still unclear in the mouse, is the nucleoplasmin *Npm2*. *Npm2-/-* oocytes, as previously described are a model for global chromosome condensation defects at the end of oocyte growth, which however does not impair meiotic progression. In *Xenopus*, Npm2 removes sperm protamines, facilitates nuclear assembly and replication of the paternal genome. In contrast, sperm decondensation occurs normally in *Npm2* maternal mutant mouse embryos; however, no nucleoli are visible in zygotes, first mitosis is delayed followed by fragmentation and death of most embryos (Burns *et al.*, 2003; De La Fuente *et al.*, 2004a). Interestingly, a recent study shows that maternal nucleoli are essential for embryonic development (Ogushi *et al.*, 2008).

Basonuclin was knocked down in growing oocytes using a transgenic RNAi approach. This is not strictly a maternal effect gene as oogenesis is also affected. Basonuclin is a zinc-finger protein involved in the transcription of rRNA. In oocytes, Basonuclin co-localizes with RNAPI activity in the nucleus, however, Basonuclin is also abundant in the nucleoplasm and interacts with RNAPII promoters. In Basonuclin depleted oocytes, RNA polymerase I and II mediated transcription and normal oocyte morphology were affected. However, some oocytes do mature and are capable to support fertilization. In the resulting embryos chromatin decondensation of the paternal PN is decreased, chromatin is frequently observed to

fragment and DNA is unequally distributed between daughter cells resulting in embryonic arrest at the 2-cell stage (Ma *et al.*, 2006).

Brg1 encodes a catalytic subunit of the SWI/SNF-related complexes. Conditional deletion of *Brg1* in oocytes or depletion in early embryos resulted in incomplete ZGA (~1/3 of embryonically expressed genes were down regulated at the 2-cell stage) that was linked to decrease in H3K4me3 levels and a 2-cell arrest phenotype (Bultman *et al.*, 2006).

At the onset of first genome activation Transcription intermediary factor 1 α (Tif1α) translocates from the cytoplasm into the pronuclei to sites of active transcription, that are also enriched in chromatin remodelers, such as Brg1 and Snf2h (Torres-Padilla and Zernicka-Goetz, 2006). Ablation of Tif1α by microinjection of dsRNAs or anti-Tif1α antibodies induces mislocalization of active RNAPII and the chromatin remodelers. Using a ChIP cloning approach authors identify 18 Tif1α targets in the zygote. In Tif1α-depleted embryos some of these target genes were found misregulated at the 4-cell stage. Therefore, Tif1α has no general effect on transcription but only affects certain genes. Moreover, it is unclear whether transcriptional misregulation of these targets occurs at the 1-cell or rather at the 2-cell stage during major ZGA. Nevertheless, ablation of Tif1α results in embryonic arrest at the 2/4-cell stage, therefore qualifies as a maternal effect gene (Torres-Padilla and Zernicka-Goetz, 2006).

Stella (*PGC7*) is a gene specifically expressed in PGCs, oocytes, pre-implantation embryos and pluripotent cells. It encodes a protein with a Sap-like domain and a splicing factor motif-like structure, suggesting possible roles in chromosomal organization or RNA processing (Aravind and Koonin, 2000; Sato *et al.*, 2002). As previously discussed, Stella protects the female PN and imprinted loci from active DNA de-methylation in the zygote (Nakamura *et al.*, 2007). *Stella*-/- females are infertile, due to pre-implantation embryonic lethality (Payer *et al.*, 2003).

Recently the pluripotency factor *Oct4* has been shown to behave as a maternal effect gene. The first indication came from a study that tried to dissect the molecular basis of the differential developmental competence of embryos that are produced from NSN GV oocytes or SN GV oocytes (Zuccotti *et al.*, 2008). While SN GV oocyte derived embryos developed normally, NSN GV-embryos did not progress beyond the 2-cell stage. Oct4 expression was revealed as a major difference between the two GV groups. Interestingly, the absence of Oct4 in NSN oocytes was also proposed to cause down-regulation of Stella in NSN oocytes, as it does in ES cells (Levasseur *et al.*, 2008; Loh *et al.*, 2006). Another study reports morpholino-mediated *Oct4* knockdown in zygotes, resulting in embryonic arrest around the 8-cell stage. Expression profiling of 2-cell *Oct4* depleted embryos revealed that Oct4 at this stage is predominantly an activator of key genes mainly involved in transcription and translation (Foygel *et al.*, 2008).

CTCF is the only known protein required for insulator activity in vertebrates. It can bind both DNA and itself, providing means by which CTCF molecules bound at remote sites in the genome can be brought together physically. According to the model for insulator activity, this would prevent enhancer-promoter interactions between elements on different chromatin loops, while facilitate interactions of those within the

same loop (Engel and Bartolomei, 2003; Wallace and Felsenfeld, 2007). CTCF binding sites were shown on the X chromosome, at imprinted loci and even at boundaries of active and inactive chromatin (Barski *et al.*, 2007; Chao *et al.*, 2002; Fitzpatrick *et al.*, 2007; Yoon *et al.*, 2005). Depleting maternal CTCF in growing oocytes results in misregulation of hundreds of genes in oocytes (overrepresented categories related to embryogenesis), problems in oocyte meiotic maturation and mitotic defects in the embryo that are accompanied by defects in ZGA (Wan *et al.*, 2008).
The maternal effect of *Dicer* was described in chapter 2.7.2.5.

2.7.4. First differentiation events in the mouse embryo

In the mouse the first three cleavage divisions give rise to eight morphologically indistinguishable embryonic blastomeres. During the 8-cell stage the embryo undergoes a process called compaction, which converts the embryo with eight clearly visible blastomeres into a tightly packed cell aggregate (**Figure 2**). Compaction is signaled by a marked increase in cell-to-cell contact between blastomeres, driven by formation of adherens junctions, consisting of E-cadherin and catenin complexes, and tight junctions (Eckert and Fleming, 2008; Johnson and McConnell, 2004). Upon compaction, an apical-basal polarity emerges in all blastomeres that are manifested in polarized distributions of various membrane and cytoplasmic factors along the axis from the surface (apical) to the center (basal) of the embryo (Johnson and McConnell, 2004). The 4^{th} cleavages generate a 16-cell embryo, where for the first time some cells in the center loose contact with the surface of the embryo. By the 32-cell stage these inner cells loose polarity, while the outer ones maintain it. After the 5^{th} cleavage, small cavities start to form between the blastomeres, which continually expand and fuse with each other to eventually form a single blastocoelic cavity. At this point the embryo is comprised of around 100 cells (divisions of blastomeres are asynchronous, leading to non-2^n blastomere numbers in the mouse embryo) and is known as the blastocyst (Cockburn and Rossant, 2010).

The first differentiation event in mammalian development is the establishment of two distinct cell lineages: the trophectoderm (TE) and the inner cell mass (ICM) that are clearly defined by the blastocyst stage. The outer TE, which will give rise to extraembryonic tissues of the developing conceptus, possesses the characteristics of an epithelium. The epithelium starts to form at the late 8-cell stage, is completed by the 32 cell-stage and is marked by the previously mentioned AJ and TJ formation, which are necessary to seal off the blastocyst cavity. On the other hand, the aggregate formed by the internal cells of the blactocyst form the ICM, which will give rise to all cell types of the embryo proper (Cockburn and Rossant, 2010; Johnson and McConnell, 2004).

The 32-cell stage is the first known time point when internal and external blastomeres of the embryo are morphologically distinguishable, inner cells being un-polarized in contrast to polarized outer cells. However,

in terms of developmental fate, when do inner and outer cells become committed to ICM and TE fates, respectively? Many studies have addressed this question by various micromanipulation techniques, leading to the general conclusion that blastomere fate in not determined until the 5th cleavage.

For example, external blastomeres of a 16-cell embryo can give rise to ICM when transplanted internally and internal blastomeres transplanted externally develop into TE (Johnson and Ziomek, 1983; Rossant and Vijh, 1980; Ziomek and Johnson, 1982). Moreover, an interesting study made use of a technique where surface cells of intact embryos were labeled with a dye and therefore could be identified and traced after dissociation and aggregation of a new embryo. When isolated internal or external cells of 16-cell embryos were aggregated, embryos developed readily to blastocyst stage, expressed appropriate markers and even produced viable mice after being transplanted into surrogate mothers. On the other hand, the same experiment performed on 32-cell embryos yielded strikingly different results. Aggregated external cells formed only TE, while internal cells did eventually form a blastocyst-like embryo but then failed to implant (Suwinska *et al.*, 2008). These results clearly demonstrate that a significant change occurs in the developmental potential of blastomeres around the 5th cleavage so that cell fates become fixed by the 32-cell stage.

Interestingly, genome wide gene expression analyses of different pre-implantation stages of embryogenesis revealed the second most drastic gene expression transition (after the major ZGA at the 2-cell stage) between morula (around 32-cell stage) and blastocyst stage, demonstrating that cell fate commitment is accompanied by major gene expression changes. It would be very interesting to look in more detail into these gene expression profiles and try to pinpoint important factors involved in cell fate determination (Hamatani *et al.*, 2004).

Irreversible fate determination only at the 32-cell stage does not however exclude the possibility of certain earlier blastomeres having a developmental preference towards a certain lineage. It has been proposed that already at the 2-cell stage the two blastomeres show preference towards contributing to one or the other half of the blastocyst (Gardner, 2001; Piotrowska *et al.*, 2001). However, further independent studies have clearly proven that this is not the case (Alarcon and Marikawa, 2003; Chroscicka *et al.*, 2004; Kurotaki *et al.*, 2007; Motosugi *et al.*, 2005). Another model proposes that a certain blastomere in the 4-cell embryo preferentially gives rise to TE on the half of the blastocyst that is on the opposite side of the ICM. This hypothesis has been supported by a number of observations. Apart from lineage tracing experiments, it has been noted that when these particular blastomeres are isolated and aggregated into an embryo, such embryos do not develop as efficiently as embryos aggregated from other blastomeres (Piotrowska-Nitsche *et al.*, 2005; Piotrowska-Nitsche and Zernicka-Goetz, 2005). In addition, another study shows that this particular blastomere has reduced levels of H3R26me compared to the other blastomeres, and that this may interfere with ICM gene activation (Torres-Padilla *et al.*, 2007). Nevertheless, this model is also under heavy debate. Just to mention one example, this certain blastomere only exists in about half of embryos (a subgroup that arises from specific asynchronous divisions of the 2nd cleavage, termed ME-patterned

embryos); therefore this developmental bias is not universal and raises the question about its general importance in embryo patterning. An interesting way to approach this would be to determine expression profiles of individual early blastomeres and searching for molecular signatures of biased development.

A few transcription factors have been identified to be essential for the formation of the first two lineages. Oct4, well known from ES cell work, is strongly expressed in the ICM (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1990). *Oct4* mutant embryos can develop to a blastocyst-like stage, however, on a closer look, the inner cells of such an embryo express TE markers (Nichols *et al.*, 1998). Also, inactivation of *Oct4* in ES cells results in differentiation into cells with TE characteristics (Niwa *et al.*, 2000). Thus, Oct4 is essential in repressing TE differentiation of ICM cells.

Another key transcription factor essential for maintaining pluripotency of the ICM and ES cells is the homeodomain containing Nanog. Nanog has been shown to repress primitive endoderm (the third cell lineage arising in the mouse blastocyst) differentiation of the ICM (Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Rodda *et al.*, 2005).

Caudal-related homeobox2 (Cdx2) is a TE specific transcription factor. Cdx2 expression commences at the 8-cell stage and at this point is co-expressed with Oct4 in all blastomeres. By the 16 to 32-cell stages, Cdx2 expression increases in outer cells and decreases in inner cells, and by the blastocyst stage is exclusively TE specific (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008). This change in Cdx2 expression is the first known molecular distinction to occur in the mouse embryo. However, *Cdx2* null embryos are capable of forming a blastocyst with TE-like cells, so loss of *Cdx2* does not prevent TE formation (Beck *et al.*, 1995; Ralston and Rossant, 2008; Strumpf *et al.*, 2005). This indicates that mechanisms up-stream of Cdx2 must exist that will define TE commitment. Cdx2 however is essential to maintain TE fate, as *Cdx2* null late blastocysts loose epithelial integrity and fail to maintain the blastocyst cavity and TE derivatives cannot be derived from *Cdx2* null embryos (Strumpf *et al.*, 2005).

The TEA domain containing transcription factor Tead4 has been shown to act upstream of Cdx2. Interestingly, while expressed in both ICM and TE, *Tead4* deficiency only affects TE formation, accompanied by the absence of Cdx2 and the presence of Oct4 and Nanog in all cells of the embryo (Nishioka *et al.*, 2008; Yagi *et al.*, 2007).

A candidate down-stream target of Cdx2 is the T-box transcription factor Eomes, also specific for TE. *Cdx2* mutant embryos express lower levels of Eomes, while *Eomes* mutant embryos express normal levels of Cdx2. *Eomes* was shown to be required for late stage trophoblast differentiation in embryos (Ciruna and Rossant, 1999; Russ *et al.*, 2000; Strumpf *et al.*, 2005).

Taken together, molecular mechanisms, including transcription factors or epigenetic mechanisms responsible for first cell fate determination are currently unknown and will be topics of exciting new discoveries.

2.8. Cell cycle and checkpoints in the embryo

2.8.1. The first two cell cycles of the embryo

Parameters of cell cycles of the pre-implantation mouse embryo are known to differ in many ways from the well conserved pattern of somatic cell cycles. Especially the first two mitotic cell cycles that each last for about 20 hours. Cell cycle progression of the early embryo takes place in the absence of transcription and is therefore reliant on maternal mRNAs and proteins provided by the oocyte. The overwhelming maternal contribution of cell cycle regulators has made it difficult to study cell cycle regulation of the first cleavage divisions.

Although the timing of cell cycle phases can differ greatly between mouse strains (Molls *et al.*, 1983), the first S-phase is shown to start 4-10 hpf, lasting for 4-8 hours. Replication is always initiated first in the male PN (Abramczuk and Sawicki, 1975). G2 is estimated to last for 3-5 hours and is followed by the first M-phase. Interestingly, the duration of the first M-phase (120 min) is almost twice as long as the second mitosis (70 min). This increase seems to be due to a transient metaphase arrest independent of the spindle assembly checkpoint (SAC) (Sikora-Polaczek *et al.*, 2006). The second G1-phase is very short (1-2 hours), followed by a 6 hour S-phase and then a very long G2-phase (12 hours) (Gamow and Prescott, 1970; Luthardt and Donahue, 1975; Molls *et al.*, 1983). Interestingly, it is during this prolonged G2-phase that major ZGA takes place (Flach *et al.*, 1982). The following divisions of the pre-implantation embryo are much faster, on average lasting for 10 hours each (Barlow *et al.*, 1972; MacQueen and Johnson, 1983).

2.8.2. Checkpoints

At key transitions during cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are referred to as cell cycle checkpoints (Hartwell and Weinert, 1989). Cells can arrest at checkpoints due to external stimuli (stress signals, lack of growth factors or nutrients) or internal signals stemming from for example DNA damage or replication fork stalling. In the later cases, checkpoints induce a delay in cell cycle and therefore allow time for cells to repair DNA or finish replication, or - if the damage cannot be repaired or S-phase is not complete - checkpoints can trigger apoptosis to eliminate genetically instable cells. Defects in checkpoints can lead to chromosome segregation problems and aneuploidy, resulting in cell death later on.

The **G1/S checkpoint** is where eukaryotes typically arrest the cell cycle if environmental conditions make cell division impossible or if the cell passes into G0 for an extended period.

Cell cycle progression is coupled with the sequential activation of of cyclin dependent kinases (Cdks) by their regulatory components, the cyclins. During normal G1-phase progression Cdk4,6/Cyclin D complexes form, they phosphorylate the tumour suppressor retinoblastoma (Rb), this in turn relieves the inhibition of the transcription factor E2F. E2F is then able to stimulate expression of Cyclin E which then interacts with Cdk2 and promotes G1/S-phase transition.

The G1/S checkpoint is mainly exerted by action of the Cdk inhibitor p16 (Ink4a). p16 can bind to Cdk4,6 and inhibit its interaction with Cyclin D. Another Cdk inhibitor causing G1/S arrest is p21 (Waf1/Cip1). After exposure of cells to genotoxic agents p21 is induced by p53-dependent transactivation. The elevated p21 binds and inactivates Cdk4,6/Cyclin D and Cdk2/Cyclin E complexes. The action of both inhibitors results in hypophosphorylation of RB and cell cycle arrest (Stewart and Pietenpol, 2001).

The **G2 cell cycle checkpoint** is an important control measure that allows suspension of the cell cycle prior to chromosome segregation. Entry into mitosis is controlled by the activity of the cyclin dependent kinase Cdc2 (Cdk1) (Nurse, 1990). Maintenance of the inhibitory phosphorylations on Cdc2 (on T14 andY15) is essential for G2 checkpoint activation. ATM and ATR indirectly modulate the phosphorylation status of these sites in response to DNA damage. The response to DNA damage inducing irradiation is mediated primarily by ATR (Graves *et al.*, 2000) with ATM playing a back-up role; the response to replication blocks is primarily controlled by ATR.

It should be noted that the stage of the cell cycle when the DNA damage occurs may influence whether the response is mediated through ATR or ATM (Abraham, 2001). In any case, upon DNA damage, ATM or ATR kinases are responsible for early steps of damage sensing and inducing a signaling cascade leading to checkpoint activation, DNA repair or apoptosis (Zhou and Elledge, 2000). Downstream kinases Chk1 and Chk2 are activated by ATR- and ATM-dependent phosphorylation, respectively, which then phosphorylate the dual specificity phosphatase Cdc25C on position Ser216 (Furnari *et al.*, 1997; Matsuoka *et al.*, 1998; Peng *et al.*, 1997; Sanchez *et al.*, 1997). Phosphorylation of this residue creates a binding site for the 14-3-3 proteins. The 14-3-3/Cdc25C protein complexes are sequestered in the cytoplasm, thereby preventing Cdc25C from activating Cdc2 through removal of the T14 and Y15 inhibitory phosphorylations (Lopez-Girona *et al.*, 1999). This results in the maintenance of the Cdc2/Cyclin B complex in its inactive state and blockage of entry into mitosis.

ATM and ATR kinases phosphorylate a variety of targets including p53 and serine 139 on H2AX (γH2AX). γH2AX is a well known marker of DNA damage that forms nuclear foci at sites of damage (Rogakou *et al.*, 1999; Rogakou *et al.*, 1998). The exact function of γH2AX is not known, but it has been suggested to function as a platform to recruit checkpoint and DNA repair proteins (Downey and Durocher, 2006). Depending on the nature of the lesion, co-localization of γH2AX can occur with DNA repair proteins such as 53BP1, Mre11, Rad50 and Nbs1 (Bekker-Jensen *et al.*, 2006; Downey and Durocher, 2006). Although

originally thought to be a molecular sensor for DSBs, the involvement of γH2AX in recognizing other types of DNA damage and even replication fork stalling is becoming evident (Ewald *et al.*, 2007; Fernandez-Capetillo *et al.*, 2004; Marti *et al.*, 2006; Ward and Chen, 2001).

During DNA replication, DNA damaging agents or stalled replication forks - which can potentially collapse and cause under-replication of chromosome regions, therefore constitute potential sources for genomic instability - can induce the **intra-S-phase checkpoint** which will reduce or arrest DNA synthesis (Bartek *et al.*, 2004; Lambert *et al.*, 2005). This might be achieved by inhibiting further initiation events and/or by slowing progression of existing replication forks. The current knowledge of the intra-S-phase checkpoint mainly comes from experimentally inducing the checkpoint response in cells by means of DNA damaging agents or replication inhibitors such as hydroxyurea (HU) that reduces the production of deoxyribonucleotides. However there is indication that checkpoint proteins might have roles even in normal S-phase regulation, although the level of the checkpoint activation might not be easily detected (Brown and Baltimore, 2000; Liu *et al.*, 2000). As mentioned before ATR is the main kinase to function following DNA replication-dependent damage. ATR phosphorylates Chk1 on Ser317 and Ser345 residues (Chen and Sanchez, 2004), which then inhibits the Cdc25A phosphatase from dephosphorylating the Tyr15 residue of the S-phase cyclin-dependent kinase Cdk2 (Busino *et al.*, 2004). Thus, in response to replication stress, the inactive Cdk2 is unable to promote S-phase progression.

2.8.2.1. Checkpoints in the early embryo

Mouse pre-implantation embryos in general are hypersensitive to ionizing radiation. This is attributed to a deficiency in the function of checkpoints or DNA repair mechanisms. If DNA repair is not functioning properly in the embryo when they are irradiated, the cell cycle stops, but the DNA damage will not be repaired. Consequently, the cell cycle remains arrested and the embryos eventually die. If checkpoint mechanisms do not function properly, the cell cycle will not stop, not allowing sufficient time for DNA repair. Consequently, the cells will divide heterogeneously and die later.

A difficulty in studying damage response in conventional cellular systems is that the effect of DNA damage itself and the effect of cellular responses are hard to distinguish in irradiated cells. The separate entities of the parental PN in the mouse zygote give a unique opportunity to analyze these effects separately. DNA damage can be delivered through irradiated sperm, while the response can be analyzed in the damage-free female PN (Shimura *et al.*, 2002a).

It was shown by analyzing cell cycle progression of zygotes produced with this method that the onset of DNA replication is not delayed compared to wild-type. The rate of DNA synthesis however, is suppressed throughout S-phase. By prolonging S-phase, about half of the sperm-irradiated zygotes managed to

synthesize a full DNA content, while the other half failed to do so. Regardless of the DNA content, all zygotes cleaved to become 2-cell embryos (Shimura *et al.*, 2002a; Shimura *et al.*, 2002b).

These results indicated two things, first, that mouse zygotes do not possess a G1/S checkpoint, and second, that they do have an intra-S-phase checkpoint, however, this checkpoint is not too strong and it does not induce apoptosis, as it allows embryos to proceed with inappropriate DNA content.

Two explanations have been found for the lack of Rb-dependent G1/S checkpoint. In one study, *Rb* mRNA and protein were barely detectable before the blastocyst stage, which could account for the lack of the checkpoint. In line with this, induction of Rb expression in zygotes by injecting an Rb-containing plasmid induced arrest before the morula stage (Iwamori *et al.*, 2002). Another study found phosphorylated forms of RB throughout pre-implantation development, suggesting that rather PTMs regulate Rb activity than expression (Xie *et al.*, 2005). Interestingly, ES cells also lack somatic-type Rb-dependent G1/S checkpoint (Savatier *et al.*, 1994). A further study shows that actually the G1/S Cdk inhibitor p21 is not functioning at these early stages (Adiga *et al.*, 2007b). Sperm-irradiated mouse embryos – apart from S-phase delay – progress relatively normally up to E2.5, however by E3.5 they start to show a cleavage delay. This start of cleavage delay coincides with the activation of p21 expression. Moreover, cleavage delay is not apparent in p21-deficient embryos, but chromosome instability and apoptosis were more pronounced as in wild type control embryos (Adiga *et al.*, 2007b).

The intra-S-phase checkpoint was shown to be dependent on p53, as p53-deficient zygotes did not suppress DNA replication (Shimura *et al.*, 2002a). Although p53 is expressed at very high levels in early embryos, its apoptotic activity is non-functional until post-implantation stages (Jurisicova *et al.*, 1998). Interestingly, ES cells also lack p53-dependent G1/S checkpoint control despite the presence of p53 in these cells too (Aladjem *et al.*, 1998). In addition, based on immunofluorescent stainings, activated ATM also seems to be involved in this checkpoint. However, its function is not to phosphorylate p53, but possibly other yet unknown components of the pathway. In somatic cells, ATM involvement has been show at S-phase checkpoint (Gottifredi and Prives, 2005) and p53 has also been shown to accumulate after HU induced replication fork stalling (Gottifredi *et al.*, 2001; Nayak and Das, 2002).

The G2/M checkpoint in embryos is a bit more controversial. The previously described sperm-irradiated embryo studies did not detect any G2/M delay. Another study reports that about half of X-irradiated zygotes arrested at G2-phase and never developed into 2-cell embryos. However, some of them had undergone late mitosis without cytokinesis and engaged in a new S-phase (Jacquet *et al.*, 2002). A further report demonstrates mechanisms induced in 1- and 2-cell embryos in response to DSB inducing γ-irradiation (Yukawa *et al.*, 2007). Zygotes and 2-cell embryos were irradiated at the G2-phase of their cell cycle and subsequently checkpoint proteins and DNA repair were analyzed. In summary, they observe a delay with both 1- and 2-cell stage embryos cleaving to the next stage. They conclude that a G2/M checkpoint is operating at these stages; however, it may not function strongly, as almost all embryos continue to develop

but run into problems around the blastocyst stage. The G2/M checkpoint can induce a delay in cleavage, but this delay is not long enough to allow complete repair of DSBs. They observe activation of ATM and DNA protein kinase (DNA-PK) in response to irradiation, but they do not detect γH2AX at the 1- and 2-cell stages (Yukawa *et al.*, 2007).

The absence of γH2AX in DNA damage induced and non-induced 1- and 2-cell mouse embryos has been simultaneously shown by another group. Early embryos showed γH2AX only after the 2-cell stage in response to γ-irradiation during the first two cell cycles (Adiga *et al.*, 2007a). A different γH2AX-pattern in non-induced early embryos was demonstrated recently (Ziegler-Birling *et al.*, 2009). γH2AX is very high in zygotes right after fertilization, is reduced to only a few foci at the 2-cell stage and is abundant again at the 4-cell stage. They also report γH2AX on mitotic chromatin at all cleavage divisions. Importantly, these γH2AX patterns are independent of any DNA damage process, as they do not co-localize with 53BP1. These contradicting γH2AX results can be due to different antibodies and/or different staining protocols used.

To summarize, although our understanding of checkpoints and DNA repair mechanisms in the early embryo is very limited, it seems that there are two checkpoints operating: one in S-phase and another at G2/M. However these checkpoints are weak and do not provide sufficient protection for the embryo when DNA damage is induced.

2.8.3. Control of DNA re-replication in mouse pre-implantation embryos

In late mitosis and early interphase, many proteins are required to prepare the next round of DNA replication in the cell cycle. These include members of the pre-replication complex (pre-RC), like the origin recognition complex (ORC), Cdc6 and Cdt1 which associate with replication origins on chromatin before the minichromosome maintenance (MCM) complex can be added, so that the origin is licensed for replication (Bell and Stillman, 1992; Coleman *et al.*, 1996; Maiorano *et al.*, 2000). It is essential that DNA replication occurs only once every cell cycle, therefore important mechanisms have evolved to prevent re-replication. One key mechanism includes inhibition of licensing by the pre-RC by inhibition through Geminin. Geminin starts to be expressed at S-phase, after licensing took place in G1. It binds to Cdt1, inhibiting its association with replication origins and therefore blocking re-replication (Wohlschlegel *et al.*, 2000). Geminin is present throughout G2- and M-phases and is targeted for proteolytic degradation by the anaphase promoting complex (APC) (McGarry and Kirschner, 1998). Its inactivation frees Cdt1, which can then license the next round of DNA replication.

Developmentally programmed endoreduplication in mammals is only known to happen twice: when trophoblast stem (TS) cells differentiate into trophoblast giant (TG) cells (Cross, 2005) and when bone

marrow megakaryoblasts differentiate into megakaryocytes (Ravid *et al.*, 2002). This requires orchestered inhibition of mitosis and simultaneous licensing of pre-RCs. The mitotic Cdk1 was shown to be inhibited by the Cdk inhibitor p57 (Kip2) during TS to TG transition, while p21 served to prevent apoptosis of these cells (Ullah *et al.*, 2008). The involvement of Geminin in this process was not examined.

Geminin depletion in human cancer cells or *Drosophila* cells in culture induces random re-replication of DNA with formation of giant cells accompanied by ATR, Chk1 DNA damage signaling pathway (Melixetian *et al.*, 2004; Mihaylov *et al.*, 2002; Tachibana *et al.*, 2005; Zhu and Dutta, 2006b). These cells arrest at G2-phase and die soon after. Involvement of Geminin in endocycles comes from an observation in *Drosophila*, where oscillating levels of Geminin was shown to accompany endocycles (Zielke *et al.*, 2008). Thus, the primary role of Geminin appears to be suppression of re-replication at inappropriate cell cycle stages.

In mouse, zygotic deficiency for Geminin also results in DNA re-replication from the 8 cell stage onwards, presumably from the time the maternal stock of Geminin is depleted (Gonzalez *et al.*, 2006; Hara *et al.*, 2006). Embryos develop large nuclei containing more that 2n DNA and express at least some genes characteristic of TE. However, these Geminin-deficient embryos also contain damaged DNA and undergo apoptosis, suggesting that they have undergone DNA re-replication rather then endoreduplication. These results nevertheless indicate that Geminin-mediated cell cycle safeguarding is functional at pre-implantation stages.

2.9. Scope of the thesis

Our laboratory is interested in understanding how chromatin-based epigenetic mechanisms regulate gene expression programs during gametogenesis and early embryonic development in the mouse, with a special focus on the possible transgenerational role of inherited epigenetic information. A certain chromatin state is inherited from the oocyte and the sperm to the early embryo. Although early embryonic development is associated with extensive chromatin remodeling and re-programming of certain epigenetic marks to produce a totipotent embryo, some epigenetic modifications are fatefully retained during early embryogenesis (Reik *et al.*, 2001; Surani, 2001). Dynamic chromatin changes during pre-implantation development indicate that mechanisms are present in the early embryo to retune certain epigenetic settings. However, inefficiency of reproductive cloning by nuclear transfer highlights that the correct “parental priming” of chromatin is a prerequisite for normal embryonic development to occur (Dean *et al.*, 2003). We focus on the importance of this inherited epigenetic information, its role in establishing proper embryonic events, such as chromatin remodeling and zygotic genome activation.

At the time when I started my PhD studies Polycomb proteins had just started to receive increasing attention. PcG proteins were known for a long time to maintain the silenced state of *Hox* genes throughout development, but apart from a handful of other targets (Gil *et al.*, 2004; Jacobs *et al.*, 1999a; Molofsky *et al.*, 2005), genome-wide mapping of Polycomb targets was not yet published. It was known that core subunits of PRC2 and Rnf2 of PRC1 were essential for embryonic development, as mutants lacking embryonic *Ezh2*, *Eed*, *Suz12* or *Rnf2* arrested development at post-implantation stages (Faust *et al.*, 1995; O'Carroll *et al.*, 2001; Pasini *et al.*, 2004; Voncken *et al.*, 2003). Initial studies of adult stem cells revealed that multipotent stem cell fate is at least in part governed by PcG proteins (Bruggeman *et al.*, 2005; Jacobs *et al.*, 1999a; Molofsky *et al.*, 2005; Sharpless and DePinho, 1999; Valk-Lingbeek *et al.*, 2004). These findings already outlined an exciting function of Polycomb proteins in developmental processes. It was however, only shortly after that the central role of Polycomb proteins in proper lineage specification became clear: genome-wide ChIP-Chip studies of Polycomb binding and characterization of Polycomb mutant ES cells revealed that Polycomb-mediated silencing targets important lineage specific factors in ES cells and that this repression is essential for directing proper differentiation (Boyer *et al.*, 2006; Bracken *et al.*, 2006; Chamberlain *et al.*, 2008; Lee *et al.*, 2006; Leeb and Wutz, 2007; Pasini *et al.*, 2007; Shen *et al.*, 2008; van der Stoop *et al.*, 2008). These findings prompted us to investigate the role of Polycomb proteins during the crucial phase of developmental, when two highly specialized gametes fuse and form a totipotent embryo.

Previous data from our laboratory demonstrated that Polycomb proteins are present in the mouse oocyte and are inherited by the embryo. My project therefore was designed to address the transgenerational role of PcG proteins, in particular the role of PRC1. We knew from previous studies that embryos maternally and zygotically deficient for *Rnf2* develop similarly as *Rnf2* zygotically deficient embryos, suggesting no major role for PRC1 in early embryonic development. This was very surprising in the light of the important role for PRC1 in ESCs (Endoh *et al.*, 2008; van der Stoop *et al.*, 2008) and during differentiation (Voncken *et al.*, 2003) and raised the suspicion of possible functional redundancy between *Rnf2* and its paralog *Rnf1*. To address this issue, we produced embryos maternally and zygotically double deficient for *Rnf1* and *Rnf2*.

Taken together, the players involved in defining chromatin states and thereby regulating gene expression programs in the germline and during early embryonic development are not well known. Given its important role in directing development, the Polycomb silencing pathway was an attractive candidate to examine in this process.

3. Results (published manuscript or manuscript in preparation)

3.1. Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation

nature genetics

Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation

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In mammalian males, the first meiotic prophase is characterized by formation of a separate chromatin domain called the sex body[1]. In this domain, the X and Y chromosomes are partially synapsed and transcriptionally silenced, a process termed meiotic sex-chromosome inactivation (MSCI)[2,3]. Likewise, unsynapsed autosomal chromatin present during pachytene is also silenced (meiotic silencing of unsynapsed chromatin, MSUC)[2,4,5]. Although it is known that MSCI and MSUC are both dependent on histone H2A.X phosphorylation mediated by the kinase ATR, and cause repressive H3 Lys9 dimethylation[4], the mechanisms underlying silencing are largely unidentified. Here, we demonstrate an extensive replacement of nucleosomes within unsynapsed chromatin, depending on and initiated shortly after induction of MSCI and MSUC. Nucleosomal eviction results in the exclusive incorporation of the H3.3 variant, which to date has primarily been associated with transcriptional activity. Nucleosomal exchange causes loss and subsequent selective reacquisition of specific histone modifications. This process therefore provides a means for epigenetic reprogramming of sex chromatin presumably required for gene silencing in the male mammalian germ line.

The nucleosome core particle comprises an octamer of four histone proteins (H2A, H2B, H3 and H4) each present twice, around which 146 base pairs of DNA are folded. Functioning as a carrier of post-translational modifications (PTMs), which regulate gene transcription and chromatin architecture, the nucleosome is at the center of chromatin dynamics and epigenetic memory[6]. A further diversification of chromatin is enabled by the use of histone variants[7]. The canonical H3.1 and H3.2 proteins are expressed during S phase and deposited during replication by a DNA replication–dependent nucleosome assembly pathway[8]. Beside H3.1 and H3.2, two H3 variants exist. CENP-A, a highly specialized variant, is only present at the centromere. Mammalian H3.3 differs from H3.1 and H3.2 at only five and four amino acid positions, respectively, and is expressed throughout the cell cycle. It is deposited by a DNA replication–independent nucleosome assembly pathway[8–10].

Pachytene spermatocytes, characterized by fully synapsed homologous autosomal chromosomes, have been reported to undergo extensive changes in chromatin configuration[1,5,11–13]. Chromosome-wide, replication-dependent nucleosome assembly is completed after premeiotic S phase. Loss of H3.1 and H3.2 therefore becomes a marker for nucleosome eviction. To study nucleosomal dynamics during mouse meiosis, we performed immunofluorescence analysis using an antibody that, on the basis of one amino acid difference in the amino tail, specifically detects H3.1 and H3.2 but not H3.3 (ref. 9). In early pachytene spermatocytes, abundances of H3.1/H3.2 are equal in autosomal and sex body chromatin. At later stages, however, we observed a progressive disappearance of H3.1 and H3.2 in the XY body, eventually resulting in complete loss of H3.1 and H3.2 from the sex chromosomes for the remainder of spermatogenesis (**Fig. 1a**). To establish whether this loss was also reflected in the overall levels of histones and in nucleosome structure, we used antibodies directed against the C terminus of H3 (detecting H3.1, H3.2 and H3.3) and an antibody that specifically recognizes nucleosomes[14]. Signal intensities of both antibodies underwent a transient reduction at sex chromatin of pachytene up to early diplotene (**Fig. 1b** and **Supplementary Fig. 1** online) and returned to autosomal levels when the XY axial elements developed their bulged appearance, a typical feature of mid-late diplotene spermatocytes[15]. These data suggest that *de novo* replication-independent nucleosome assembly of nucleosomes and H3.3 incorporation takes place during MSCI.

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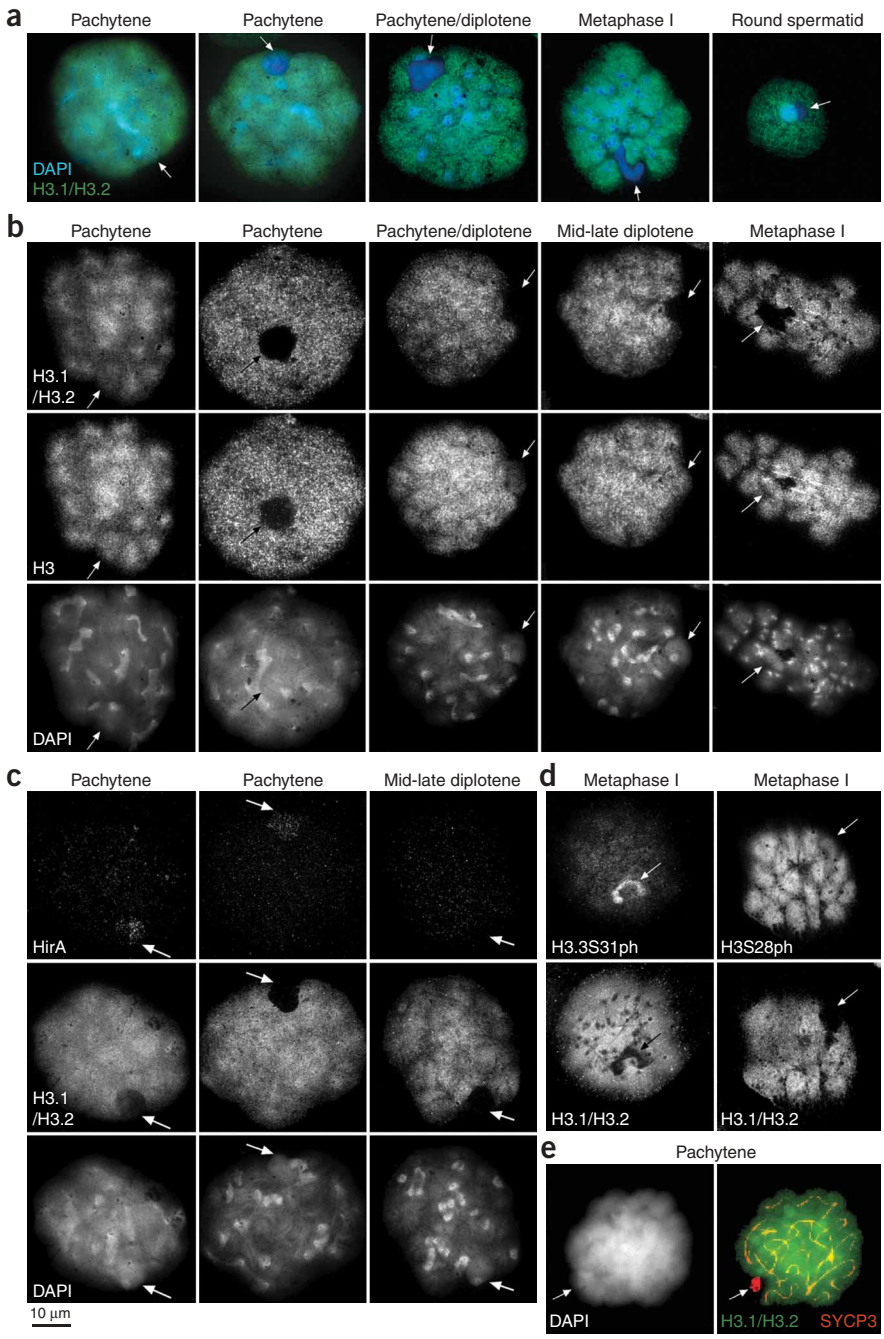

Figure 1 Gradual removal of H3.1 and H3.2 in the XY body in spermatocytes during meiotic prophase I. Arrows indicate sex chromosomes. (**a**) Spermatocytes in progressing stages of prophase I, metaphase I and a round spermatid stained by antibody to H3.1/H3.2 (green) and DAPI (blue). We observed a gradual loss of H3.1/H3.2 from the XY body, resulting in sex chromatin lacking H3.1 and H3.2. (**b**) Spermatocytes in progressing stages of prophase I and metaphase I stained by antibody to H3.1/H3.2 (upper panels), antibody to H3 (middle panels) and DAPI (lower panels). Whereas loss of H3.1 and H3.2 is persistent, staining with antibody to H3 only shows a temporary decrease in signal. (**c**) Localization of the replication-independent nucleosome assembly histone chaperone HirA in pachytene and diplotene spermatocytes (upper panels) combined with H3.1/H3.2 (middle panels) and DAPI (lower panels) staining. HirA is enriched in the XY body in pachytene and early diplotene spermatocytes and diminishes to autosomal levels or lower in mid-late diplotene spermatocytes. (**d**) Presence of H3.3 was confirmed by localization of the H3.3-specific modification phosphorylated Ser31. Metaphase I spermatocytes stained for H3.3Ser31ph and H3Ser28ph are shown on the left and right, respectively. Histone H3.3–specific phosphorylation of Ser31 is enriched in XY chromatin, whereas phosphorylation of H3.1 and H3.2 or H3.3 on Ser28 is equally present in XY and autosomal chromatin. (**e**) Localization of H3.1 and H3.2 (green) in a human pachytene spermatocyte. SYCP3 (red) labels lateral elements of the synaptonemal complex and axial elements of the sex chromosomes; DAPI labels DNA. Loss of H3.1 and H3.2 from the XY chromatin, as identified by typical SYCP3 configuration, is observed during prophase I. Scale bar, 10 μm.

To investigate whether H3.1 and H3.2 removal and *de novo* nucleosome deposition occur simultaneously, we localized the H3.3•H4-specific chaperone HirA[16]. Concomitant with the removal of H3.1 and H3.2, we observed a relative increase of signal in the XY body (**Fig. 1c**), suggesting that H3.1 and H3.2 removal coincides with *de novo* H3.3•H4 deposition. HirA levels in the XY body were equal to or lower than autosomal levels in mid-late diplotene spermatocytes (**Fig. 1c** and **Supplementary Table 1** online).

Replication-independent nucleosome assembly by HirA is predicted to yield H3.3-containing nucleosomes[16]. As no specific antibody against unmodified H3.3 is available, we examined the H3.3-specific phosphoepitope H3.3S31ph. This modification is prominently present during mitosis in human cells[17] and meiosis of the urochordate *Oikopleura dioica* (A. Schulmeister, M. Schmid and E.M. Thompson, unpublished data). At diakinesis (**Fig. 1d**) and meiosis II, the antibody to H3.3S31ph bound to all chromosomes, but to a much greater degree in sex chromosomes, confirming the enrichment of H3.3-containing nucleosomes in XY chromatin. In contrast, phosphorylation of Ser28, which also occurs during mitosis and meiosis in H3.1, H3.2 and H3.3, showed a similar labeling index for autosomal and XY chromatin (**Fig. 1d**).

To establish whether the depletion of H3.1 and H3.2 is conserved in humans, we performed a double staining, combining antibodies to H3.1/H3.2 and the synaptonemal complex protein 3 (SYCP3) on human spermatocytes. As in mice, we observed loss of H3.1 and H3.2 from sex chromosomes at pachytene in human spermatocytes (**Fig. 1e**).

To estimate the onset and duration of H3.1 and H3.2 removal, we carried out double stainings with an antibody to γH2A.X, which stains the sex chromosomes from the zygotene-pachytene transition onward[18]. This event preceded loss of H3.1 and H3.2 (**Fig. 2a**). The histone H1 testis variant (H1t) is present from mid-pachytene onward[19]. A strong H1t signal always correlated with lack of H3.1 or H3.2 staining (**Fig. 2b**). To more precisely determine the timing of remodeling (**Fig. 2c**), we selected pachytene spermatocytes, as

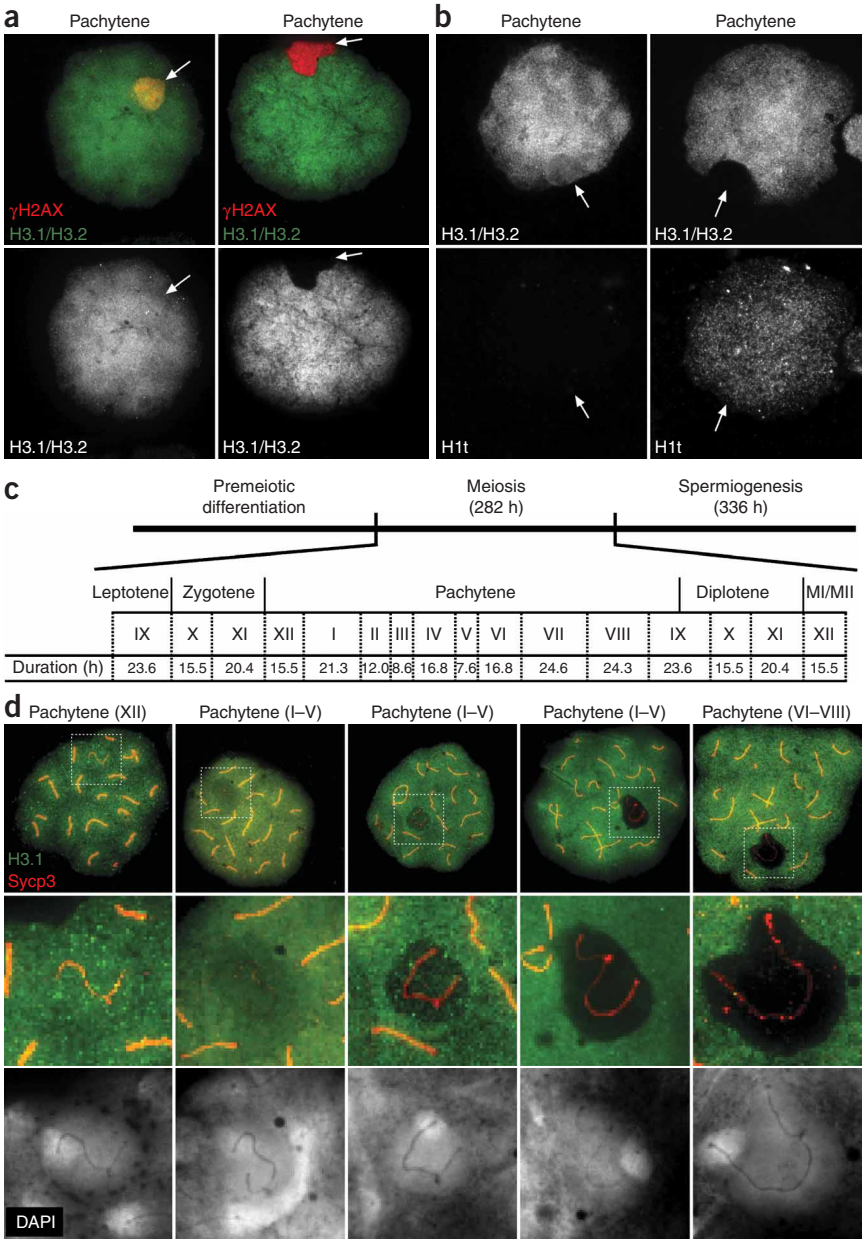

Figure 2 Timing of onset and duration of H3.1 and H3.2 removal. Arrows indicate sex chromosomes. (**a**) Localization of γH2A.X (red) and H3.1 and H3.2 (green) in pachytene spermatocytes. Phosphorylation of H2A.X at Ser139 in the XY body precedes loss of H3.1 and H3.2. (**b**) Localization of H1t and of H3.1 and H3.2 in pachytene spermatocytes. Loss of H3.1 and H3.2 precedes full expression of H1t. (**c**) Schematic representation of spermatogenesis, showing the sequence of epithelial stages (roman numerals) and their duration (hours)[20]. We adapted the pachytene-to-diplotene transition from a previously published study[15]. (**d**) Determination of the start and completion of H3.1 and H3.2 removal. We stained spermatocytes with antibody to H3.1/H3.2 (green), antibody to Sycp3 (red) and DAPI. Pachytene spermatocytes were identified on the basis of synaptonemal complex morphology ($n = 246$). Roman numerals in brackets denote the corresponding stages of the seminiferous epithelium.

defined by complete synaptonemal complexes, and scored them for the H3.1/H3.2 status of the sex body (**Fig. 2d**). Ten percent of 246 spermatocytes showed no difference in signal intensity between the XY body and autosomal bivalents, 42% showed a decreased signal and 48% showed a complete loss of H3.1 and H3.2 from the sex chromatin. Given that in mice the pachytene stage lasts for 6.6 d (159 h), these quantifications show that the decrease in H3.1 and H3.2 labeling is first detectable approximately 16 h after the zygotene-pachytene transition. It is expected, however, that the actual replacement process starts somewhat earlier. Histone H3.1 and H3.2 removal was completed at 3.5 d, coinciding with upregulation of autosomal transcription[3] (**Supplementary Table 2** online). In terms of the Oakberg scheme of stages of the seminiferous epithelium, we conclude that removal of H3.1 and H3.2 occurs during stages I–V (**Fig. 2c**)[20].

To address the kinetics of *de novo* H3.3 incorporation into XY chromatin during MSCI, we generated two transgenic mouse models, one with a gene encoding V5-tagged H3.3 and the other with a gene encoding hemagglutinin (HA)-tagged H3.1 (**Supplementary Fig. 2** online) and studied the spatial and temporal appearance of the tagged histones. In both a high- and a low-expressing transgenic line (**Supplementary Fig. 2**), the meiotic H3.3-V5 signal became visible in chromatin of the X chromosome at mid-pachytene, after desynapsis of the axial elements of the XY chromosomes had begun (**Fig. 3a–e**; **Supplementary Table 3** online). This was later than detection of HirA in the XY body (**Fig. 1c** and **Supplementary Table 1**), probably as a result of a lower sensitivity of the H3.3-V5 staining. The intensity of the signal progressively increased, and the staining extended to chromatin of the Y chromosome during late pachytene. For autosomal chromatin, we observed a concomitant rise that may relate to the increasing autosomal transcription observed at this stage[3]. The H3.3-V5 signal reached its maximal intensity at the diplotene stage in both autosomal and sex chromatin (**Fig. 3f–j**). In round spermatids, both sex chromosomes could be easily recognized by their accumulation of H3.3 (**Fig. 3j**). The behavior of HA-tagged H3.1 supports the findings with the monoclonal antibody to H3.1 and H3.2, though with a reduced sensitivity for stages before late pachytene as a result of a low expression of the transgene (**Fig. 3k**).

Structural differences between H3.1-, H3.2- and H3.3-containing nucleosomes are expected to be small[8], suggesting that the replacement of H3.1 and H3.2 by H3.3 as such is not the prime aim of the observed nucleosomal exchange. Eviction of H3.1 and H3.2 may follow from the need to remove certain histone PTMs carried by the H3•H4 dimers. To test this hypothesis, we studied the temporal dynamics of methylated forms of H3 and H4 in the XY body from early pachytene spermatocytes up to round spermatids.

We detected all 13 modifications (mono-, di- and trimethylated H3K4; mono-, di- and trimethylated H3K9; mono-, di- and

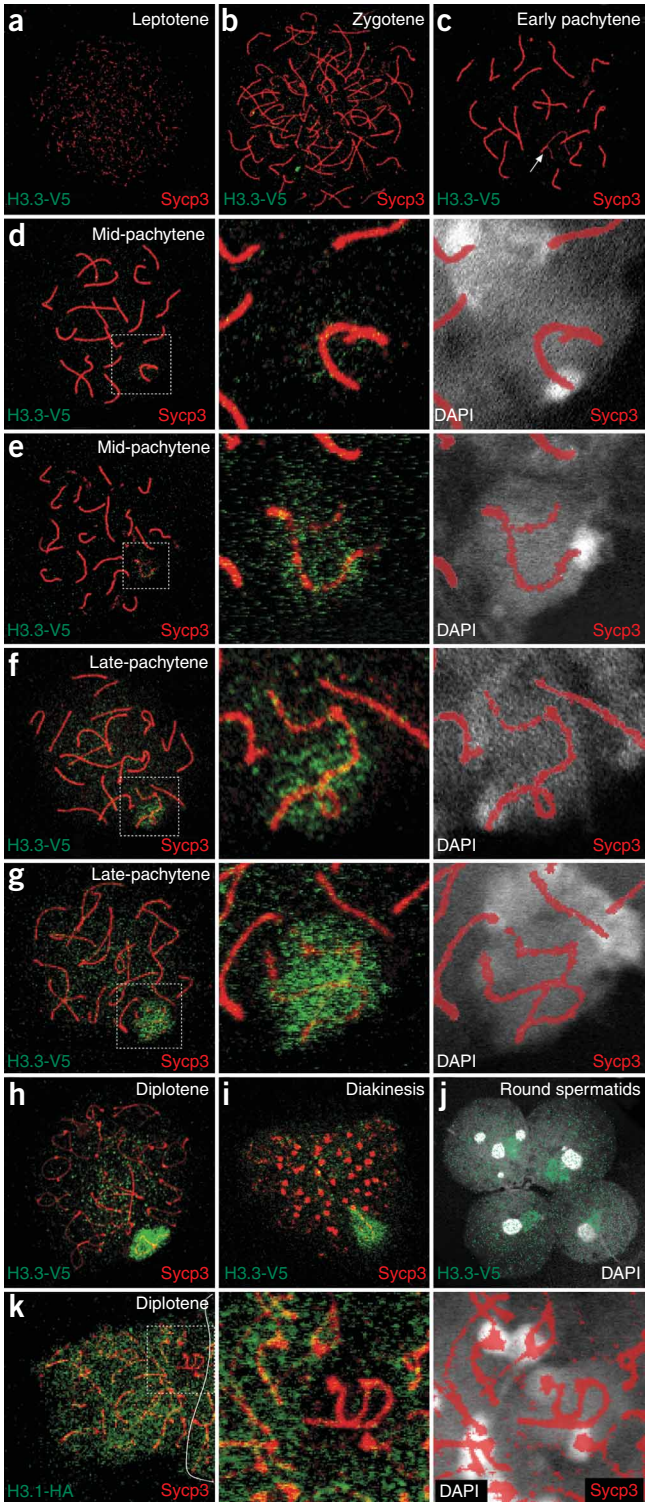

Figure 3 Progressive incorporation of H3.3 at sex chromosomes and autosomes during mouse meiotic prophase. All H3.3-V5 images are from the high-expressing transgenic line TgN(H3.3-V5)1Apet. Spermatocytes were staged on the basis of morphological criteria of chromosome axial and lateral elements (**Supplementary Table 3** online), visualized by staining with antibody to Sycp3 (red). DNA was stained by DAPI (white). (**a–d**) From leptotene onward, H3.3-V5 (green) became visible throughout chromatin only in midpachytene spermatocytes. (**d–g**) In the sex chromosomes, H3.3-V5 incorporation was first detected at X-linked chromatin, and later during late pachytene also at Y-linked chromatin. (**h,i**) H3.3-V5 labeling increased up to the diplotene stage and remained detectable during both meiotic divisions. (**j**) H3.3-V5 labeled X and Y territories[4,12,13] in haploid round spermatids. (**k**) H3.1-HA staining (green) at XY chromosomes was lost in diplotene spermatocytes of transgenic line TgN(H3.1-HA)1Apet.

trimethylated H3K27; dimethylated H3K79; and mono-, di- and trimethylated H4K20) in varying degrees of intensity in early pachytene XY chromatin (**Table 1**, **Fig. 4** and **Supplementary Fig. 3** online). Loss of H3.1 and H3.2 coincided with loss of all histone PTMs, with the exception of the monomethylated forms of H3K9 and H4K20.

We distinguished four patterns of histone lysine methylation dynamics from early pachytene to haplophase (**Table 1**): (i) the mark was lost and reappeared gradually during stages VI–XII (six cases; for example, trimethylated H3K9 in **Fig. 4a**); (ii) the mark was lost and reappeared at haplophase (two cases; for example, dimethylated H3K79 in **Fig. 4b**); (iii) the mark accumulated during nucleosome replacement, was absent in mid-late diplotene (stages X–XI) and reappeared during the meiotic divisions or haplophase (two cases; for example, monomethylated H4K20 in **Fig. 4c**); (iv) the mark was lost from the sex chromosomes (three cases; for example, trimethylated H4K20 in **Fig. 4d**). The temporary absence of certain histone modifications as a consequence of nucleosome replacement clarifies variations between reported staining patterns of these methylated lysine residues during pachytene[5,11–13]. Moreover, it is conceivable that it largely precludes H2A.Z from being incorporated into sex chromatin during pachytene and diplotene whereas autosomal chromatin is amenable to this process[13]. In conclusion, nucleosomal replacement during pachytene causes loss of almost all studied H3- and H4-associated methyl marks and leads to a selective reappearance of specific histone modifications in later meiotic and postmeiotic stages.

To assess whether nucleosome replacement is a sex chromosome–specific trait, or whether asynapsed autosomal segments also undergo this process, we analyzed primary spermatocytes from mice that are double heterozygous for two semi-identical reciprocal translocations[21]. In such T(1;13)70H/T(1;13)1Wa spermatocytes, translocation bivalents (**Fig. 5a–c**) often contain asynapsed autosomal chromosome segments that are subjected to MSUC[5] and positioned adjacent to the XY bivalent. Localization of Sycp3 and H3.1/H3.2 showed eviction of H3.1 and H3.2 in such asynapsed autosomal regions (**Fig. 5d–f**). Loss of H3.1 and H3.2 also occurred when asynapsed autosomal and sex chromatin were localized in separate domains, indicating that nucleosomal replacement is a general feature of MSUC and independent of sex body formation (**Fig. 5g**). Notably, in ~40% of late pachytene and early diplotene spermatocytes, we observed a residual H3.1/H3.2 signal in asynapsed autosomal segments or adjacent sex chromosomes, or both (**Fig. 5e** and **Supplementary Table 4** online). Thus, the capacity for nucleosomal replacement seems to be limited in spermatocytes. As sex bodies containing asynapsed autosomal chromatin show increased levels of transcription[22], nucleosomal replacement is probably a prerequisite for proper gene silencing in the context of MSCI and MSUC.

According to recent reports, MSUC and MSCI are initiated in late zygotene cells by targeting of the PI3-kinase ATR via BRCA1 to asynapsed chromatin[23,24] that later phosphorylates H2A.X (γH2A.X)[18] and ultimately leads to sex body formation in early pachytene spermatocytes. Timely induction of MSCI and MSUC by ATR targeting to asynapsed chromatin depends on the capacity of cells to monitor the state of synapsis at the zygotene-pachytene

Table 1 Temporal dynamics of histones and histone PTMs in sex chromatin during pachytene, diplotene, the meiotic divisions and in round spermatids

Staining pattern	Antibody	Early pachytene (stage XII)	Mid pachytene to mid diplotene (stages VI–X)	Mid to late diplotene (stages X–XI)	Meiotic divisions (stage XII)	Round spermatids[c]	Image in figure
	H3.1/H3.2	+	–	–	–	–	**Fig. 1**
	H3	+	+/–	+	+	+	**Fig. 1**
i	H3K4me1	+/–	+/–[a]	+/–	+	+/–	**Supplementary Fig. 3**
	H3K4me2	+/–	–	–	+	+/–	**Supplementary Fig. 3**
	H3K4me3	+/–	–	–	+/–	+	**Supplementary Fig. 3**
	H3K9me2	+	+[a]	+	+	+	**Supplementary Fig. 3**
	H3K9me3	+	+[a]	+	+	+	**Fig. 4a** and **Supplementary Fig. 3**
	H4K20me2	+/–	–	+/–[a]	+/–	–	**Supplementary Fig. 3**
ii	H3K27me1	+	–	–	–	+	**Supplementary Fig. 3**
	H3K79me2	+	–	–	–	+	**Fig. 4b**
iii	H3K9me1	+/–	+/–[b]	–	–	+/–	**Supplementary Fig. 3**
	H4K20me1	+/–	+[b]	–	+/–	+	**Fig. 4c** and **Supplementary Fig. 3**
iv	H3K27me2	+	–	–	–	–	**Supplementary Fig. 3**
	H3K27me3	+/–	–	–	–	–	**Supplementary Fig. 3**
	H4K20me3	+	–	–	–	–	**Fig. 4d**

Corresponding stages of the seminiferous epithelium are denoted between brackets. See Methods and **Supplementary Figures** and **Tables** for substaging of spermatocytes. Stage I–V spermatocytes in the process of H3.1 and H3.2 removal showed reduced levels of all histone PTMs except for monomethylated H3K9 and monomethylated H4K20 and are not referred to in this table. Variation between spermatocytes within a stage was limited with the exception of monomethylated H3K4, H3K9, H4K20 and di- and trimethylated H3K4 in stage XII pachytene spermatocytes. At this stage, levels of these modifications were heterogeneous and varied from faint to absent. me1, monomethylated; me2, dimethylated; me3, trimethylated.
[a]*De novo* appearance of histone PTM after temporary previous absence. [b]*De novo* appearance during stages I–V. [c]Period of appearance of histone modifications in sex chromatin of round spermatids differed between marks. +, abundant; +/–, faintly present; –, not present.

transition[2,25]. For example, in *Sycp1*$^{-/-}$ spermatocytes that do not finalize synaptonemal complex formation, staining patterns of ATR and γH2A.X do not differ between sex chromosomes and autosomes or between the zygotene, pachytene and diplotene stages[25]. Accordingly, such mutants do not form sex bodies. To determine whether nucleosome replacement depends on induction of MSUC and MSCI, we probed *Sycp1*$^{-/-}$ zygotene, pachytene and diplotene-like spermatocytes[25] with antibodies specific for H3.1/H3.2 and Sycp3. We did not obtain any evidence for nucleosome exchange at XY or autosomal chromatin (**Fig. 5h** and **Supplementary Table 4**).

Together, our data on eviction of H3.1 and H3.2 in wild-type, T(1;13)70H/T(1;13)1Wa and *Sycp1*$^{-/-}$ spermatocytes and on incorporation of H3.3 in transgenic H3.3-V5 spermatocytes show that nucleosome replacement is a general feature of MSUC, initiated shortly after induction of MSUC in early pachytene cells (**Supplementary Fig. 4**).

A function of γH2A.X in DNA double-strand break repair is to attract chromatin remodelers[26], and an analogous role for γH2AX in MSUC is therefore probable (as elimination of H2A.X results in failure to induce MSCI[27]). The accumulation in the XY body[1] of DNA repair proteins, such as Mre11 and Rad50, could therefore be connected to MSUC-induced nucleosome replacement. Hence, we propose that during MSUC these repair proteins facilitate chromatin remodeling rather than being instrumental in DNA repair.

In summary, we describe massive chromatin remodeling of the sex chromosomes to be a feature of human and mouse male meiotic prophase I. The unique chromatin composition obtained in this process potentially serves a role in MSCI and postmeiotic functioning of the sex chromosomes when a selective gene reactivation of MSCI-subjected genes occurs[12].

METHODS

Mice. We made initial observations in male F1(CBA/B6) mice. We used homozygous T(1;13)70H male mice on a Swiss random-bred background for collecting all data presented here. We used male T(1;13)70H/T(1;13)1Wa double heterozygous mice, also on a Swiss random-bred background, to study translocation chromosome–involved autosomal asynapsis[21]. Procedures involving mice were approved by the animal ethics committee of the Radboud University Nijmegen Medical Centre and conformed to Dutch Council for Animal Care and US National Institutes of Health guidelines. Generation, handling and housing of transgenic H3.3-V5 and H3.1-HA mice conformed to the Swiss Animal Protection Ordinance, chapter 1. We obtained human testicular tissue as remnant material from a diagnostic testicular biopsy with informed consent.

Surface-spread preparations. We obtained nuclear spreads as previously described[28], with some modifications[24]. Briefly, we obtained a suspension of spermatogenic cells which we treated with a hypotonic buffer (17 mM sodium citrate, 50 mM sucrose, 30 mM Tris HCl, pH 8.2). After centrifugation, we carefully resuspended the pellet in a 100 mM sucrose solution and applied it over a PFA-coated glass slide (1% PFA, 0.15% Triton-X-100, pH 9.2–9.5). We kept the slides for 2 h in a humidified atmosphere. After 1.5 h, we opened the box and washed slides with 0.08% photoflow (Kodak).

Fluorescent immunostaining. We stained surface-spread slides as previously described[24]. Briefly, we blocked slides for 1 h at 37 °C. After blocking, we applied primary antibodies diluted in blocking solution. We followed priming for 40 min at 37 °C by overnight incubation at 4 °C. The next day, we washed slides, after which we blocked nuclei for 1 h at 37 °C. We diluted secondary antibodies in blocking solution and incubated them for 2 h at 37 °C. After washing in PBS, we incubated slides with DAPI and mounted them with Vectashield.

Antibodies. We used the monoclonal antibody #34 to localize H3.1/H3.2 at a dilution of 1:1,500 (ref. 9). The monoclonal antibody #32 recognizes nucleosomes and we used it at a dilution of 1:2,000 (ref. 14). We purchased

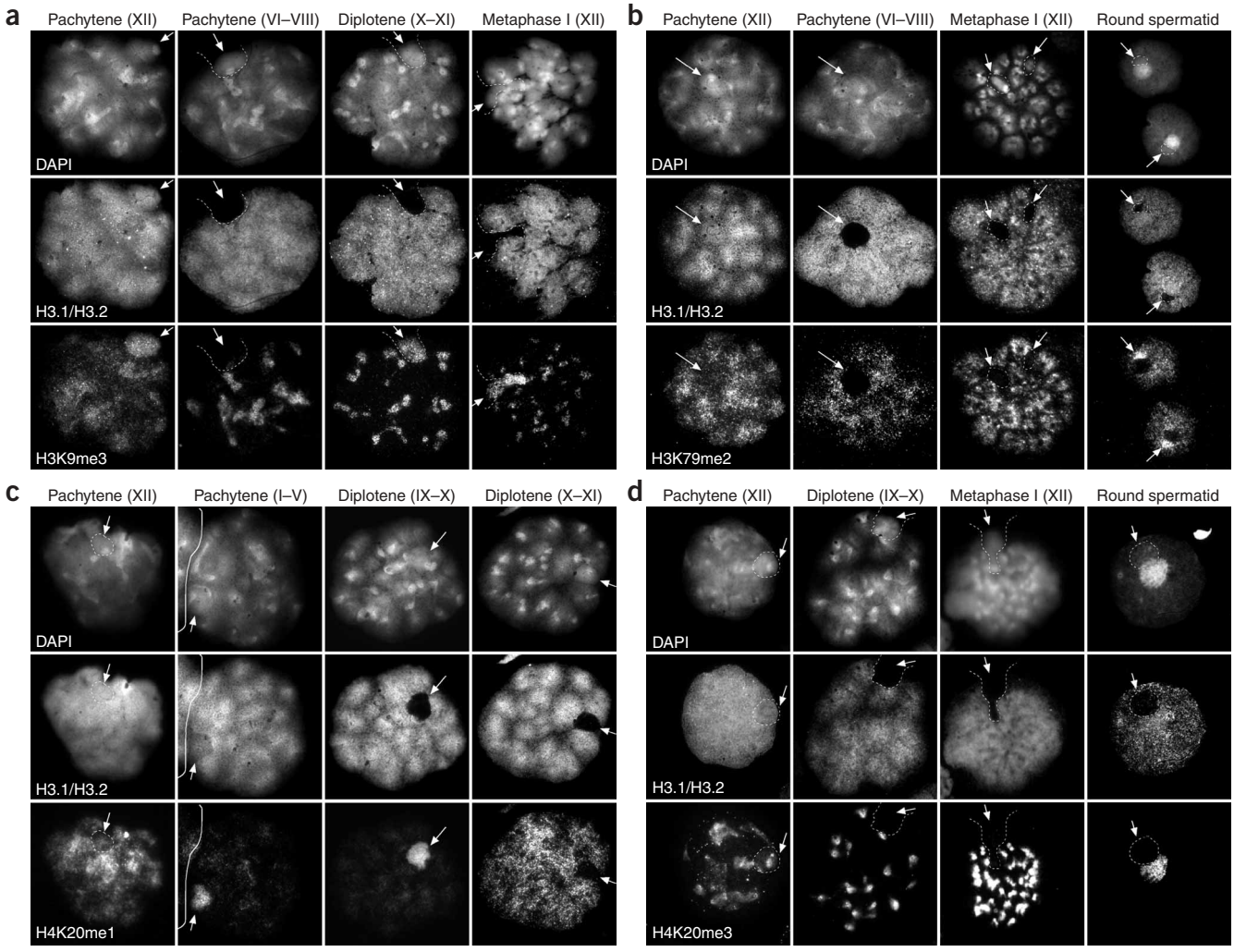

Figure 4 Dynamics in histone lysine-methylation patterns in relation to nucleosomal exchange at the XY body (**a–d**). Progressing stages of spermatogenesis are shown from left to right. Nuclei were stained with DAPI (upper panels) and with antibodies against H3.1/H3.2 (middle panels) and the indicated post-translational histone modification (lower panels). Arrows and dotted white lines indicate the position and shape of sex chromosomes. (**a**) Trimethylated H3K9: before removal of H3.1 and H3.2 removal, trimethylated H3K9 was enriched in the sex body. Loss of H3.1 and H3.2 depleted most of this marker, though some signal remained present in the heterochromatic region of the X chromosome. By stages X and XI, a prominent signal had accumulated in the XY chromatin, which persisted throughout the meiotic divisions and haplophase (**Supplementary Fig. 3**). (**b**) Dimethylated H3K79: in stage XII pachytene spermatocytes, dimethylated H3K79 was present in XY chromatin, although less prominently than in the autosomes. A complete loss of signal was observed with removal of H3.1 and H3.2. A signal accentuating the sex chromatin reappeared in round spermatids. (**c**) Monomethylated H4K20: in stage XII pachytene spermatocytes, monomethylated H4K20 was reduced in sex chromatin as compared to autosomes. During nucleosome replacement, levels of this marker increased vastly, were then absent in stage X–XI diplotene and reappeared in round spermatids (**Supplementary Fig. 3**). (**d**) Trimethylated H4K20 was present prominently in X heterochromatin and the pseudoautosomal region and faintly throughout the euchromatin of the sex chromosomes in stage XII pachytene spermatocytes[30]. A complete loss of signal was observed in concert with removal of H3.1 and H3.2. No return of this marker was observed up to the round spermatid stage.

polyclonal rabbit antibodies to Pan-H3 (ab1793; 1:500) and H3.3S31ph (ab2889, 1:20) from Abcam. We used polyclonal rabbit antibody D34, which recognizes HirA, at a dilution of 1:100 (P. Adams, Fox Chase Cancer Center). We used polyclonal rabbit antibody to Sycp3 in a 1:400 dilution (C. Heyting, Wageningen University and Research Centre). We purchased the mouse monoclonal antibody against γH2A.X from Upstate Biotechnology (clone JBW301; 1:10,000). We used polyclonal rabbit antibody against histone H1t in a 1:100 dilution (P. Moens, York University). We used polyclonal rabbit antibodies to mono-, di- and trimethylated H3K9 at a dilution of 1:250; antibodies to mono-, di- and trimethylated H3K27 at a dilution of 1:250; antibody to monomethylated H4K20 at 1:1,000; antibody to dimethylated H4K20 at 1:50; and antibody to trimethylated H4K20 at 1:250 (T. Jenuwein, Research Institute of Molecular Pathology and the Vienna Biocenter). We purchased rabbit polyclonal antibodies for mono-, di- and trimethylated H3K4 from Abcam (respectively: ab8895, 1:100; ab7766, 1:100; ab8580, 1:1,500). We used polyclonal rabbit antibody to detect dimethylated H3K79 in a 1:500 dilution (F. van Leeuwen, Netherlands Cancer Institute). We used antibody to V5 (Invitrogen) at a dilution of 1:500, and antibody to HA (Roche) in a 1:100 dilution. To identify the epitopes for the pan-H3, dimethylated H3K79, H3.3S31ph, V5-specific and HA-specific antibodies, we first incubated slides in 4 M HCl for 6 min before blocking, after which we extensively washed slides in PBS. We detected primary antibodies by labeling with Molecular Probes A11001 Fluor 488–conjugated goat antibody to mouse IgG (H+L) and A11012 Fluor 594–conjugated goat antibody to rabbit IgG (H+L). We used both at a 1:500 dilution.

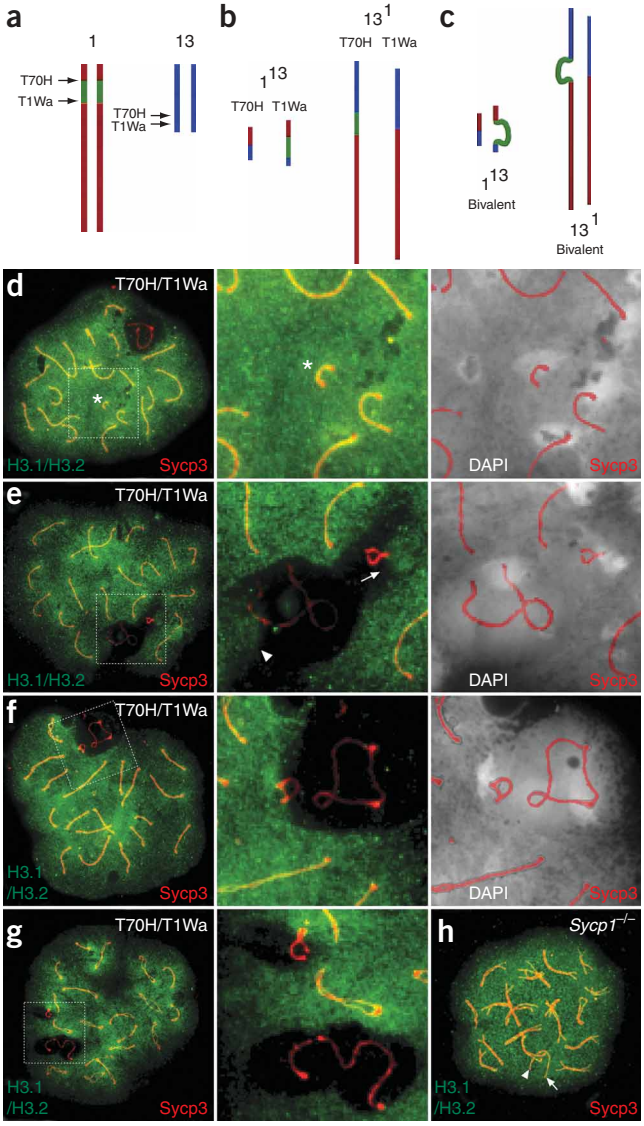

Figure 5 Localization of H3.1 and H3.2 in asynapsed autosomal chromatin of the T(1;13)70H and T(1;13)Wa translocation–containing spermatocytes (**a–g**) and *Sycp1*$^{-/-}$ spermatocytes (**h**). Nuclei were stained for H3.1 and H3.2 (green), Sycp3 (red) and DAPI. The second and third columns are higher-magnification views, except for **h**. (**a**) Localization of T70H and T1Wa breakpoints. (**b**) Double translocation heterozygosity leads two semi-identical 'homologous' chromosomes: 1^{13} and 13^1. (**c**) During early meiotic prophase I, the nonhomologous segments (green) are initially asynapsed. In the 13^1 bivalent, this segments readjusts during late zygotene and early pachytene, whereas in the 1^{13} bivalent, it frequently remains totally or partially asynapsed (diagram reproduced from ref. 18). (**d**) When full heterologous synapsis was accomplished, a horseshoe-like synaptonemal complex was produced (indicated by asterisk). No loss of H3.1 and H3.2 was observed from the 1^{13} bivalent. (**e**) Synapsis on one end of the 1^{13} bivalent yielded a fork conformation. Removal of H3.1 and H3.2 was observed in the asynapsed regions. Synapsed chromatin of the same bivalent retained H3.1 and H3.2 (indicated with arrow). In spermatocytes carrying a partially synapsed 1^{13} bivalent, an incomplete loss of H3.1 and H3.2 in the XY chromatin was more frequently observed (indicated by arrowhead). (**f**) Synapsis occurring on both ends of the 1^{13} bivalent resulted in the loop conformation. H3.1 and H3.2 were lost only in the intermediate asynapsed region but not in synapsed extremities of the bivalent. (**g**) Loss of H3.1 and H3.2 was also observed when the sex chromosomes and the 1^{13} bivalent were localized in separate domains. Such separation was a rare event (3 out of 221 pachytene or diplotene spermatocytes). (**h**) In *Sycp1*$^{-/-}$ diplotene-like spermatocytes, no loss of H3.1 and H3.2 was observed from the sex chromosomes (arrow indicates X, arrowhead indicates Y).

Analysis and image capture. We analyzed a minimum of 100 pachytene or diplotene nuclei, or round spermatid nuclei, per staining. We performed all stainings except those with the nucleosome antibody in combination with the antibody to H3.1 and H3.2. As immunofluorescence stainings were done in combination with DAPI staining, the identification of first meiotic prophase stages relied on DAPI staining pattern, in particular the morphology of the sex body. We made the assumption that pachytene had commenced when a sex body could be identified with DAPI. More precise staging was based on antistaining of the axial elements of the XY chromosomes by DAPI[15,29]. The DAPI staining characteristics of centric heterochromatin that change over prophase I constituted the other criterion[29] (**Fig. 1** and **Supplementary Fig. 1**). The pachytene-diplotene transition could not be precisely determined with this approach. We grouped diakinesis and metaphase I (stage XII) as metaphase I. Images were collected on a Zeiss Axioplan fluorescence microscope. Pictures were captured by a Zeiss AxioCam MR camera on Axiovision 3.1 software (Carl Zeiss). Adobe Photoshop 7.0 was used to reduce background when necessary.

Spermatocytes from H3.3-V5 and H3.1-HA transgenic mice were co-stained with antibody to Sycp3 and either antibody to V5 or antibody to HA. Staging criteria for substages of pachytene are described in **Supplementary Table 3** online.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

G.W.v.d.H., A.A.H.A.D., E.P., A.H.F.M.P. and P.d.B. conceived and designed the experiments. G.W.v.d.H., A.A.H.A.D., E.P. and M.G. performed the experiments. G.W.v.d.H., A.A.H.A.D., E.P., A.H.F.M.P. and P.d.B. analyzed the data. P.P. generated histone-tagged transgenic mice. L.R. was responsible for human material. J.v.d.V. contributed H3.1- and H3.2-specific antibodies. G.W.v.d.H., A.A.H.A.D., D.G.W., J.v.d.V., A.H.F.M.P. and P.d.B. contributed to the writing of the manuscript. J.V.d.V. and A.H.F.M.P. contributed equally to this work.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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3.2. Polycomb function during oogenesis is required for early embryonic development

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3.2.1. Abstract

In many sexual reproducing organisms, early embryonic development is driven by factors provided by the oocyte to exert the maternal-to-embryonic transition. The nature of the maternal contribution and the mechanisms specifying it are largely unknown. Polycomb proteins are evolutionarily conserved chromatin modifying proteins that are thought to maintain cellular identity and developmental potential by transcriptionally repressing genes that promote differentiation during development. Here we report the role of Rnf1 (Ring1a) and Rnf2 (Ring1b), two core components of the Polycomb Repressive Complex 1 (PRC1), in defining the maternal contribution. Embryos deficient for maternal *Rnf1* and *Rnf2* expression failed to develop beyond the two-cell stage. Numerous developmental regulator genes that are established Polycomb targets in other cellular systems are derepressed in *Rnf1/Rnf2* double mutant (dm) fully grown oocytes. Translation of aberrant maternal transcripts is, however, delayed until after fertilization, resulting in the inappropriate presence of differentiation factors during the otherwise totipotent stage of early embryogenesis. Transfer of maternal pro-nuclei between control and *Rnf1/Rnf2* maternally deficient early zygotes revealed, however, that the developmental arrest is not only due to maternal transmission of inappropriate cytoplasmic factors, but also to an altered chromatin state inherited from the *Rnf1/Rnf2* dm oocyte. Together, these data reveal an essential role for Polycomb during oogenesis in defining cytoplasmic and nuclear maternal contributions that are required for proper initiation of embryonic development. The data suggest that Polycomb acts in the germline and early embryogenesis to sustain developmental potency across generations.

3.2.2. Materials and Methods

Mice

The generation of conditionally deficient *Rnf2* mice was performed as previously described (Puschendorf *et al.*, 2008; van der Stoop *et al.*, 2008) and also illustrated in Supplementary Figure 1.

Maternally deficient *Rnf2* zygotes were generated by crossing $Rnf2^{F/F}$; *Zp3*-cre/+ females with $Rnf2^{F/F}$ males. Maternally and zygotically deficient *Rnf2* embryos were generated by crossing $Rnf2^{F/F}$; *Zp3*-cre/+ females with $Rnf2^{F/F}$; *Prm1-cre*/+ males. Wild-type control embryos were generated by $Rnf2^{F/F}$ females and $Rnf2^{F/F}$ males.

Generation of maternally or maternally and zygotically double deficient embryos for *Rnf1* and *Rnf2* was performed as described above but on a $Rnf1^{-/-}$ background (del Mar Lorente *et al.*, 2000). Control embryos used in experiments are generated form littermate females lacking *Zp3*-cre and therefore are $Rnf1^{-/-}$ (unless specifically stated wildy-type). Mice were maintained on a mixed background of 129/Sv

and C57BL/6J. All experiments were performed in accordance with the Swiss animal protection laws and institutional guidelines.

Collection, *in vitro* fertilization and culture of mouse oocytes and embryos

Mouse oocytes and embryos were derived from superovulated 5-10 week old females according to standard procedures (Hogan *et al.*, 1994). Fully-grown germinal vesicle (GV)-intact oocytes were collected 46 h after PMSG injection (5 U, Intervet) in M2 medium (Sigma) containing 2.5 µM milrinone (Sigma). For Germinal Vesicle Break Down (GVBD) and meiotic maturation experiments GV oocytes were transferred into M16 medium (Sigma) without milrinone. Oocytes were harvested at indicted time points.

Metaphase II-arrested eggs were collected from PMSG- and hCG-primed (5 U each, Intervet) mice 14 h after hCG injection. Late M-II oocytes were collected 18 h after hCG injection. Embryos were harvested from superovulated females mated to appropriate males in FHM medium (Chemicon) at indicated time points after hCG injection: late zygotes (26 h), early 2-cell (36 h), mid 2-cell (42 h), late 2-cell (48 h), and blastocyst stage embryos (94 h). Where precise timing of progression of embryonic stages was required, oocytes were fertilized *in vitro*. Sperm was obtained from 10-16 week old control males, and M-II oocytes used for IVF were collected from appropriate females 14 h after hCG injection. Sperm capacitation was carried out in HTF containing 9 mg/ml BSA for 2h. IVF was performed in capacitation medium for 2 h and thereafter the embryos were cultured in FHM or KSOM medium plus amino acids (Chemicon) in a humidified atmosphere of 5% CO_2 in air until required. Zygotes were substaged according to morphology of pronuclei using criteria as defined previously (Adenot *et al.*, 1997; Santos *et al.*, 2002). In brief, PN0 refers to oocytes immediately after fertilization, PN1 pronuclei are small and reside at the periphery of the embryo, PN2 pronuclei have an increased size and have started to migrate towards the center of the embryo, PN3 pronuclei have migrated towards the center, large PN4 pronuclei were close to each other in the center of the embryo and PN5 refers to large central pronuclei.

Meiotically incompetent growing oocytes were collected from 12-14 day old mice. For expression analysis by qRT-PCR ovaries were dissected and washed in Ca^{2+}- and Mg^{2+}-free CZBT medium (CMF-CZBT) (85.35 mM NaCl, 4.83 mM KCl, 1.18 mM KH_2PO_4, 25.12 mM sodium bicarbonate ($NaHCO_3$), 10 µg/mL gentamicin, 10 µg/mL phenol red, 0.27 mM sodium pyruvate, 7 mM taurine, 0.11 mM EDTA , 31.3 mM sodium lactate, 0.1% polyvinylalcohol (PVA)), transferred to CMF-CZBT containing 1mg/ml collagenase (Worthington Biochemical Corp) and 0.2mg/ml DNaseI (Sigma) and dissociated by repeated pipeting. Oocytes free of follicle cells were washed in CMF-CZBT and staged primary (diameter 50-60 µm) or secondary (diameter >60 µm) growing oocyte based on size before harvesting (Pan *et al.*, 2005).

Antibodies

For immunofluorescence analyses of Polycomb group proteins, the following antibodies were used : monoclonal anti-Rnf2 (Atsuta *et al.*, 2001), 1:400), monoclonal anti-Rnf1 (Vidal;, 1:100), polyclonal anti-Bmi1 (van Lohuizen, 1:400), monoclonal anti-Bmi1 (van Lohuizen, 1:100), polyclonal anti-Pc1 (Otte, 1:500).
Other antibodies used: monoclonal anti-RNAPII (8WG16, 1:5), polyclonal anti-Gata4 (SC-9053, 1:100), monoclonal anti-Pax6 (Hybridoma Bank, 1:1000), polyclonal anti-Eomes (Abcam 23345, 1:100) and monoclonal anti-Myc (Ascites 9E10, 1:200).

Immunofluorescence

Before fixation of oocytes and embryos, the zona pellucida was removed by incubation in acidic tyrode for 30 seconds. Embryos were washed twice in FHM, fixed for 15 min in 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with 0.2% Triton-X 100 in PBS for 15 min at room temperature (RT). Fixed embryos were blocked at least 4 hours at RT in 0.1% Tween-20 in PBS containing 2% BSA and 5% normal goat serum, and were then incubated with primary antibodies in blocking solution overnight at 4ºC. Double antibody stainings were accomplished by mixing appropriate different primary and different secondary antibodies for simultaneous incubation. Embryos were washed three times for 30 min in 0.1% Tween-20 in PBS containing 2% BSA before application of secondary antibodies. For detection, anti-rabbit IgG-Alexa 488, anti-mouse IgG-Alexa 488 and anti-mouse IgG-Alexa 555 (Molecular Probes) secondary antibodies were diluted 1:500 in blocking solution and embryos were incubated for 1 h at RT followed by three washing steps for 30 min in 0.1% Tween-20 in PBS containing 2% BSA in the dark. Embryos were mounted in Vectashield containing DAPI (Vector) (Santos *et al.*, 2005).
Ovaries form 12-14 day old mice were dissected, washed in CMF-CZBT and frozen in Tissue-Tek O.C.T.^{TM} compound (Sakura Finetek) on dry ice. 10µm thick cryo-sections were cut from frozen blocks with Microm HM355S. Cryo-sections were fixed on slides with 2% paraformaldehyde in PBS (pH 7.4) for 10 min on ice, permeabilized in 0.1% Triton-X100 in 0.1% sodium citrate for 15 min and blocked for 30 min in 0.1% Tween-20 in PBS containing 2% BSA and 5% normal goat serum at RT. Incubation with primary and secondary antibodies as well as mounting was performed as described above.
If not stated otherwise in figures, at least 10 oocytes or 5 embryos were analysed for each IF staining.

Microscopy and image analysis

Immunofluorescence stainings of embryos were analyzed using a laser scanning confocal microscope LSM510 META (Zeiss) and LSM510 software. For embryos, either a z-series 1.3 µm slices was recorded or one confocal slice through the maximal radius of each (pro)nucleus was scanned. Images were

analysed using Imaris (Bitplane) software and exported as TIFF files. DIC images were recorded with a 2.45 Zeiss Z1 microscope by transferring embryos into a drop of immersion oil and placing the 40x objective directly in the drop.

Quantitative real-time RT-PCR

For RNA isolation, oocytes and embryos were pooled from several mice and RNA was isolated from batches of 20-50 oocytes or embryos. Total RNA was extracted using the PicoPure™ RNA Isolation Kit (KIT0202) according to the manufacturer's instructions (Stratagene) with the addition of adding 100 ng E.Coli rRNA as carrier and a bacterial probe set as spike (GeneChip®Eukaryotic Poly-A RNA Control Kit). The bacterial spike stock (as provided by the manufacturer) was diluted 1:2000 and 1 µl was added per 1 oocyte or embryo. Reverse transcription was performed from total RNA corresponding to 20-25 oocytes or embryos using random primers (200 ng) and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. For quantitative real time PCR reactions, cDNA corresponding to 0.4 oocytes or embryos was used as a template. Real time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystem) and ABI Prism 7000 Real time PCR machine. All real time PCR measurements were normalized against endogenous LnmB1 and to exogenous bacterial spike gene Thr. All real time PCR experiments were performed on at least 2 biological replicates form independent isolations.

Primers used:

Rnf1 (F: 5'-GCCATCATGGATGGTACAGAG, R: 5'-TATTCCTCCCGGCTAGGGTAG),

Rnf2 (F: 5'-AGGCAATAACAGATGGCTTGG, R: 5'-GAGAGCCTGCTGATTGTTGTG),

Cbx2 (F: 5'-GTAGTCCCAAAGCCCAGTCAG, R: 5'-CAAGTGCCTACATCAGCTTGC),

Bmi1 (F: 5'-AAGACCGAGGAGAAGTTGCAG, R: 5'-CCCAGAGTCACTTTCCAGCTC),

Phc1 (F: 5'-GCCTTCTTCAGGATTGACTGG, R: 5'-GATCACCACTTGCTTCTGCTG),

Phc2 (F: 5'-CAGTGCTCTACCACGCATGTC, R: 5'-GCTGGATGTTGGGACTCTTG),

Phc3 (F: 5'-GTACCTGCAGCAGATGTACGC, R: 5'-CTGCAGACTGACAGGAAGGTG),

Ezh2 (F: 5'-AGCCTTGTGACAGTTCGTGC, R: 5'-TTTAGAGCCCCGCTGAATG),

Eed (F: 5'-ACCAGCCATTGTTTGGAGTTC, R: 5'-ACCTCCGAATATTGCCACAAG),

Gata4 (F: 5'-TCTCTGCATGTCCCATACCA, R: 5'- AGAAAGTCCCAGAGCCAGGT),

Pax6 (F: 5´-GCACATGCAAACACACATGA, R: 5´-ACTTGGACGGGAACTGACAC),

Eomes (F: 5'-AAGCCCGATATTATAACGGTGAGA, R: 5'-GCTTGTTGGTCACAGGTTGCT),

Klf4 (F: 5'-CCAGACCAGATGCAGTCACAAG, R: 5'-ACGACCTTCTTCCCCTCTTTG),

Gata6 (F: 5'-GGGCCTTGTCTGCTAAGGAAG, R: 5'-CCACGAACGCTTGTGAAATG),

Nestin (F: 5'-CAGCAACTGGCACACCTCAA, R: 5'-CCCAAGGAAATGCAGCTTCA),

Sox2 (F: 5'-CGAGATAAACATGGCAATCAAATG, R: 5'-AACGTTTGCCTTAAACAAGACCAC)

Bmp4 (F: 5'-TCGAGGCGACACTTCTACAG, R: 5'- CCTGGGATGTTCTCCAGATG),

Hoxa1 (F: 5'-CACGTAGCCGTACTCTCCAAC, R: 5'-CACCAAGAAGCCTGTCGTTC),

Thr (F: 5'-ATTGCCGACTGATGAAGACAG, R: 5'-ACAAATCTGCGGTCCGTTTAG),

LnmB1 (F: 5'-GCCCTGGACATGGAGATCAGC, R: 5'-CGACTGGAGGACGCTCTGGA).

Expression profiling of GV oocytes and data analysis

GV oocytes were pooled from several mice and RNA was isolated from batches of 50 oocytes, 3 biological replicates per genotype. RNA was isolated using the PicoPure™ RNA Isolation Kit (KIT0202) according to the manufacturer's instructions (Stratagene). The quality of the RNA was assessed using the Agilent 2100 bioanalyzer and RNA 6000 Pico Chip. The extracted RNA was converted into OmniPlex WTA cDNA libraries and amplified by WTA PCR using reagents supplied with the TransPlex Whole Transcriptome Amplification kit (WTA1, Sigma, USA) following the manufacturer's instructions with minor modifications. The obtained cDNA was purified using the GeneChip cDNA Sample Cleanup Module (Affymetrix). The labeling, fragmentation and hybridization of cDNA was performed according to Affymetrix instructions (GeneChip Whole Transcription Sense Target Labeling technical manual, Rev. 2) with minor modifications. Samples were hybridized to Mouse Gene 1.0 arrays from Affymetrix.

Quality control and background normalization was performed using Refiner 4.5 from Genedata AG (Basel, Switzerland). Expression value estimates were obtained using the GC-RMA implementation in Refiner 4.5. Quantile normalization and median scaling of the genes called present (detection P-value <0.05) was performed in Expressionist pro 5.0 (Genedata AG). Statistically significant changes were identified using N-way ANOVA (P>0.05). To identify transcripts that are differentially expressed between control and mutant mice, we defined a criterion of a 1.5-fold and greater difference plus a p-value of <0.05. The P-values reported for enriched GO terms were obtained using GO Stat (http://gostat.wehi.edu.au).

Microinjection of *Rnf2* mRNA

N-terminally myc-tagged Rnf2 open reading frame (accession number NM_011277) was cloned into a pcDNA3.1-polyA vector. The plasmid was linearized and *in vitro* transcribed using the mMessage mMachine T7 kit (Ambion, AM1344). The synthesized mRNA was diluted to the optimal concentration

using nuclease-free water (not DEPC-treated; Ambion, AM9937). 2-4 pl mRNA (50ng/ul) was microinjected into the cytolplasm of *in vitro* fertilized zygotes using the Eppendorf FemtoJet injector system. Zygotes were then cultured in M16 (Sigma, M7292) under a 5% CO2 atmosphere at 37ºC, and fixed at various stages as indicated in the figure legends.

Pronucear transfer experiments

Fertilized eggs were collected at 22-24 h post hCG injection in M2 medium (Sigma) after a brief treatment with 1 mg/ml of hyaluronidase in phosphate-buffered sodium (PBS, pH 7.5) to separate them from the surrounding follicular cells. After collection embryos were transferred to FHM medium (Millipore) and kept at 37°C under 5%CO_2 until subsequent manipulations.

Pro-nucleus exchanges were performed between 24 and 28 h post hCG. For the micromanipulation, embryos were transferred in M2 medium containing 5µg/ml Cytochalasin B and placed in a chamber on the stage of an inverted microscope (Nikon) equipped with micromanipulators (Nikon-Narishige MO-188). The maternal pronuclei from donor embryos (visualized under differential interference contrast) were identified using both size and position in respect to the polar body. The polar bodies were removed using an enucleation/injection pipette containing low viscosity silicone oil. The maternal pronuclei were aspirated into the pipette and subsequently re-injected into the perivitelline space of receiver embryos, from which the maternal pronuclei had been previously removed. The receiver embryos were transferred into an electroporation chamber containing a solution of Mannitol (Sigma) 0.3M/0.3%BSA (Sigma) sterilized by filtration on 0.22 µm filters. The electroporation was performed using a BTX electro cell manipulator 200 (BTX). To operate the fusion between the exogenous maternal pronuclei and the receiver embryos, the embryos were positioned so that the pronucleus-embryo axis was perpendicular to that of the electrodes. The space between the electrodes was 250 µm, and 2 pulses of 40µsec each at 30V were performed. After the fusion procedure, embryos were transferred in 50µl drops of FHM medium under mineral oil and kept in culture at 37°C under 5% CO_2.

Comparison to expression patterns during oocyte development and early embryogenesis

Defining expression states

To relate data to expression status genes in different stages of oogenesis and embryogenesis, we processed data from publicly available Affymetrix CEL files (Pan *et al.*, 2005; Zeng *et al.*, 2004; Zeng and Schultz, 2005) using Expressionist pro 5.0 (Genedata AG). Expression values were estimated using the RMA-Bioconductor function and their distributions were standardized by quantile normalization and scaled by transforming the median expression value to 20.

Probe sets with a detection P-value < 0.04 (Affymetrix default) in at least three out of four replicates (Pan *et al.*, 2005; Zeng *et al.*, 2004; Zeng and Schultz, 2005) were considered to be expressed and annotated with Gene symbols. Lists of genes expressed at a given stage were compared to current data set using the Gene symbol for mapping. For analysis of absolute expression levels the mean of replicates was calculated for each probe set and the highest value was taken in case of multiple probe sets per gene.

Defining expression profiles

To identify classes of genes with similar expression profiles during oogenesis and embryogenesis, we first selected probesets with an expression value >10 (oogenesis) or >20 (embryogenesis) in at least one developmental stage. Probes with significantly changing expression levels between developmental states (P-value < 0.05 in N-way ANOVA analysis) were assigned to different expression profiles using the self organizing map (SOM) clustering algorithm. We combined profiles with similar changes in expression states during development. In brief, for oogenesis, expression levels in primordial, primary, secondary, small antral and large antral oocytes were analyzed (Pan *et al.*, 2005). Probe sets expressed throughout the five stages were termed “stable”, genes not detected at either stage were termed “not detected”, probe sets expressed in primordial oocytes only and probe sets showing decreasing expression during to course of oogenesis were termed “early expressed” and finally, probe sets expressed at all stages except in primordial oocytes and probe sets showing increasing expression during oogenesis were termed “late expressed”. For embryogenesis, expression levels in GV oocytes, 1-cell, 2-cell, 8-cell embryos and blastocysts were analyzed (Zeng *et al.*, 2004). To distinguish between maternally provided transcripts and *de novo* transcription in 2-cell stage embryos, we compared expression levels in embryos treated and untreated with the transcription inhibitor α-amanitin (Zeng and Schultz, 2005). Probe sets not detected at any stage were termed “not detected”, probe sets expressed in GV oocytes were termed “maternal”, probe sets showing α-amanitin sensitive expression at the 2-cell stage were termed “early expressed” and probe sets showing α-amanitin sensitive expression at the 8-cell or blastocyst stage were termed “late expressed”. Probe sets in groups of combined profiles were annotated with gene symbols. Groups were compared to current data set based on mouse gene symbols.

Matching to published data sets

For each comparison a data set of genes common in both array platforms (current data on Mouse Gene 1.0 array and each published array platform) was determined and only these genes were used in subsequent analyses.

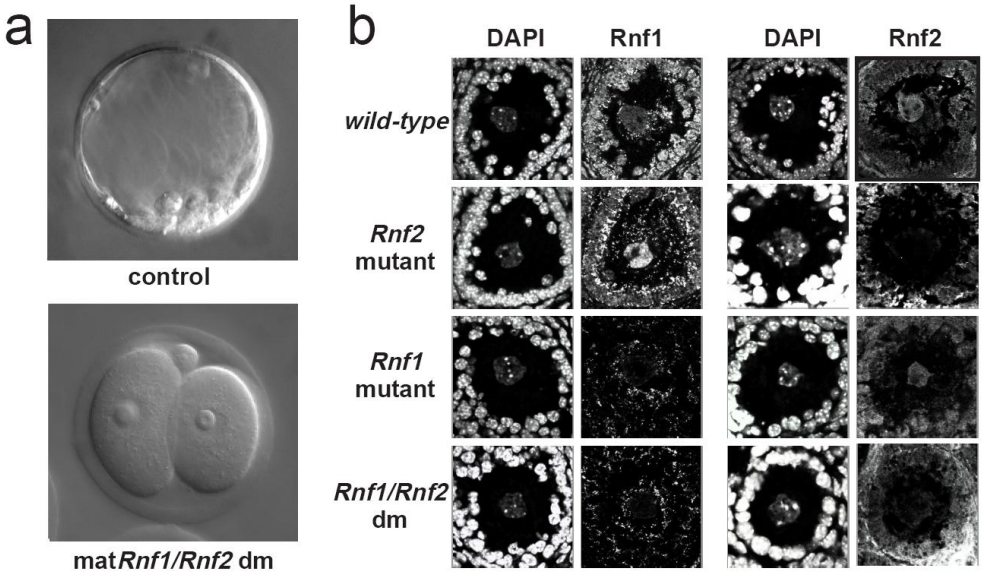
Comparison to maps of Polycomb proteins and H3K27me3 in mouse ES cells and expression in PRC1/PRC2 deficient mouse ES cells

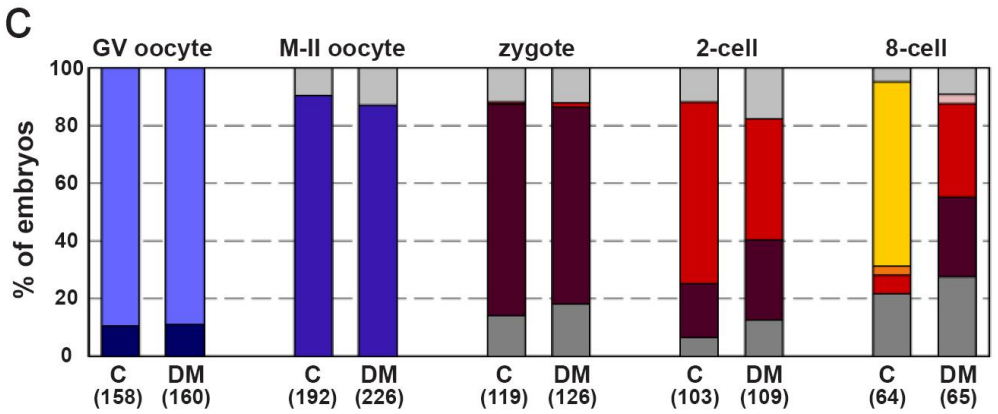
To compare our expression data to published studies, we processed data from publicly available Affymetrix CEL files using Expressionist pro 5.0 (Genedata AG) or from provided supplementary excel files. (Ku *et al.*, 2008; Leeb *et al.*, 2010) Probe sets were matched between platforms based on gene IDs or gene symbols. For each comparison only probe sets common to both platforms were taken into account.

3.2.3. Results and discussion

In mammals, fusion of two highly differentiated germ cells leads to the formation of a totipotent pre-implantation embryo. Acquisition of totipotency concurs with extensive remodeling of chromatin states of parental genomes (commonly referred to as “epigenetic reprogramming”), changes in maternally provided transcriptomes and proteomes, and zygotic genome activation (Evsikov and Marin de Evsikova, 2009; Santos and Dean, 2004; Tadros and Lipshitz, 2009). Genomes in mature germ cells are marked by various active and repressive histone modifications, including tri-methylation of histone H3 at lysine 27 (H3K27me3) mediated by the Polycomb Repressive Complex 2 (PRC2), suggesting a transcriptional regulatory function for PcG proteins during gametogenesis and possibly across generations (Arpanahi *et al.*, 2009; Brykczynska, in press.*;* Hammoud *et al.*, 2009; Puschendorf *et al.*, 2008; Santos *et al.*, 2005). Components of PRC2, like *Ezh2*, *Eed* and *Suz12*, as well as of PRC1, such as *Rnf2* (*Ring1b*), *Bmi1*, *Phc2* and *Cbx2*, are maternally provided. Both complexes are required for genome-wide establishment and propagation of repressive chromatin on paternal and maternal genomes, respectively, in early embryos. Nonetheless, pre-implantation embryos maternally and zygotically deficient for *Rnf2* or *Ezh2,* which lack detectable levels of PRC1 or of PRC2 proteins and H3K27me3 respectively, develop similarly as *Rnf2* or *Ezh2* zygotically deficient embryos, suggesting no major role for maternally provided PRC1 or PRC2 for early embryonic development (Supplementary Fig. 1) (Puschendorf *et al.*, 2008; Terranova *et al.*, 2008). However, loss of *Rnf2* or *Ezh2* function may be compensated by low expression of the *Rnf1* (*Ring1a*) or *Ezh1* paralogs, as observed in embryonic stem cells (Endoh *et al.*, 2008; Shen *et al.*, 2008; van der Stoop *et al.*, 2008).

To address the definite function of maternal PRC1 for oogenesis and early embryogenesis, we performed loss-of-function analysis of *Rnf1* and *Rnf2* during oogenesis by conditionally deleting *Rnf2* in growing oocytes that are constitutively deficient for *Rnf1* (*Rnf1*$^{-/-}$*; Rnf2*$^{F/F}$*; Zp3*-cre), hereafter referred to as *Rnf1/Rnf2* dm oocytes (Supplementary Fig. 1). When fertilizing such oocytes with sperm deficient for *Rnf1* and *Rnf2* (from *Rnf1*$^{-/-}$*; Rnf2*$^{F/F}$*; Prm1*-cre males), we observed that the development of embryos (deficient for maternal and zygotic *Rnf1* and *Rnf2* expression) beyond the two-cell stage was abrogated. Similarly, even when fertilized by wild type sperm, maternal *Rnf1/Rnf2* dm (mat*Rnf1/Rnf2* dm) embryos got arrested at the two-cell stage, demonstrating the maternal-effect nature of the phenotype (Fig. 1a).


a
control
matRnf1/Rnf2 dm
b
DAPI
Rnf1
DAPI
Rnf2
wild-type
Rnf2 mutant
Rnf1 mutant
Rnf1/Rnf2 dm


c
GV oocyte
M-II oocyte
zygote
2-cell
8-cell
% of embryos
100
80
60
40
20
0
C (158)
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DM (226)
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DM (126)
C (103)
DM (109)
C (64)
DM (65)

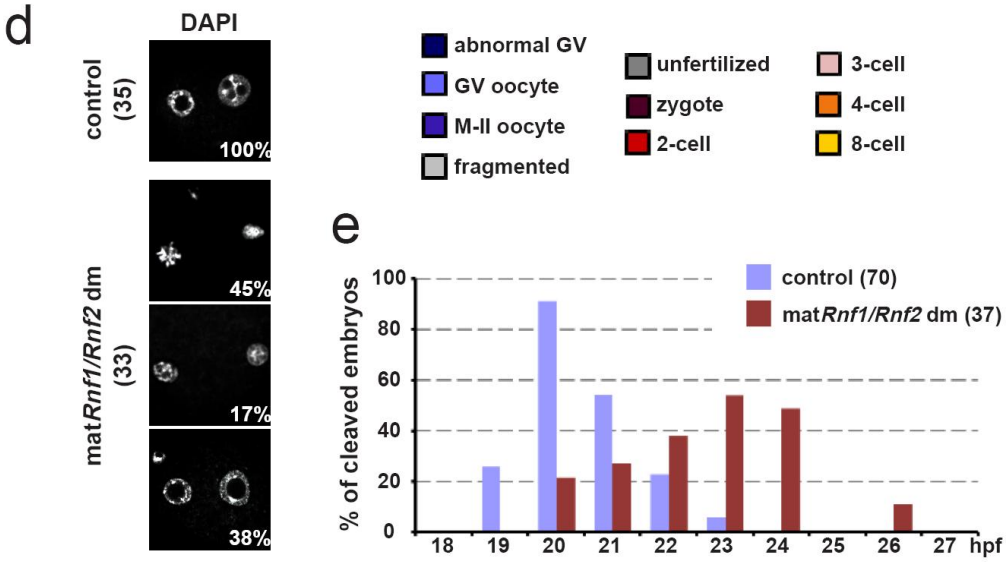

d
DAPI
control (35)
100%
matRnf1/Rnf2 dm (33)
45%
17%
38%
abnormal GV
GV oocyte
M-II oocyte
fragmented
unfertilized
zygote
2-cell
3-cell
4-cell
8-cell
e
% of cleaved embryos
100
80
60
40
20
0
18
19
20
21
22
23
24
25
26
27
hpf
control (70)
matRnf1/Rnf2 dm (37)

Figure 1 | Loss of maternal *Rnf1* and *Rnf2* delays early embryonic development and impairs progression beyond the two-cell stage.
a, Differential interference contrast image of mat*Rnf1* mutant (further used as control) and mat*Rnf1/Rnf2* dm embryo at embryonic day 3.5. **b,** Immunofluorescent (IF) staining for Rnf1 and Rnf2 of wild-type, *Rnf2* mutant, *Rnf1* mutant and *Rnf1/Rnf2* dm growing oocytes (secondary oocytes) surrounded by follicle cells in sections of ovaries from 12-14 day old mice. Due to constitutive deletion, granulosa cells lack Rnf1 in *Rnf1* mutant ovaries. **c,** Detailed developmental progression analysis of control (C) and mat*Rnf1/Rnf2* dm (DM) oocytes and embryos at indicated developmental stages. All embryos carry wild-type paternal alleles for *Rnf1* and *Rnf2*. **d,** DAPI staining of control and mat*Rnf1*/*Rnf2* dm zygotes 5 hours post *in vitro* fertilization showing pronuclear stages. 100% refers to number of fertilized embryos analyzed. **e,** Timing of first cleavage division of control and mat*Rnf1*/*Rnf2* dm embryos. X-axis represents hours post *in vitro* fertilization. 100% refers to number of embryos that progress to two-cell stage. Total numbers of oocytes/embryos analyzed at each stage are shown in brackets.

To investigate at which developmental stage *Rnf1* and *Rnf2* are required for supporting embryonic development, we analyzed mRNA and protein levels of PRC1 members in mutant and control oocytes and early embryos. While *Rnf2* and most other PRC1 members (*Cbx2*, *Bmi1*, *Phc1*) were continuously expressed during oogenesis and maternally provided to one- and two-cell embryos, *Rnf1* was only moderately expressed in primary and secondary oocytes, and barely detectable in fully grown oocytes (Supplementary Fig. 1). Furthermore, apart from *Rnf1* and *Rnf2*, we measured no major change in transcript levels of other PRC1 components in single or double deficient oocytes and embryos. In wild-type early embryos, we observed nuclear localization of PRC1 components Rnf2, Bmi1 and Cbx2 (and not of Rnf1) by immunofluorescence whereas we failed to detect these proteins in mat*Rnf1/Rnf2* dm as well as in mat*Rnf2* single mutant embryos, despite abundance of the *Bmi1* and *Cbx2* mRNAs (Supplementary Fig 2). During oogenesis, we detected several PRC1 members, including Rnf1 and Rybp, in nuclei of wild-type growing oocytes. Notably, Rnf1 protein levels were up-regulated in *Rnf2* single mutant growing oocytes, reminiscent to *Rnf2* deficient ES cells (Fig. 1b). Moreover, *Rnf1* expression during oocyte growth is able to compensate for Rnf2 in stabilizing the PRC1 complex as we detected Cbx2, Bmi1 and Rybp in nuclei of *Rnf2* single mutant oocytes (Supplementary Fig. 2). These data therefore suggest that the two-cell arrest displayed by mat*Rnf1/Rnf2* dm embryos is primarily caused by loss-of-function of both Ring finger proteins during oogenesis.

To characterize the nature of the two-cell arrest phenotype, we assessed the development of *Rnf1/Rnf2* dm oocytes and mat*Rnf1/Rnf2* dm embryos in more detail. Upon hormonal stimulation, *Rnf1*$^{-/-}$*; Rnf2*$^{F/F}$*; Zp3*-cre and control littermates produced a similar number of phenotypically normal germinal vesicle (GV) oocytes (Fig 1c). When released into meiotic maturation, *Rnf1*/*Rnf2* dm GV oocytes showed a delay in germinal vesicle break down, and in condensation and alignment of chromosomes on the metaphase-I plate, possibly due to impaired spindle formation (Supplementary Fig. 3). Likewise, although the majority of *Rnf1*/*Rnf2* dm oocytes completed the first meiotic division (Fig. 1c), spindle formation and chromosome congression were impaired at the metaphase-II stage as well. Prolonged *in vitro* culture promoted spindle formation and chromosome alignment (Supplementary Fig. 3). Nevertheless, despite the delay,

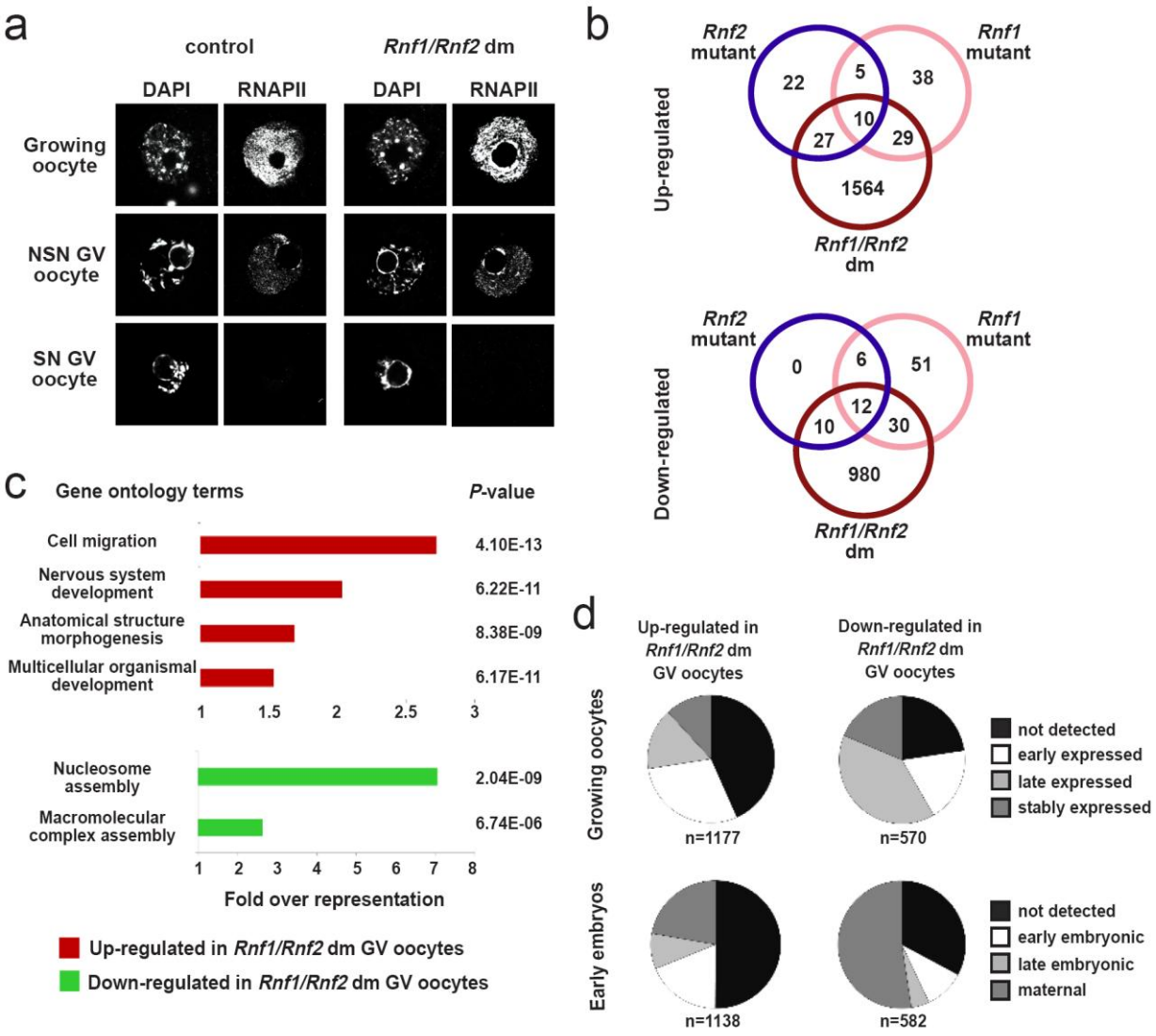


Figure 2 | Massive transcriptional misregulation in *Rnf1/Rnf2* dm oocytes.
a, IF staining for RNA polymerase II (RNAPII) in control and *Rnf1/Rnf2* dm growing oocytes and fully grown germinal vesicle (GV) stage oocytes (with a non-surrounded nucleolus (NSN) or surrounded nucleolus (SN)). **b,** Venn diagram showing overlap among genes up- or down-regulated (>1.5-fold; $P < 0.05$) in *Rnf2* mutant (blue), *Rnf1* mutant (pink) and *Rnf1/Rnf2* dm (red) GV oocytes compared to wild-type. **c,** Gene Ontology (GO) analysis of genes up- (red) or down-regulated (green) in *Rnf1/Rnf2* dm GV oocytes. Fold over-representation indicates the observed percentage of up-regulated genes in a particular GO category over the percentage expected on the basis of all GO-annotated genes on the array. *P*-values indicate significance of enrichment. **d,** Classification of genes up- and down-regulated in *Rnf1/Rnf2* dm GV versus wild-type oocytes according to their expression patterns over subsequent stages of oogenesis (upper panels) and pre-implantation development (lower panels) in wild-type condition as measured by expression profiling (outlined in Supplementary Fig. 1) (Pan *et al.*, 2005; Zeng *et al.*, 2004; Zeng and Schultz, 2005).

Rnf1/Rnf2 dm oocytes completed meiosis as we isolated equivalent numbers of one-cell embryos from *Rnf1*$^{-/-}$*; Rnf2*$^{F/F}$*; Zp3*-cre and control littermates (Fig. 1c). Under *in vitro* fertilization conditions, *Rnf1/Rnf2 dm* oocytes displayed a moderate increase in fertilization frequency (controls: 63% (n=101); dm: 87% (n=73)) and a 3-fold increase in polyspermia occurrence (controls: 11% (n=101); dm: 32% (n=73)) (Supplementary Fig. 2), suggesting impaired function of the zona pellucida (Gardner and Evans, 2006). Furthermore, compared to control embryos, the formation and subsequent maturation of maternal and paternal pronuclei was delayed in mat*Rnf1/Rnf2* dm zygotes (Fig. 1d; Supplementary Fig. 3). Correspondingly, the first cleavage division occurred with a 2.5 hour delay *in vitro* (Fig. 1e) and reduced number of mutant embryos entered the two-cell stage *in vitro* and *in vivo* (Fig. 1c). Finally, development of mat*Rnf1/Rnf2* dm embryos ceased before entrance into the second cleavage division, as we failed to detect signs of chromatin condensation, spindle formation, and genome-wide acquisition of phosphorylation at histone H3 serine 10, a marker of late G2/M phase chromatin (Supplementary Fig. 3).

To dissect the mechanism underlying the maternal effect caused by *Rnf1/Rnf2* deficiency during oogenesis, we first determined by IF staining for RNA polymerase II (RNAPII) that genome-wide transcriptional shut down, normally occurring in fully grown GV oocytes, was correctly achieved in double deficient oocytes (Fig 2a). Second, to examine gene-specific expression defects during oocyte growth we performed mRNA profiling of GV oocytes that naturally store the majority of transcripts produced during the growing phase for subsequent meiotic maturation and early embryogenesis. Compared to wild-type oocytes, we observed that 2662 genes were misexpressed over 1.5 fold in *Rnf1/Rnf2* dm oocytes with over 60% being up-regulated. In contrast, only 181 and 92 genes were misregulated in *Rnf1* and *Rnf2* single mutants, respectively ($P < 0.05$) (Fig. 2b, Supplementary Fig. 4, Supplementary Table 1). For nine genes tested, we validated by quantitative PCR (qPCR) analyses that up-regulated gene expression was restricted to oocytes double deficient for *Rnf1* and *Rnf2* (Fig. 3a, 3c; Supplementary Figs. 4, 5). For genes up-regulated in *Rnf1*/*Rnf2* dm oocytes, gene ontology (GO) analyses revealed among others a significant over-representation of developmental gene functions, consistent with a role of PRC1 in repressing developmental master regulators in ES cells and during development (Fig. 2c; Supplementary Table 2) (Azuara *et al.*, 2006; Bernstein *et al.*, 2006; Boyer *et al.*, 2006; Lee *et al.*, 2006). In addition to several *Hox* genes, known as classical Polycomb targets, we found that many genes marking lineage specification during pre-implantation development, like *Sox2*, *Fn1*, *Gata4*, *Klf4*, *Ahcy*, *Gata6*, *Msx2*, *Eomes*, *Fgf2r*, *Krt8* and *Tcfap2a* were up-regulated (Guo *et al.*). Among loci down-regulated in *Rnf1/Rnf2* dm GV oocytes we found the majority of genes encoding the four core histones involved in nucleosomal assembly (Fig. 2c), providing a possible explanation for the delay in pronuclear formation. Consistent with reduced *Atrx* transcript levels, we observed reduced Atrx protein levels in *Rnf1/Rnf2* dm GV and meiotically maturing oocytes (data not shown). The meiotic maturation defects observed in *Rnf1/Rnf2* dm oocytes are reminiscent of those reported for *Atrx* deficiency (De La Fuente *et al.*, 2004b).

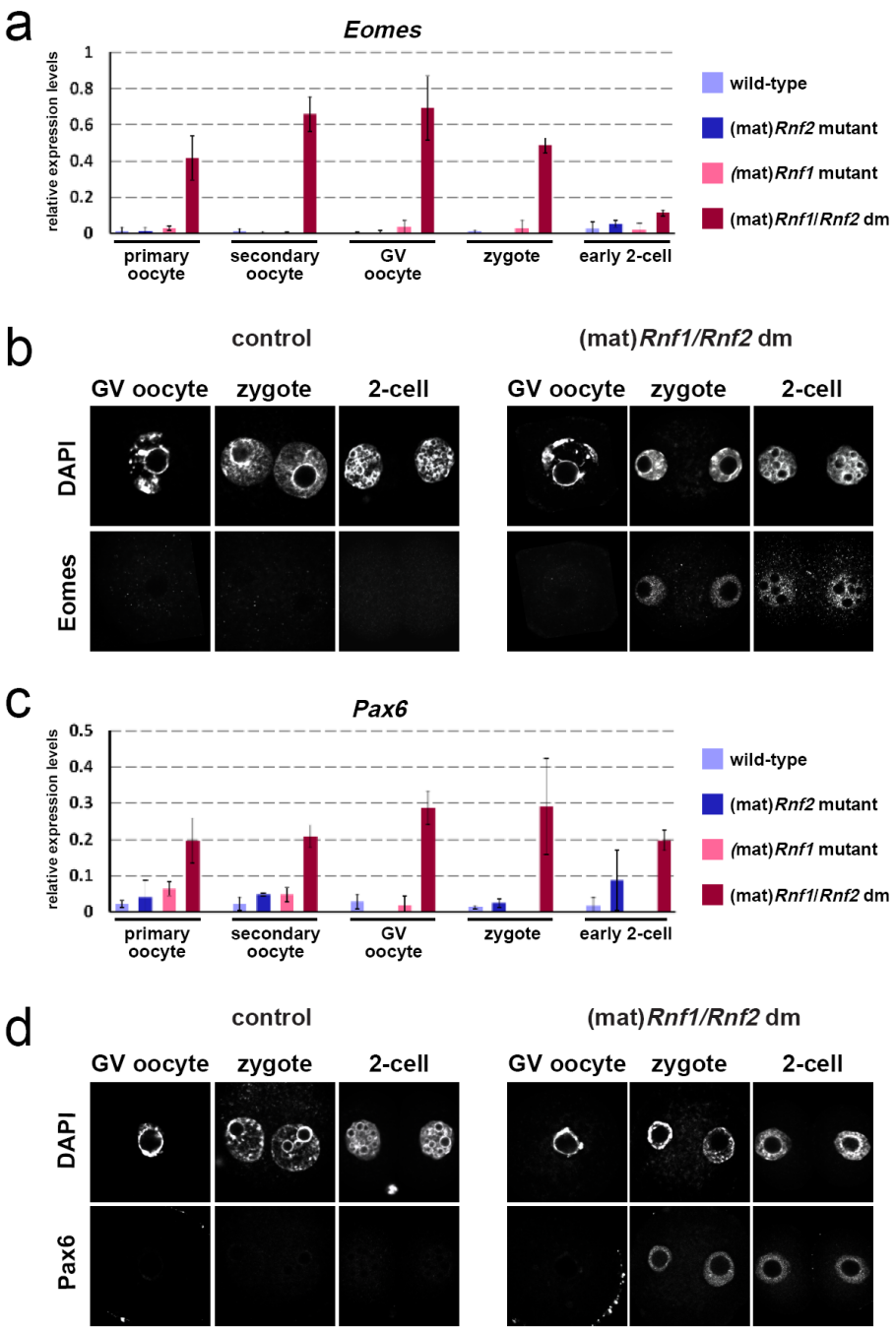


Figure 3 | Transcripts of developmental regulators de-repressed in *Rnf1/Rnf2* dm oocytes are only translated after fertilization.

a, c, Real-time PCR analysis of *Eomes* (**a**) and *Pax6* (**c**) transcripts in wild-type (light blue), (mat)*Rnf2* mutant (dark blue), (mat)*Rnf1* mutant (pink) and (mat)*Rnf1/Rnf2* dm (red) growing oocytes (primary and secondary), GV oocytes, zygotes and early 2-cell embryos. Transcript levels were normalized to *LnmB1* control. Error bars indicate standard deviation based on 2-3 biological replicates. **b, d,** IF staining for Eomes (**b**) and Pax6 (**d**) in control and (mat)*Rnf1/Rnf2* dm GV oocytes, zygotes and early 2-cell embryos.

To identify when *Rnf1* and *Rnf2* serve transcriptionally regulatory functions during oogenesis, we compared the expression status of genes misregulated in *Rnf1/Rnf2* dm GV oocytes to the temporal expression pattern of genes during subsequent stages of normal oogenesis and embryogenesis, as measured by genome-wide transcriptome analyses (Fig. 2d) (Pan *et al.*, 2005; Zeng *et al.*, 2004; Zeng and Schultz, 2005). In contrast to genes down-regulated, the majority of genes up-regulated in *Rnf1/Rnf2* dm GV oocytes are either never or only early expressed during normal oogenesis (Fig. 2d). Consistently, qPCR analyses confirmed that *Eomes*, *Pax6* and *Gata4* were already up-regulated in *Rnf1/Rnf2* dm primary oocytes, concurrent with *Zp3*-cre-mediated deletion of *Rnf2* (Fig. 3a, 3c; Supplementary Figs. 1, 5). Furthermore, substantially more genes up- than down-regulated in *Rnf1/Rnf2* dm GV oocytes are never expressed or become up-regulated during normal pre-implantation development. Interestingly, 78% of up-regulated genes that are never expressed during normal oogenesis (n = 510), are also never expressed during pre-implantation development. Comparison to transcriptomes of ES cells deficient for *Eed* and/or *Rnf2* revealed that up to 24% and 7% of genes up- or down-regulated in *Rnf1/Rnf2* dm GV oocytes are similarly misexpressed in deficient ES cells (data not shown) (Leeb *et al.*, 2010). Finally, we related the gene expression status in *Rnf1/Rnf2* dm oocytes to the occupancy of various Polycomb proteins and H3K27me3 at promoters in mouse ES cells (Ku *et al.*, 2008). We observed for genes up- versus down-regulated that 15% versus 5% were Rnf2 targets while 38% versus 14% were bound by at least one Polycomb protein (Rnf2, Ezh2, Suz12) or H3K27me3 in ES cells (data not shown). Together, these data suggest that a substantial fraction of genes up-regulated in *Rnf1/Rnf2* dm GV oocytes are direct targets of PRC1 throughout oogenesis as well as during early embryogenesis and in ES cells.

To investigate the fate of developmental gene transcripts up-regulated in *Rnf1/Rnf2* dm GV oocytes, we analysed mRNA and protein expression of *Eomes*, *Pax6* and *Gata4* in zygotes and two-cell embryos (Fig 3; Supplementary Fig. 5). We failed to detect protein of all three genes in control oocytes and embryos, as well as in *Rnf1/Rnf2* dm oocytes, despite elevated transcript levels in the latter sample. In mat*Rnf1/Rnf2* dm zygotes and two-cell embryos, however, we detected nuclear localization of Eomes, Pax6 and Gata4 protein concurrent with a progressive decrease in transcript levels, indicating translation of these aberrant maternal messages only upon fertilization (Fig 3; Supplementary Figs. 4, 5). These data suggest that the impairment of *Rnf1/Rnf2*-mediated transcriptional repression during oogenesis is, at least in part, functionally suppressed in GV oocytes via translational repression of aberrant maternal transcripts, a widely-conserved gene regulatory mechanism functioning during gametogenesis and the maternal-to-embryonic transition in a variety of species (Bettegowda and Smith, 2007; Rajyaguru and Parker, 2009; Vardy and Orr-Weaver, 2007).

It is unclear to what extent the developmental arrest observed for mat*Rnf1/Rnf2* dm two-cell embryos is due to inheritance of an aberrant set of maternally provided transcripts (representing gain- and loss-of-functions), to inheritance of aberrantly programmed chromatin states on the maternal genome and/or to impaired genome activation potentially resulting from PRC1 deficiency during the two-cell stage. To

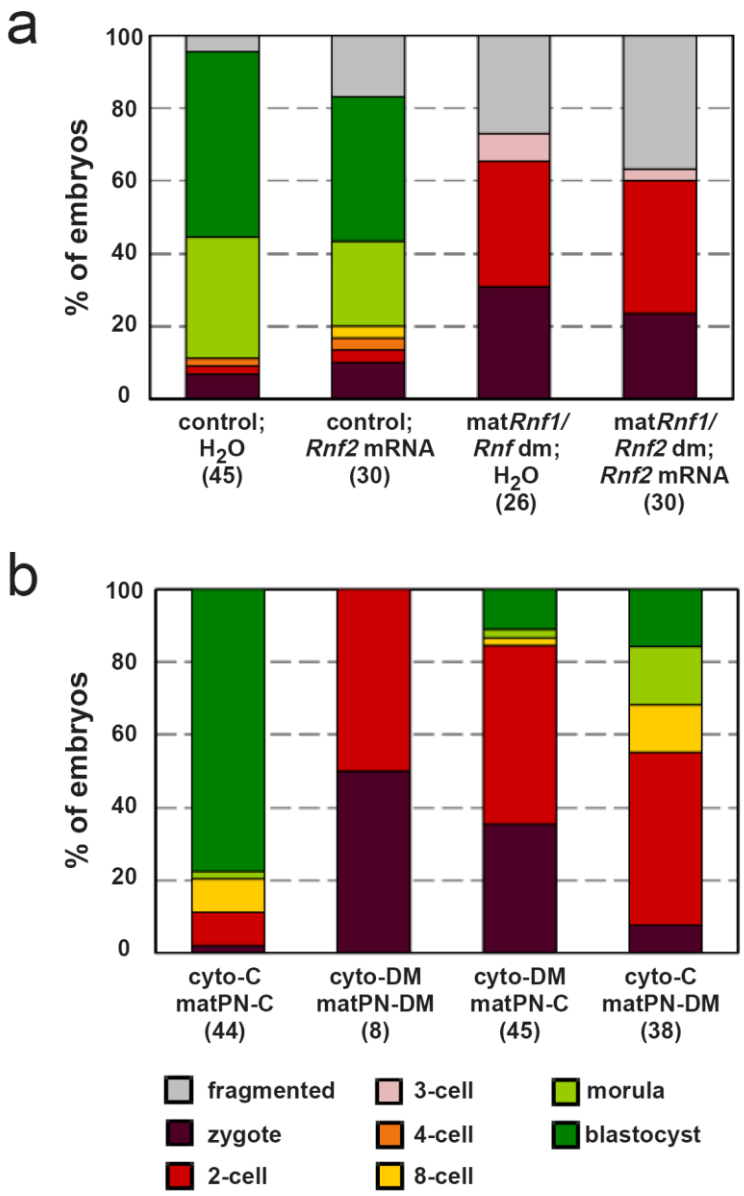


Figure 4 | *Rnf1*/*Rnf2* expression during oogenesis defines maternal cytoplasmic and nuclear contributions required for embryonic development.
a, Diagram showing developmental potential scored at embryonic day 3.5 of control and mat*Rnf1/Rnf2* dm early zygotes microinjected with buffer only or *Rnf2* mRNA. **b,** Diagram showing developmental potential scored at embryonic day 4.5 of reconstructed zygotes with exchanged maternal PN (matPN). Control groups include control (mat*Rnf1* mutant) matPN transferred into control (matPN enucleated) zygote (cyto-C/matPN-C) and mat*Rnf1/Rnf2* dm matPN transferred into mat*Rnf1/Rnf2* dm zygote (cyto-DM/matPN-DM). Experimental groups include control (mat*Rnf1* mutant) matPN transferred into mat*Rnf1/Rnf2* dm zygote (cyto-DM/matPN-C) and mat*Rnf1/Rnf2* dm matPN transferred into control zygote (cyto-C/matPN-DM). Total number of injected or matPN transferred embryos shown in brackets.

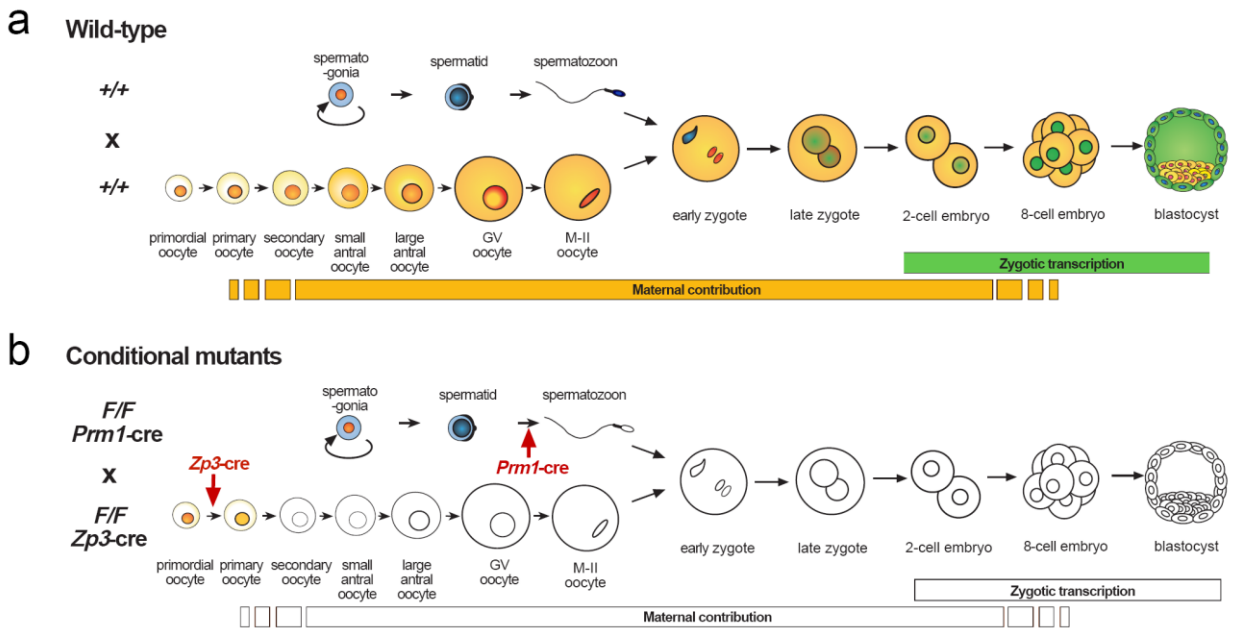
address whether exogenous *Rnf2* expression could alleviate the two-cell arrest of mat*Rnf1/Rnf2* dm embryos we microinjected *Rnf2* mRNA into such early zygotes. We noticed nuclear localization of myc-tagged Rnf2 in control and mat*Rnf1/Rnf2* dm zygotes and two-cell embryos as well as a re-appearance of Cbx2 in the (pro)-nuclei of mat*Rnf1/Rnf2* dm embryos, arguing for reconstitution of a chromatin-bound PRC1 complex (Supplementary Fig. 6). Nonetheless, development of mat*Rnf1/Rnf2* dm embryos beyond the two-cell stage was not restored, indicating that *Rnf1*/*Rnf2* function during oocyte growth is required to ensure proper programming of the oocyte for early embryonic development (Fig. 4a).

To assay the relative contribution of transmission of aberrant maternal transcripts and proteins versus a potential abnormally-programmed chromatin state of the maternal genome to the two-cell arrest, we exchanged maternal pro-nuclei (matPN) between control and mat*Rnf1/Rnf2* dm early zygotes, thereby generating diploid hybrid embryos. To first evaluate the efficacy of the experimental procedure, we assayed the developmental potential of reconstructed embryos in which matPNs had been exchanged between control zygotes or between mat*Rnf1/Rnf2* dm zygotes. Embryos reconstructed with cytoplasm and a matPN from different control zygotes (cyto-C/matPN-C) developed into morulae and blastocysts with 80% efficiency, as observed for naturally produced embryos. All embryos reconstructed with cytoplasm and a matPN from different mat*Rnf1/Rnf2* dm zygotes (cyto-DM/matPN-DM) showed the expected one- to two-cell arrest (Fig. 4b; Supplementary Fig. 6). As these reconstructed embryos recapitulated the developmental phenotypes observed in naturally generated embryos, we subsequently exchanged matPNs between control and mat*Rnf1*/*Rnf2* dm genotypes. Notably, 87% of reconstructed hybrid cyto-DM/matPN-C embryos composed of double mutant cytoplasm and a control matPN failed to develop into morulae or blastocysts. In contrast, development of 69% of hybrid cyto-C/matPN-DM embryos reconstructed with control cytoplasm and a double mutant matPN was impaired (Fig. 4b). In both conditions, we ruled out that the observed changes in developmental potential were due to cytoplasm that was transferred along with a control or mutant matPN (Supplementary Fig. 6). The severely reduced developmental potential of cyto-DM/matPN-C hybrid embryos indicates that *Rnf1*/*Rnf2*-mediated gene regulation during oogenesis is essential to provide the oocyte the proper maternal cytoplasmic factors to support pre-implantation development. On the other hand, as illustrated by the cyto-C/matPN-DM hybrid embryos, *Rnf1* and *Rnf2* expression during oogenesis is required to program the chromatin state of the maternal genome to sustain pre-implantation development. Nonetheless, while transmission of the proper maternal cytoplasmic factors is essential for development, wild-type maternal factors can, to some extent, reprogram a refractory chromatin state inherited from *Rnf1*/*Rnf2* dm oocytes.

In summary, here we reveal an essential function for *Rnf1* and *Rnf2*, two PRC1 core components, during oogenesis to support early embryogenesis. Notably, our results challenge the classical paradigm of Polycomb functioning in the maintenance of the transcriptionally repressed state of genes, initially established by gene specific transcriptional repressors during early development. In contrast to the loss-of-zygotic-function studies in flies and mice, underlying the paradigm, our work of maternal deficiency

demonstrates that many Polycomb targets, including homeotic genes, are stably repressed by Polycomb in the female mouse germline. Our work extends comparable observations in germlines of plants and *Caenorhabditis elegans* (Capowski *et al.*, 1991; Leroy *et al.*, 2007; Luo *et al.*, 2009; Rodrigues *et al.*, 2008). Together with marking of similar target genes by PRC2-mediated H3K27me3 in mature human and mouse spermatozoa (Brykczynska, in press*.;* Hammoud *et al.*, 2009), we propose that in mammals Polycomb functions in both germ lines and in early embryos to repress expression of differentiation promoting factors, thereby maintaining cell identity and preserving developmental potency across generations.

3.2.4. Supplementary information

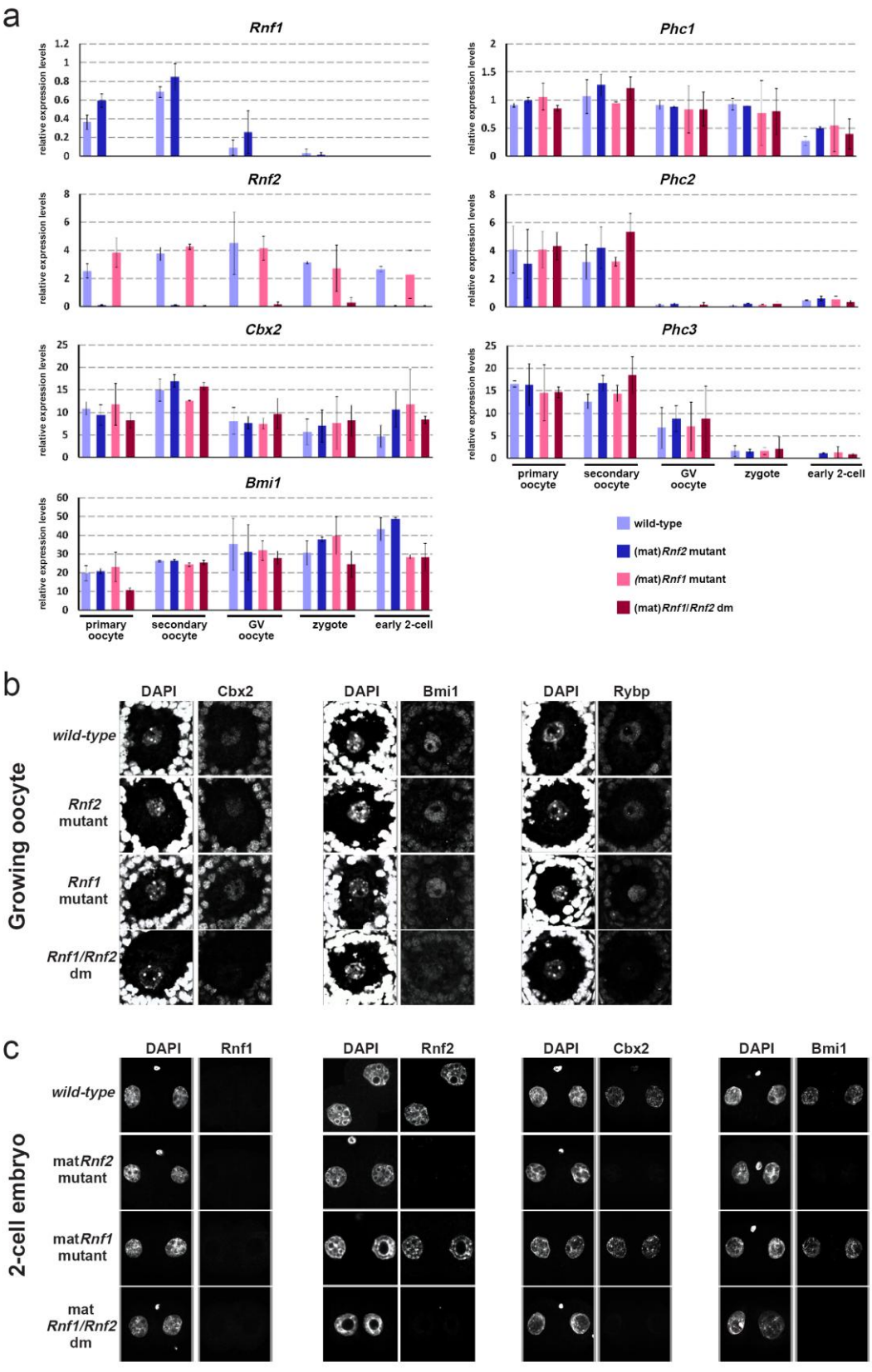


Supplementary Fig. 1. │Conditional gene disruption during gametogenesis.

a. Schematic diagram illustrating different stages of gametogenesis and early embryonic development.

b. Conditional genetic strategy to generate embryos maternally and zygotically deficient for *Rnf2* function. *Zp3*-cre drives Cre recombinase expression during oogenesis from the primary oocyte stage onwards, resulting in loss of *Rnf2* expression during the transcriptionally active growth phase of oogenesis. *Prm1*-cre drives Cre recombinase expression during the elongation phase of spermiogenesis during which transcription is largely shut-down in a genome-wide manner.

Comparative expression analyses described in Fig. 2d was based on genome-wide transcriptomic analyses in wild-type oocytes at five subsequent stages of development (from primordial oocyte to large antral follicle stage) and in wild-type GV oocytes and early embryos (late zygotes, 2-cell, 8-cell and blastocyst embryos).

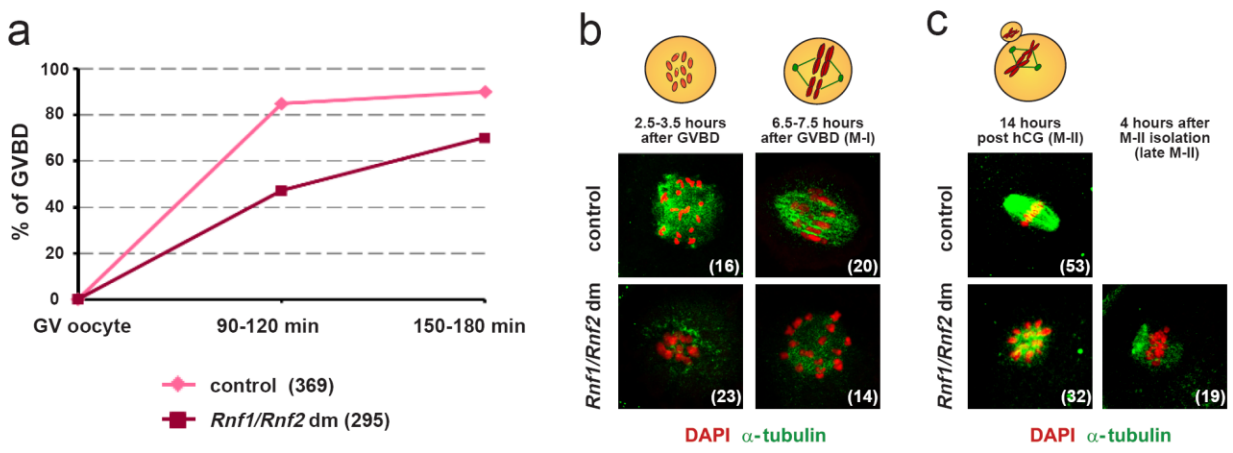

a
Rnf1
Phc1
Rnf2
Phc2
Cbx2
Phc3
Bmi1
relative expression levels
primary oocyte
secondary oocyte
GV oocyte
zygote
early 2-cell
wild-type
(mat)Rnf2 mutant
(mat)Rnf1 mutant
(mat)Rnf1/Rnf2 dm
b
Growing oocyte
DAPI
Cbx2
Bmi1
Rybp
wild-type
Rnf2 mutant
Rnf1 mutant
Rnf1/Rnf2 dm
c
2-cell embryo
Rnf1
Rnf2
Cbx2
Bmi1
wild-type
matRnf2 mutant
matRnf1 mutant
mat Rnf1/Rnf2 dm

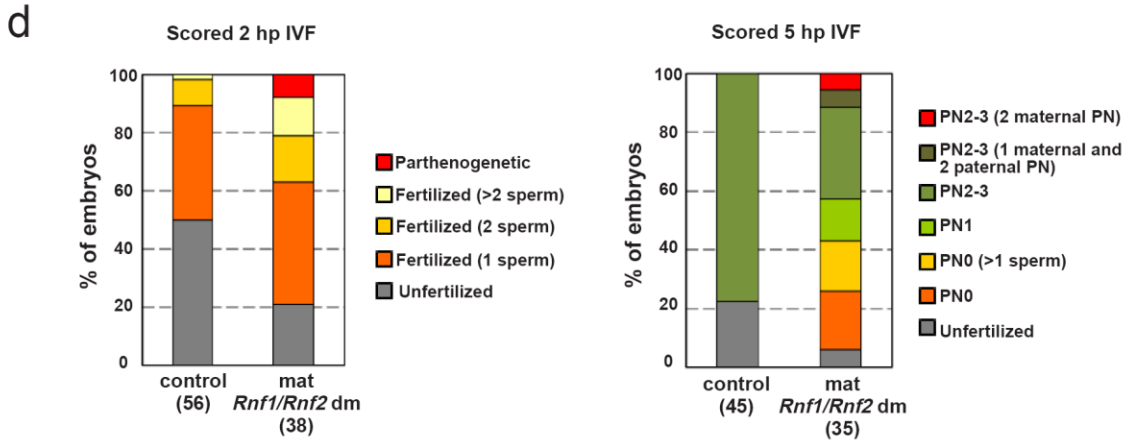
Supplementary Fig. 2. | Transcript and protein levels of PRC1 components in wild-type, mat*Rnf1*, mat*Rnf2* and mat*Rnf1/Rnf2* dm mutant growing oocytes and early embryos.

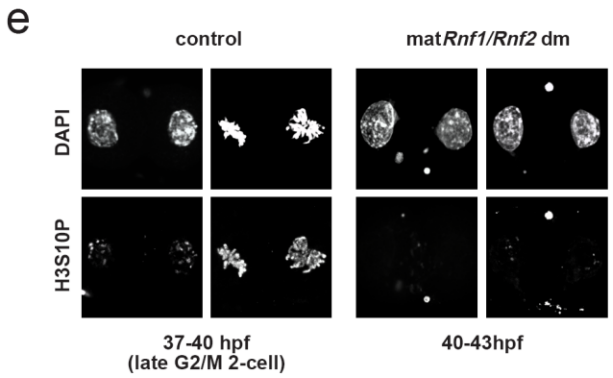
a. Real-time PCR analysis of *Rnf1*, *Rnf2* and other PRC1 members *Cbx2*, *Bmi1*, *Phc1*, *Phc2* and *Phc3* gene transcripts in wild-type (light blue), (mat)*Rnf2* mutant (dark blue), (mat)*Rnf1* mutant (pink) and (mat)*Rnf1/Rnf2* dm (red) growing oocytes (primary and secondary), GV oocytes, zygotes and early 2-cell embryos. Transcript levels were normalized to *LnmB1* control. Error bars indicate standard deviation based on 2-3 biological replicates.

b. Immunofluorescent (IF) staining for Cbx2, Bmi1 and Rybp of wild-type, *Rnf2* mutant, *Rnf1* mutant and *Rnf1/Rnf2* dm growing oocytes (secondary oocytes) surrounded by follicle cells in sections of ovaries from 12-14 day old mice. Due to constitutive deletion, granulosa cells lack Rnf1 in *Rnf1* mutant ovaries.

c. IF staining for Rnf1, Rnf2, Cbx2 and Bmi1 in wild type, mat*Rnf2* mutant, mat*Rnf1* mutant and mat*Rnf1/Rnf2* dm 2-cell embryos.


a
% of GVBD
100
80
60
40
20
0
GV oocyte
90-120 min
150-180 min
control (369)
Rnf1/Rnf2 dm (295)
b
2.5-3.5 hours after GVBD
6.5-7.5 hours after GVBD (M-I)
control
Rnf1/Rnf2 dm
(16)
(20)
(23)
(14)
DAPI α-tubulin
c
14 hours post hCG (M-II)
4 hours after M-II isolation (late M-II)
control
Rnf1/Rnf2 dm
(53)
(32)
(19)
DAPI α-tubulin


d
Scored 2 hp IVF
% of embryos
100
80
60
40
20
0
control (56)
mat Rnf1/Rnf2 dm (38)
Parthenogenetic
Fertilized (>2 sperm)
Fertilized (2 sperm)
Fertilized (1 sperm)
Unfertilized
Scored 5 hp IVF
% of embryos
control (45)
mat Rnf1/Rnf2 dm (35)
PN2-3 (2 maternal PN)
PN2-3 (1 maternal and 2 paternal PN)
PN2-3
PN1
PN0 (>1 sperm)
PN0
Unfertilized


e
control
mat Rnf1/Rnf2 dm
DAPI
H3S10P
37-40 hpf (late G2/M 2-cell)
40-43hpf

Supplementary Fig. 3. │Delay in meiotic maturation and early embryonic development of mat*Rnf1/Rnf2* dm oocytes and embryos.

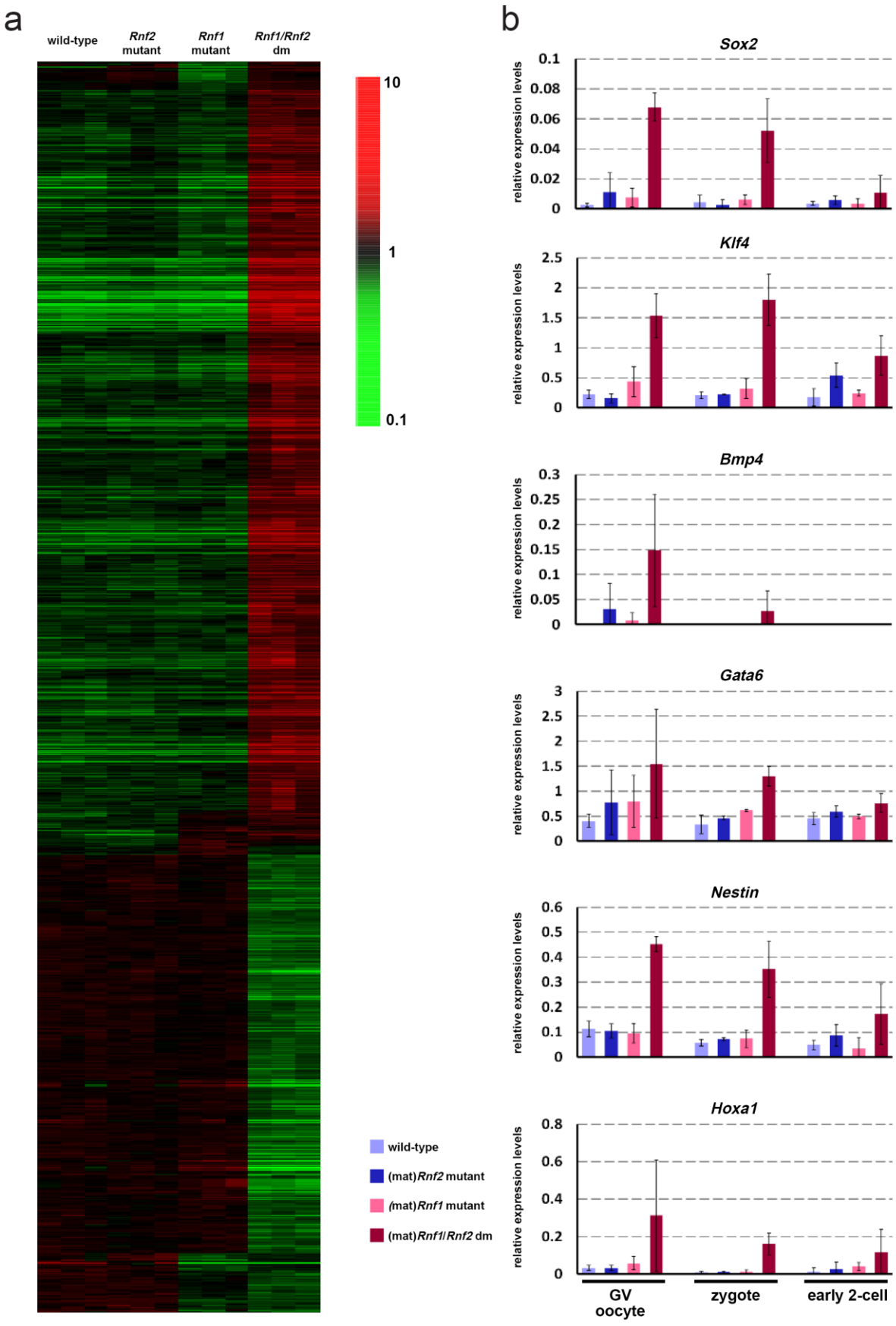
a. Chart showing percent of control and *Rnf1/Rnf2* dm GV oocytes that have undergone germinal vesicle break down (GVBD) at subsequent time points of *in vitro* culture after release from milrinone-inhibition.

b. c. Representative images of control and *Rnf1/Rnf2* dm oocytes (**b**) undergoing meiotic maturation at indicated time points after GVBD (*in vitro* culture) and of M-II oocytes (**c**) isolated at 14 hours post hCG injection and after subsequent 4 hours of *in vitro* culture (late M-II oocytes). Microtubules were visualized by IF staining with anti-α-tubulin antibody (green) and DNA was counterstained with DAPI (red).

d. Diagram showing detailed developmental progression analysis (pronuclear stage analysis) of control (mat*Rnf1* mutant) and mat*Rnf1/Rnf2* dm embryos scored at 2 hours (left panel) and 5 hours (right panel) post *in vitro* fertilization. 100% refers to number of embryos analyzed. Total number of embryos analyzed at each stage are indicated in brackets.

e. IF staining of control and mat*Rnf1/Rnf2* dm late 2-cell embryos (37-43 hours post *in vitro* fertilization) for phosphorylated H3S10, a late G2-/M-phase marker.

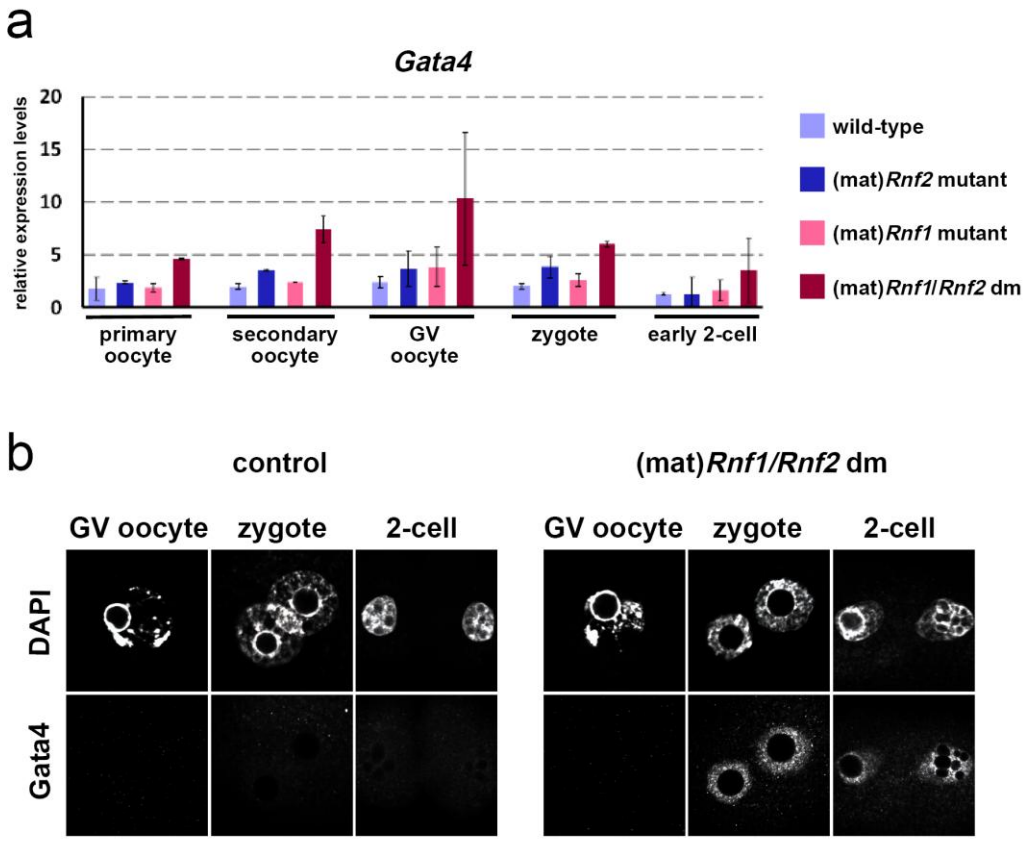
The total number of GV oocytes or embryos analyzed at each stage is indicated in brackets.


a
wild-type
Rnf2 mutant
Rnf1 mutant
Rnf1/Rnf2 dm
10
1
0.1
b
Sox2
Klf4
Bmp4
Gata6
Nestin
Hoxa1
relative expression levels
wild-type
(mat)Rnf2 mutant
(mat)Rnf1 mutant
(mat)Rnf1/Rnf2 dm
GV oocyte
zygote
early 2-cell

Supplementary Fig. 4. | Majority of genes misregulated in *Rnf1/Rnf2* dm GV oocytes are normally expressed in *Rnf1*- or *Rnf2* single mutant oocytes.

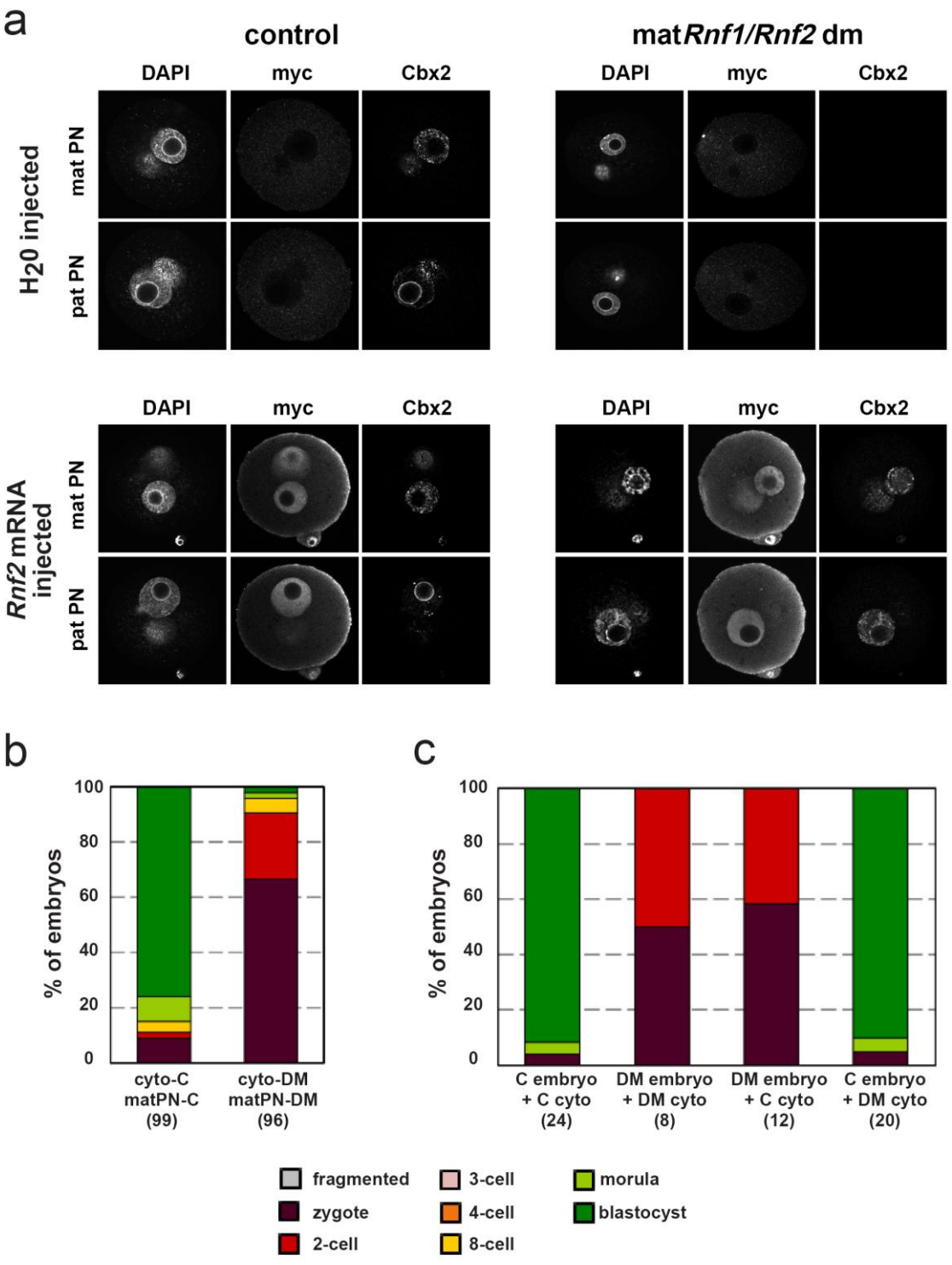
a. Heat map of relative expression levels of probe sets showing >1.5-fold misregulation in any mutant genotype compared to wild-type. For each genotype (wild-type, *Rnf2* mutant, *Rnf1* mutant and *Rnf1/Rnf2* dm) expression analysis was performed in triplicate on 50 GV oocytes per sample. Each column corresponds to a single expression microarray. For each probe set, expression is shown relative to the average expression level of that probe set across all samples. Green and red represent lower-than-average and higher-than-average signal levels, respectively.

b. Real-time PCR analysis of *Sox2*, *Klf4*, *Bmp4*, *Gata6*, *Nestin* and *Hoxa1* gene transcripts in wild-type (light blue), (mat)*Rnf2* (dark blue), (mat)*Rnf1* (pink) and (mat)*Rnf1/Rnf2* dm (red) growing oocytes (primary and secondary), GV oocytes, zygotes and early 2-cell embryos. Transcript levels were normalized to *LnmB1* control. Error bars indicate standard deviation based on 2-3 biological replicates.



Supplementary Fig. 5. | Transcripts of *Gata4*, de-repressed in *Rnf1/Rnf2* dm oocytes, are only translated after fertilization.

a. Real-time PCR analysis of *Gata4* transcripts in wild-type (light blue), (mat)*Rnf2* mutant (dark blue), (mat)*Rnf1* mutant (pink) and (mat)*Rnf1/Rnf2* dm (red) growing oocytes (primary and secondary), GV oocytes, zygotes and early 2-cell embryos. Transcript levels were normalized to *LnmB1* control. Error bars indicate standard deviation based on 2-3 biological replicates. **b.** IF staining for Gata4 in control and mat*Rnf1/Rnf2* dm GV oocytes, zygotes and early 2-cell embryos.


a
control
mat*Rnf1/Rnf2* dm
DAPI
myc
Cbx2
H20 injected
mat PN
pat PN
Rnf2 mRNA injected
b
% of embryos
cyto-C matPN-C (99)
cyto-DM matPN-DM (96)
c
C embryo + C cyto (24)
DM embryo + DM cyto (8)
DM embryo + C cyto (12)
C embryo + DM cyto (20)
fragmented
zygote
2-cell
3-cell
4-cell
8-cell
morula
blastocyst

Supplementary Fig. 6. |

a. Microinjection of *Rnf2* mRNA into mat*Rnf1/Rnf2* dm zygotes leads to *de-novo* PRC1 complex formation. IF analyses of control (left panels) and mat*Rnf1/Rnf2* dm (right panels) zygotes microinjected with water–only (upper panels) or with *Rnf2* mRNA (lower panels). Embryos are stained with anti-Myc antibody to detect injected Myc-tagged Rnf2 and with anti-Cbx2 antibody.

b. Developmental potential of non-reconstructed control (C) and mat*Rnf1/Rnf2* dm (DM) embryos assessed at E4.5, under culture conditions of matPN transfer experiment. Number of embryos analyzed is indicated in brackets.

c. Microinjection of cytoplasm obtained from control or mat*Rnf1/Rnf2* dm zygotes into control or mat*Rnf1/Rnf2* dm zygotes does not influence developmental potential. Diagram shows developmental potential assessed at E3.5 of control (C) and mat*Rnf1/Rnf2* dm (DM) embryos (not matPN transferred) microinjected with 5% cytoplasm obtained from control (C) or mat*Rnf1/Rnf2* dm (DM) embryos shortly after fertilization.

Supplementary Table 2. | Gene Ontology (GO) categories of genes up- or down-regulated in *Rnf1/Rnf2* dm GV oocytes.

Over- (green) and under-represented (red) GO categories of misregulated probe sets in *Rnf1/Rnf2* dm GV oocytes. GO categories that do not differ by 2 or more genes are shown together in one box. All GO terms shown with a P-value <0.05.

Supplementary Table 1. | Normalized expression profiling data of wild-type, *Rnf1* mutant, *Rnf2* mutant and *Rnf1/Rnf2* dm GV oocytes.

4. Unpublished results and ongoing work

4.1. Cell cycle defects in maternal *Rnf1/Rnf2* double mutant embryos

4.1.1. Introduction

Polycomb group proteins (PcG) were originally discovered as factors responsible for maintaining the silenced state of *Hox* genes (Deschamps *et al.*, 1999). Over the past years PcG proteins have been found to target many other key developmental regulators and repress cell-type-inappropriate expression of these genes (Bernstein *et al.*, 2006; Boyer *et al.*, 2006; Lee *et al.*, 2006). Although PcG proteins are usually regarded as stable repressors, they are also involved in the regulation of cell proliferation, and their mutation has been linked to cycle defects in cells or embryos (Valk-Lingbeek *et al.*, 2004). Some studies describe Polycomb mutations effecting mitosis (O'Dor *et al.*, 2006; Phillips and Shearn, 1990), while in others the exact phase of the cell cycle arrest is not even determined (Endoh *et al.*, 2008; Wang *et al.*, 2004a). The mechanisms by which Polycomb proteins may regulate the cell cycle remain largely elusive.

An exception to this is the well studied Polycomb target, the *Ink4a/Arf* locus (Bracken *et al.*, 2007; Kotake *et al.*, 2007). The *Ink4a/Arf* locus encodes two cell cycle repressors, Cdkn2a (p16) and Ink4d (p19) that are important players in the retinoblastoma (Rb) and p53-mediated pathways, respectively. Several PcG proteins were shown to target this locus (Bracken *et al.*, 2007; Dietrich *et al.*, 2007; Gil *et al.*, 2004; Jacobs *et al.*, 1999a; Voncken *et al.*, 2003) and some, such as Bmi1, Cbx7 and Cbx8 have also been functionally linked to the repression of these cell cycle inhibitors (Dietrich *et al.*, 2007; Gil *et al.*, 2004; Molofsky *et al.*, 2005). Another cyclin-dependent kinase inhibitor Cdkn1c (p57, Kip2) has also been shown to be a direct target of PRC2 (Yang *et al.*, 2009) and the paternally imprinted allele to be expressed upon the loss of *Eed* (Mager *et al.*, 2003), *Ezh2* or *Rnf2* (Terranova *et al.*, 2008). Inactivation of these Cdk inhibitors has been implicated in cancer (Jacobs *et al.*, 1999a; Jacobs *et al.*, 1999b; Yang *et al.*, 2009). Finally, Cyclin A has been described to be directly bound by Polycomb in *Drosophila* (Martinez *et al.*, 2006). Apart from direct regulation of cell cycle gene, accumulating evidence suggests that PcG proteins may exert their regulatory effect indirectly. For a more detailed review on Polycomb-cell cycle connection, see 2.6.4.6.

The early pre-implantation embryo has several unique cell cycle properties. Initially, cell cycle progression is controlled by maternal factors in the early embryo, also underlined by the observation that cell cycle regulators are over-represented among maternal messages (Hamatani *et al.*, 2004; Potireddy *et al.*, 2006). The first two cell cycles of the embryo are exceptionally long, each comprising around 20 hours.

The second cell cycle is characterized by a very short G1-phase and a prolonged G2-phase, presumably due to zygotic genome activation taking place during this period (Flach *et al.*, 1982). Finally, some studies demonstrate a non-somatic-like cell cycle regulation in the early embryo. For example, somatic cell-like patterns of proliferation controlling factors Rb, Dp-1 and max are acquired only during the third cell cycle (Xie *et al.*, 2005). Cell cycle checkpoints are also altered in early embryos: while the G1/S checkpoint seems to be non-functional altogether, an intra-S and a G2/M checkpoint can be activated in response to DNA damage. However, although these checkpoints are able to prolong S- or delay M-phases, respectively, they are not fully functional, as embryos with abnormal DNA content or damaged DNA can still escape and divide. Fully functional checkpoints are reported only to act from the morula stage onwards (Adiga *et al.*, 2007b; Shimura *et al.*, 2002a; Yukawa *et al.*, 2007).

As described in 3.2., we observed a 2-cell arrest of mat*Rnf1/Rnf2* dm embryos. A closer glance at these arrested embryos revealed that they never enter M-phase, as we failed to observe any mitotic figures. Moreover, mat*Rnf1/Rnf2* dm embryos failed to acquire chromatin-wide H3S10 phosphorylation (H3S10P), an early marker of mitotic chromatin. H3S10P is first observed in wild type embryos at late G2-phase chromatin, where it weakly labels heterochromatic foci. Only upon M-phase entry does H3S10P label the entire chromatin. Interestingly, we found that few mat*Rnf1/Rnf2* dm embryos show very weak heterochromatic H3S10P labeling at the time control embryos are already at the 4-cell stage. This indicated that the most advanced mat*Rnf1/Rnf2* dm embryo developed was to the G2-phase of the second cell cycle.

We set out to dissect cell cycle events in mat*Rnf1/Rnf2* dm embryos to gain better understanding of the cause of the 2-cell arrest phenotype in these embryos. Although we still lack a direct molecular link to Polycomb deficiency, we characterized cell cycle progression in mat*Rnf1/Rnf2* dm embryos and found that the second S-phase was severely affected. We show that S-phase in mat*Rnf1/Rnf2* dm embryos was prolonged and the rate of DNA synthesis was reduced compared to controls. Moreover our results suggest that most embryos never exit from S-phase which is also accompanied by activation of an intra-S-phase checkpoint.

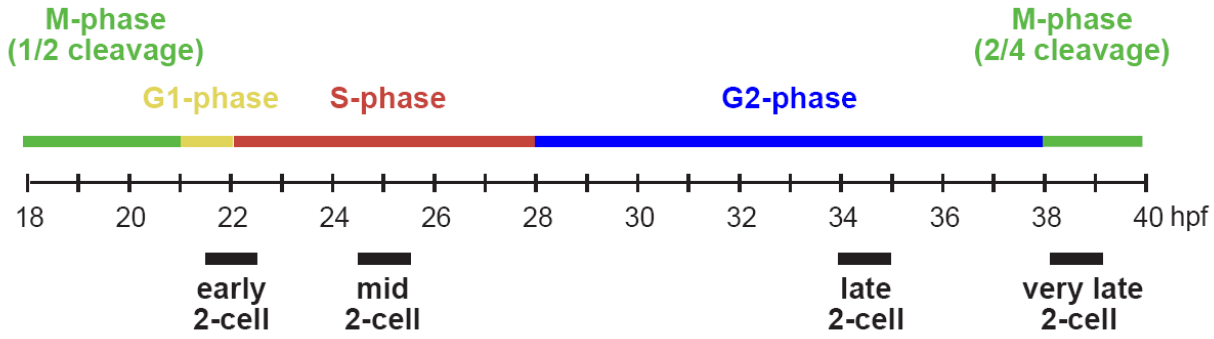
4.1.2. Materials and Methods

Mice

The generation of *Rnf1/Rnf2* double mutant (dm) oocytes and maternally (mat) *Rnf1/Rnf2* dm embryos was described in 3.2.2.

Collection, *in vitro* fertilization and culture of mouse oocytes and embryos

Collection of oocytes or embryos, in vitro fertilization (IVF) procedure and embryo culture was performed as described in 3.2.2. For a simplistic overview, time points for harvesting 2-cell embryos for RT-PCR and immunofluorescent (IF) staining purposes is shown in Supplementary Figure S1.



Supplementary Figure S1 | Time course of the second cell cycle in control embryos. Cell cycle phases for control embryos are indicated in different colors based on replication timing and BrdU incorporation experiments. Black bars show time points for embryo harvesting.

Immunofluorescence, microscopy and image analysis

Immunofluorescense analysis was performed as described in 3.2.2. Antibodies used in this study are: anti-BrdU (Sigma B8434), anti-Cdkn1c (RB-1637), anti-CyclinB1 (Sc-245), anti-Cdt1 (gift from H. Nishitani), anti-Geminin (gift from H. Nishitani), anti-γH2AX (Upstate 05-636), anti-Phospho-(Ser/Thr) ATM/ATR Substrate (Cell Signaling 2851), anti-Phospho-Ser345 Chk1 (Cell Signaling 133D3; 2348) and anti-Phospho-Thr68 Chk2 (Cell Signaling 2661).

Immunofluorescense stainings were analyzed using a laser scanning confocal microscope LSM510 META (Zeiss) and LSM510 software. For embryos, either a z-series 1.3 µm slices was recorded or one confocal slice through the maximal radius of each (pro)nucleus was scanned. Images were analysed using Imaris (Bitplane) software and exported as TIFF files.

DNA replication analysis by BrdU or EdU incorporation

Embryos were produced by IVF to minimalise developmental heterogeneity. Time intervals for culture in presence of BrdU (500µM; Sigma) and EdU (100nM; Invitrogen Click-iT™ Alexa Fluor® 488) are indicated in Figure2. Embryos were fixed (15 min in 4% paraformaldehyde in PBS) at the end of each indicated interval. For immunofluorescent analysis of BrdU standard IF protocol was used with the addition of a denaturing step (25 min, RT, 4M HCl in PBS-0.1% Triton-X 100) and a neutralizing step (0.1M Tris-HCl pH 8.5) after standard permeabilization. EdU was detected according to manufacturer's instructions (Invitrogen Click-iT™ Alexa Fluor® 488). Both BrdU and EdU was fluorescently detected using a laser scanning confocal microscope LSM510 META (Zeiss) and LSM510 software. Quantification of BrdU signals was performed by eye (+ for strong, +/- for weak and – for no BrdU incorporation), while EdU treated embryos were scanned using a laser scanning confocal microscope LSM510 META (Zeiss). The 10x objective was used with the pinhole opened to maximum to obtain one image of each embryo containing the entire depth of nuclei. Total nuclear area and total fluorescent intensity was quantified using ImageJ software. Nuclear fluorescent signal was corrected for background levels (cytoplasmic signal).

Quantitative real-time RT-PCR

RNA isolation, reverse transcription and quantitative real-time PCR was performed as described in chapter… All real time PCR measurements were normalized against endogenous LnmB1 and to exogenous bacterial spike gene Thr. All real time PCR experiments were performed on one or two biological replicates form independent isolations.

Primers used:

Cdk2 (F: 5'-CATTCCTCTTCCCCTCATCA, R: 5'-TAAGCAGGTTCTGGGGCTTA),

Cdkn1c (F: 5'-AATCTCCGCGAGGAAAGC, R: 5'-GTCTGCAGCGGACTCCAT),

Cdkn2a (F:5'-TAGAGGCTAACGGCCAGAGA, R: 5'-CCCAGAGTTCTTCCATCGTC),

CcnE1 (F: 5'-TGCTAGGTGTTTTAACTATAGGGTCA, R:5'-TCTGGAGCACTCAGTGGTGT),

CcnE2 (F: 5'-CGAGCTGTGGAGGGTCTG, R: 5'-AAACGGCTACTGCGTCTTGA),

Cdt1 (F: 5'-TAGTACCCCAGATGCCAAGG, R: 5'-GCGGAACATCTCAACTAGCA),

Geminin (F: 5'-GGAGCCCAAGAGAATGTGAA, R: 5'-CTAGCTGGTCATCCCAAAGC),

LnmB1 (F: 5'-GCCCTGGACATGGAGATCAGC, R: 5'-CGACTGGAGGACGCTCTGGA).

Expression profiling of GV oocytes and data analysis

Expression profiling and data analysis was performed as described in 3.2.2.

4.1.3. Results

Cell cycle regulators are misexpressed in *Rnf1/Rnf2* dm GV oocytes

As previously described we performed genome-wide expression profiling of *Rnf1/Rnf2* dm GV oocytes and found massive misregulation of gene expression compared to control oocytes (2662 genes misregulated > 1.5-fold compared to wild type; $p<0.05$). Although Gene Ontology analyses did not pick up significant cell cycle-related categories among misregulated genes, we still found a number of cell cycle regulators up- or down-regulated in *Rnf1/Rnf2* dm GV oocytes (Fig 1a). Interestingly, a number of positive cell cycle regulators, like cyclins and Cdk-s were up regulated, as well as two cell cycle inhibitors, such as Cdkn2a (Ink4a, p16) and Cdkn1c (p57). Remarkably, the only misregulated cell cycle genes listed in Figure 1a that are Polycomb targets in ES cells (based on Mohn et al. and Ku et al.) are Cdk6 and the two inhibitors, Cdkn1c and Cdkn2a. Other up-regulated genes may be oocyte specific Polycomb targets or mere secondary effects arising from the loss of *Rnf1/Rnf2*. We confirmed up-regulation of some cell-cycle genes by quantitative RT-PCR in GV oocytes and further examined the fate of transcript levels after fertilization in zygotes and 2-cell embryos (Fig 1b). While all five examined genes were up-regulated in *Rnf1/Rnf2* dm GV ooctytes compared to wild type, transcript levels dropped close to wild type levels by 1-cell (Cdk2) or early 2-cell stage (CcnE1, CcnE2, Cdkn1c, Cdkn2a).

DNA replication analyses in mat*Rnf1/Rnf2* dm zygotes and 2-cell embryos

To determine whether mat*Rnf1/Rnf2* dm embryos undergo DNA replication, we cultured IVF, therefore synchronized embryos in the presence or absence of BrdU during the first (4-16 hpf) or second cell cycle (19-34 hpf) (Fig 2a, right panels). Time of treatment was chosen to broadly cover the estimated S-phase of each cell cycle. Embryos were harvested at the end of each time point and BrdU incorporation was quantified by immunofluorescent staining. All control and all mat*Rnf1/Rnf2* dm zygotes and 2-cell embryos examined underwent DNA replication, while control groups without BrdU did not show any signal (Fig 2a, right panels). To further dissect the dynamics of DNA replication, we performed a time course analysis of BrdU incorporation starting from 4 hpf (G1 of 1-cell stage) to 46 hpf (G2 of 4-cell stage in wild type embryos) broken down to 3 or 6 hour intervals of BrdU treatment (Fig 2a, left panels). While control zygotes replicated rather synchronously between 7-13 hpf, mat*Rnf1/Rnf2* dm zygotes started DNA replication with a delay and in a less synchronous fashion (replication detected between 10-25 hpf). This delay in S-phase onset is consistent with the previously described delay of PN development in mat*Rnf1/Rnf2* dm 1-cell embryos. Control embryos started the second round of DNA replication shortly after cleavage to 2-cell stage. Notably, “early cleaving” mat*Rnf1/Rnf2* dm embryos (mat*Rnf1/Rnf2* dm

a

Misregulated genes in *Rnf1/Rnf2* dm GV		Values from array (*Rnf1/Rnf2* dm over control)
Cdc23	Cell division cycle 23	50/84
Cdca8	Cell division cycle associated 8	360/576
Cdkal1	CDK5 regulatory subunit associated protein 1-like 1	72/113
Cdk2	Cyclin dependent kinase 2	233/155
Cdk9	Cyclin dependent kinase 9 (CDC2-related)	226/112
Cdk6	Cyclin dependent kinase 6	97/65
Cdc2l6	Cell division cycle 2-like 6	100/66
Ccna1	Cyclin A1	21/10
Ccne1	Cyclin E1	303/84
Ccne2	Cyclin E2	473/312
Ccnm1	Cyclin M1	33/13
Ccnh	Cyclin H	260/173
Ccng1	Cyclin G1	244/97
Cdkn1c (Kip2, p57)	Cyclin-dependent kinase inhibitor 1C	287/86
Cdkn2a (Ink4a, p16)	Cyclin-dependent kinase inhibitor 2A	66/31

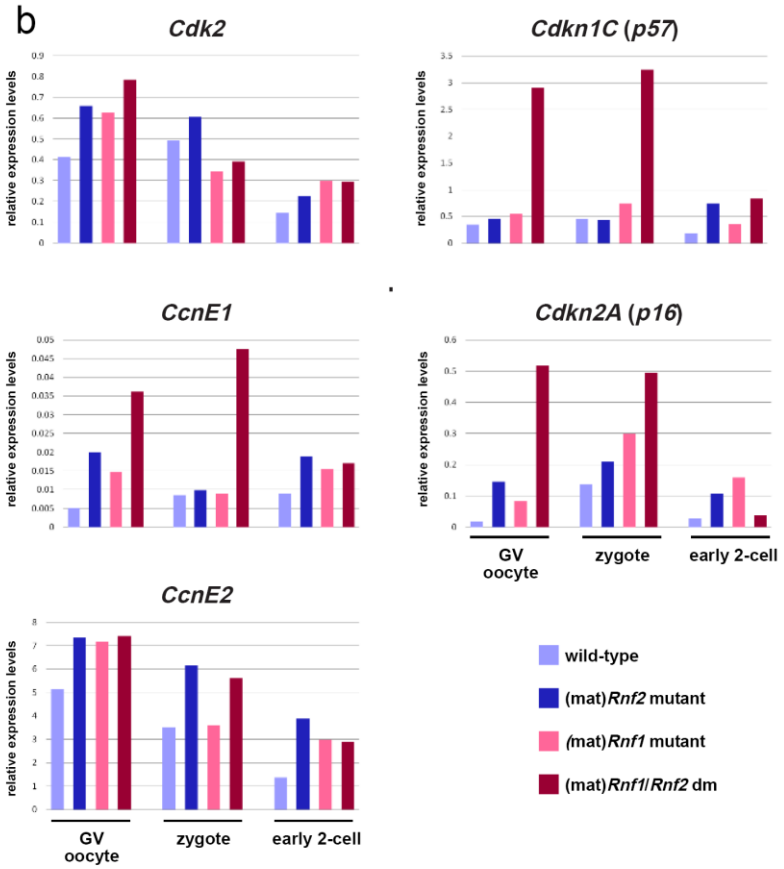
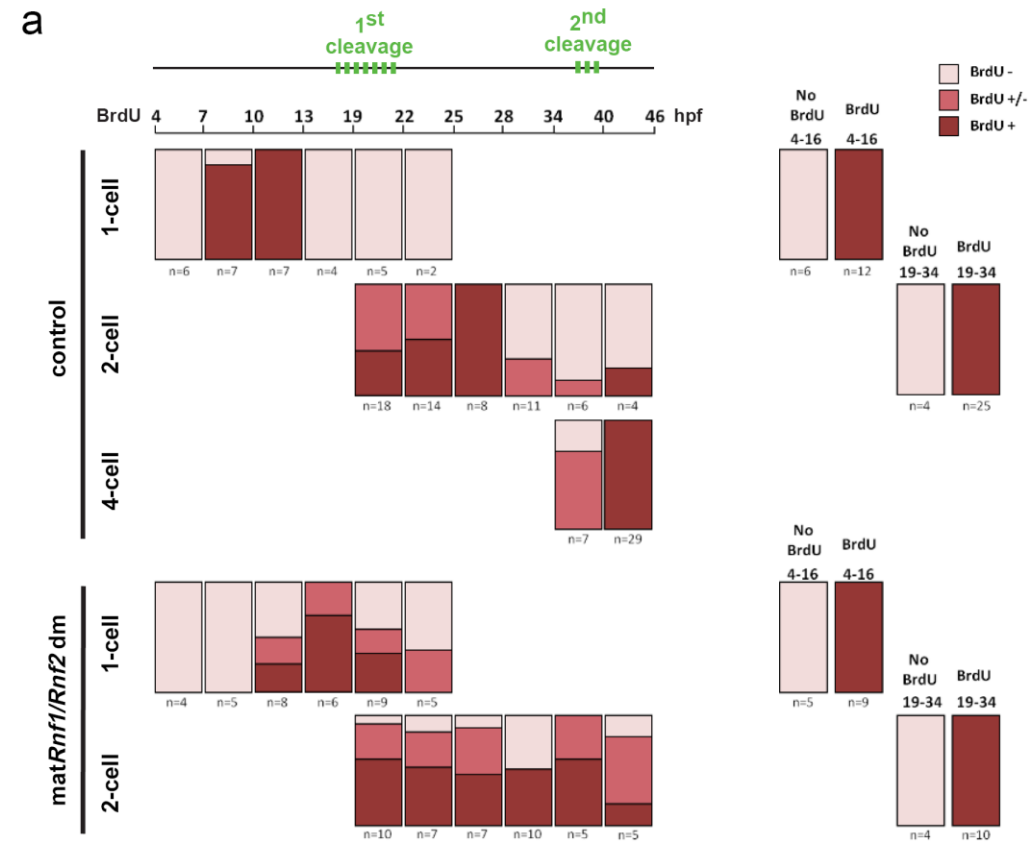


Figure 1 | Cell cycle regulators are misexpressed in *Rnf1/Rnf2* double mutant GV oocytes. a, Cell cycle regulators found to be down- (green) or up-regulated (red) more then 1.5-fold in *Rnf1/Rnf2* dm GV oocytes compared to wild-type in genome-wide expression profiling analyses **b,** Real-time PCR analysis of cell cycle regulator gene transcripts in wild-type (light blue), (mat)*Rnf2* (dark blue), (mat)*Rnf1* (pink) and (mat)*Rnf1/Rnf2* dm (red) GV oocyets, zygotes and early 2-cell embryos. Transcript levels were normalized to *LnmB1* control.

embryos that were already 2-cell at 22 hpf also readily started DNA replication, while delayed cleaving mat*Rnf1/Rnf2* dm embryos presumably started replication accordingly, also with a delay. Strikingly however, majority of mat*Rnf1/Rnf2* dm embryos did not seem to exit S-phase, as BrdU incorporation continued even up to the time when control embryos were engaged in the third round of DNA synthesis at the 4-cell stage (Fig 2a, left panels).

Next, we precisely quantified the amount of DNA replication during the 2- and “4-“cell stages by culturing embryos in the presence of another nucleotide analog, EdU and quantifying EdU incorporation by fluorescent staining (Fig 2b). We found that quantification of one round of genome replication in control embryos either at the 2- or 4-cell stage is relatively consistent and that EdU incorporation signals during G2-phase are at background levels (Fig 2b, upper panel). Interestingly, mat*Rnf1/Rnf2* dm 2-cell embryos showed on average markedly less EdU incorporation during the time of the 2-cell S-phase (Fig 2b, lower panel). Moreover, as expected from the previous experiment, EdU incorporation was not restricted to the normal interval of an S-phase, but continued at low levels at least up to the time point when control embryos finished DNA replication at the 4-cell stage.


a
1st cleavage
2nd cleavage
BrdU 4 7 10 13 19 22 25 28 34 40 46 hpf
BrdU -
BrdU +/-
BrdU +
control
1-cell
2-cell
4-cell
matRnf1/Rnf2 dm
No BrdU 4-16
BrdU 4-16
No BrdU 19-34
BrdU 19-34

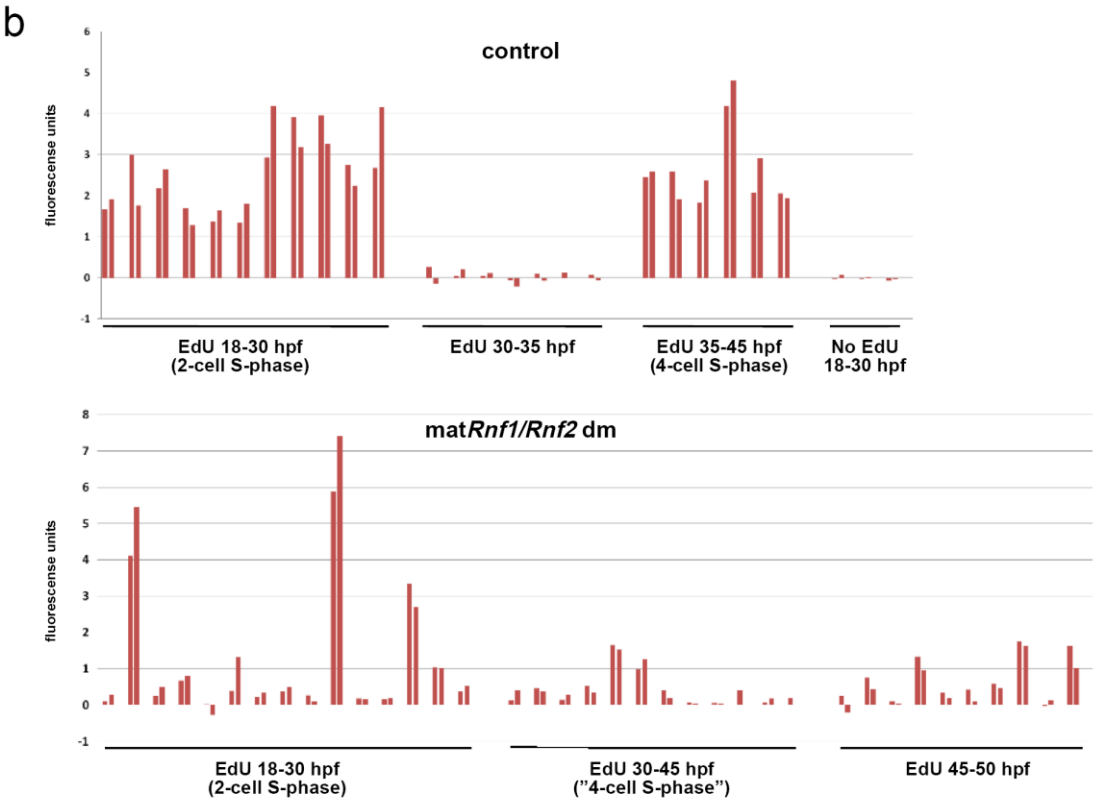

b
control
fluorescense units
EdU 18-30 hpf (2-cell S-phase)
EdU 30-35 hpf
EdU 35-45 hpf (4-cell S-phase)
No EdU 18-30 hpf
matRnf1/Rnf2 dm
EdU 18-30 hpf (2-cell S-phase)
EdU 30-45 hpf ("4-cell S-phase")
EdU 45-50 hpf

Figure 2 | DNA replication is abnormal in mat*Rnf1/Rnf2* dm zygotes and 2-cell embryos. a, Time course analysis of BrdU incorporation. Following IVF, control (upper panel) and mat*Rnf1/Rnf2* dm embryos (lower panel) were cultured in presence of BrdU at indicated intervals (top scale bar); hours post fertilization (hpf); embryos were fixed at the end of each BrdU treatment interval, stained with anti-BrdU antibody, and scored as BrdU- (light pink), BrdU+/- (dark pink) or BrdU+ (red). Each bar represents 100% of embryos analyzed at each interval, the actual number of embryos analyzed is indicated below each bar (n). Control groups of embryos incubated with no BrdU or with BrdU for the entire 1- or 2-cell stage are shown on the right side. Green dashed line shows time of cleavage divisions of control embryos. **b,** Quantification of DNA replication during the 2-cell and 4-cell stage. Control (upper panel) and mat*Rnf1/Rnf2* dm embryos (lower panel) were cultured in the presence of EdU for indicated intervals, and fixed at the end of each interval. EdU was quantified by measuring total fluorescent signal (Y axis) in each nucleus of embryos. Two adjacent bars represent the two nuclei of a 2-cell embryo or randomly chosen two nuclei of a 4-cell embryo.

Overexpression of Cdk inhibitors in *Rnf1/Rnf2* dm GV oocytes is not likely the cause of the 2-cell arrest

We observed that two cyclin dependent kinase (Cdk) inhibitors Cdkn2A and Cdkn1C were up-regulated in *Rnf1/Rnf2* dm GV oocytes. Overexpression of Cdk inhibitors seemed like an attractive explanation for the cell cycle arrest phenotype in embryos. Cdkn2A however, is an inhibitor of the G1/S-phase cyclin dependent kinases Cdk4 and 6. As mat*Rnf1/Rnf2* dm embryos enter S-phase, overexpression of this inhibitor is not a likely cause for the 2-cell arrest. This may be due to the observation that mouse pre-implantation embryos lack a G1/S checkpoint. Cdkn1C on the other hand is an inducer of the G2/M checkpoint, through inhibition of the mitotic Cdk1. As our DNA replication analyses and H3S10P stainings indicate that some mat*Rnf1/Rnf2* dm embryos may enter G2-phase, we examined whether Cdkn1C is responsible for the cell cycle arrest. Immunoflourescent stainings for Cdkn1C (Fig 3a) revealed that the inhibitor is present in mat*Rnf1/Rnf2* dm zygotes (data not shown) and early 2-cell embryos, while absent or very low in control embryos. However, Cdkn1C protein levels in mat*Rnf1/Rnf2* dm embryos decrease by mid 2-cell stage and remain at control-like levels up to 3.5 dpc (data not shown). Therefore, at the time of the arrest, Cdnk1C is not higher in mat*Rnf1/Rnf2* dm embryos then in controls, arguing that it does not play a role in the 2-cell block.

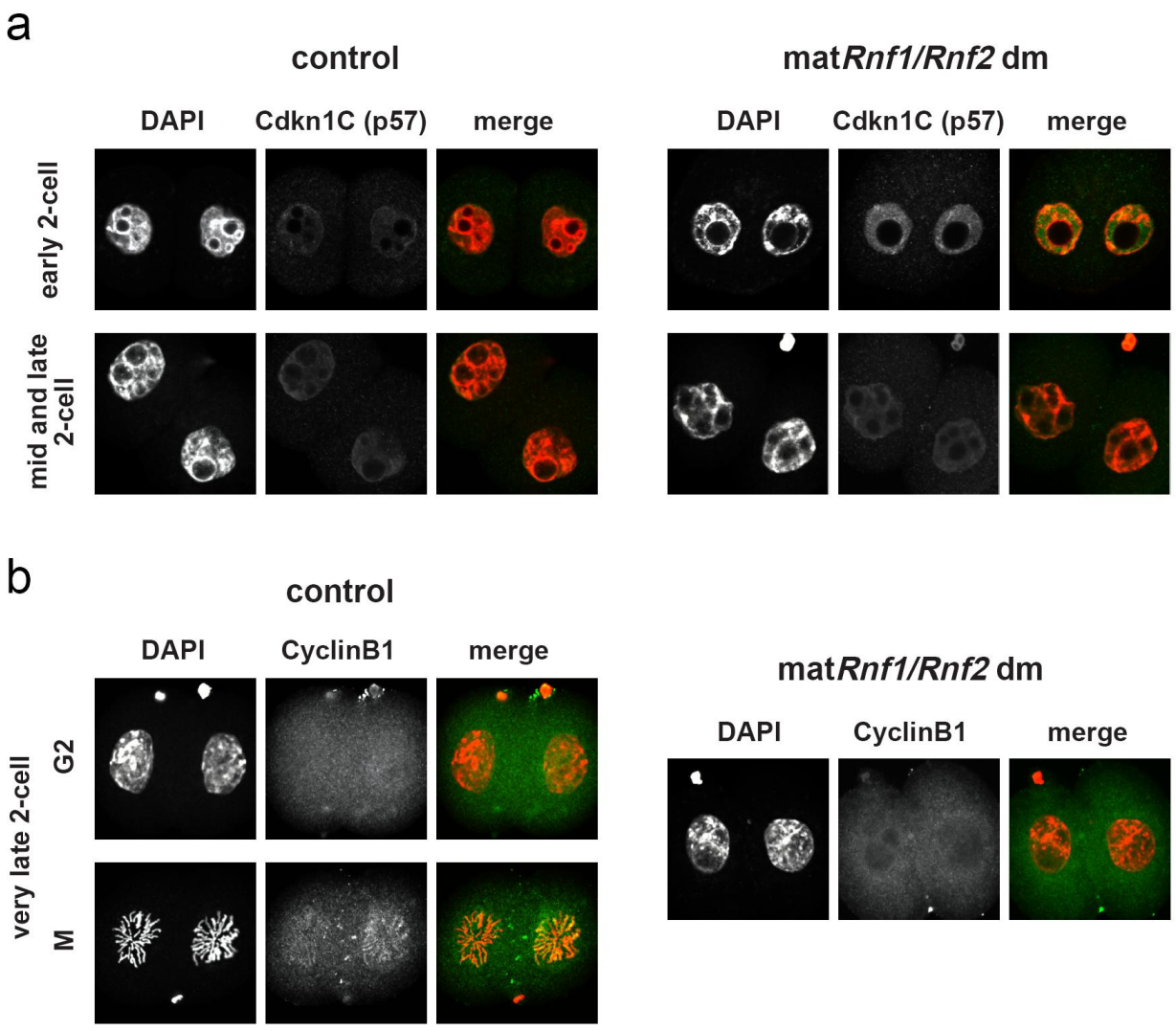


Figure 3 | Expression of cell cycle inhibitor Cdkn1c and M-phase cyclin Cyclin B1 in control and mat*Rnf1/Rnf2* dm 2-cell embryos. Control and mat*Rnf1/Rnf2* dm 2-cell embryos stained for **a,** Cdkn1c or **b,** Cyclin B1 at different time points, as indicated on the left hand side. DNA was stained with DAPI.

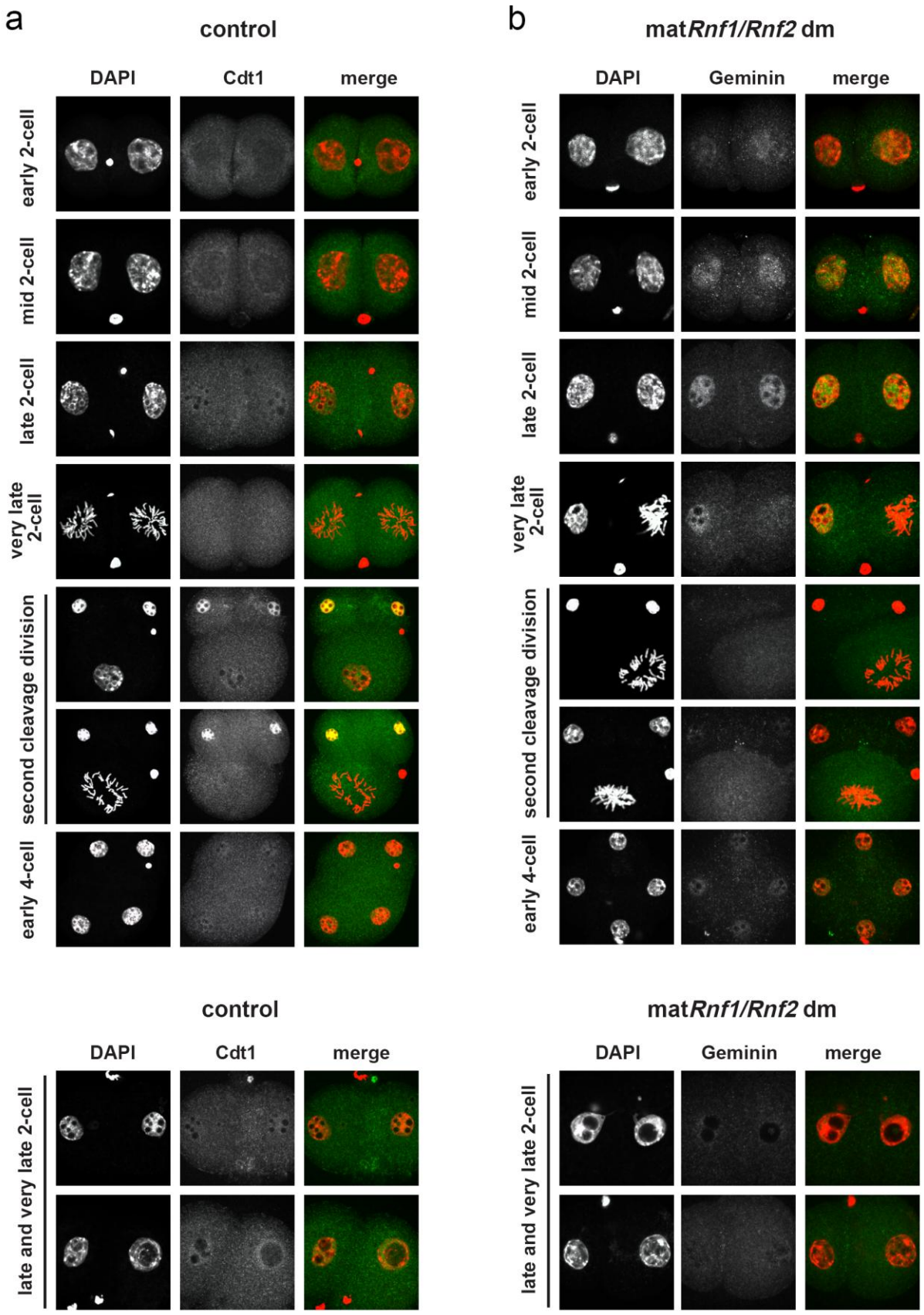
CyclinB1 does not localize to the nucleus in mat*Rnf1/Rnf2* dm 2-cell embryos

In eukaryotes, initiation of mitosis requires the activity of Cdk1 in complex with a B-type cyclin (Nurse, 1990). In 2-cell mouse embryos cytoplasmic Cyclin B1 was shown to accumulate starting from S-phase into G2-phase and to rapidly localize to the nucleus upon Ckd1 activation at the G2/M transition (Ohashi *et al.*, 2001). To address whether mat*Rnf1/Rnf2* dm embryos produce Cyclin B1 and whether Cyclin B1

localization is effected, we performed immunoflourescent stainings for Cyclin B1 in embryos at the time of the G2/M transition (Fig 3b). Control embryos showed homogenous cytoplasmic and surprisingly nuclear Cyclin B1 staining at late G2 and a clear nuclear Cyclin B1 accumulation at M-phase. Mat*Rnf1/Rnf2* dm embryos however showed overall less Cyclin B1 which failed to localize to the nucleus. As nuclear shuttling of Cyclin B1 is dependent on Cdk1 activation, these results suggest that Cdk1 remains inactive in mat*Rnf1/Rnf2* dm embryos.

De-novo replication licensing may participate in the prolonged S-phase of mat*Rnf1/Rnf2* dm 2-cell embryos

The prolonged S-phase in mat*Rnf1/Rnf2* dm embryos in theory might be due to slowing or stalling replication forks and/or to re-firing of replication origins. Cdt1 is a component of the replication licensing complex (Bell and Stillman, 1992), while Geminin is an inhibitor of Cdt1, therefore an inhibitor of re-licensing replication origins (Melixetian and Helin, 2004; Pitulescu *et al.*, 2005). To test whether ectopic replication licensing would take place in mat*Rnf1/Rnf2* dm embryos, we examined Cdt1 and Geminin expression. As reported for somatic cells, Cdt1 only localizes to the nucleus for a short time in early G1-phase in control embryos to achieve one round of replication licensing and is then excluded from the nucleus for the rest of the cell cycle (Fig 4a, upper panel). Geminin on the other hand shows an opposite pattern in control embryos, staring to accumulate in the nucleus at late G1, reaching maximal expression at late G2 and being degraded at M-phase (Fig 4b, upper panel). Strikingly, mat*Rnf1/Rnf2* dm embryos at late 2-cell stage do not show any nuclear Geminin staining (8 embryos out of 8) (Fig 4b, lower panel). While most (4 embryos out of 6) mat*Rnf1/Rnf2* dm embryos showed control-like absence of Cdt1 at the late 2-cell stage, few (2 embryos out of 6) embryos did show nuclear staining of Cdt1, albeit much weaker then early G1 controls (Fig 4a, lower panel). The absence of Geminin and the low levels of nuclear Cdt1 in some embryos suggests that re-licensing of replication origins may take place at the late 2-cell stage in mat*Rnf1/Rnf2* dm embryos, although it may not happen genome-wide, like during normal licensing in G1. Examining transcript levels of *Cdt1* (Fig 4c) and *Geminin* (Fig 4d) in wild type (light blue), mat*Rnf2* mutant (dark blue), mat*Rnf1* mutant (pink) and mat*Rnf1/Rnf2* dm (red) GV oocytes, late zygotes, early 2-cell (G1/S) and late 2-cell (G2) embryos showed that in double mutants transcript levels at neither stage was convincingly altered. These results suggest that misregulation of Cdt1 and Geminin protein levels results from post-transcriptional misregulation in mat*Rnf1/Rnf2* dm embryos. The mechanistic link of Cdt1 and Geminin misregulation to Polycomb deficiency remains elusive.


a
control
DAPI
Cdt1
merge
early 2-cell
mid 2-cell
late 2-cell
very late 2-cell
second cleavage division
early 4-cell
b
matRnf1/Rnf2 dm
DAPI
Geminin
merge
early 2-cell
mid 2-cell
late 2-cell
very late 2-cell
second cleavage division
early 4-cell
control
DAPI
Cdt1
merge
late and very late 2-cell
matRnf1/Rnf2 dm
DAPI
Geminin
merge
late and very late 2-cell

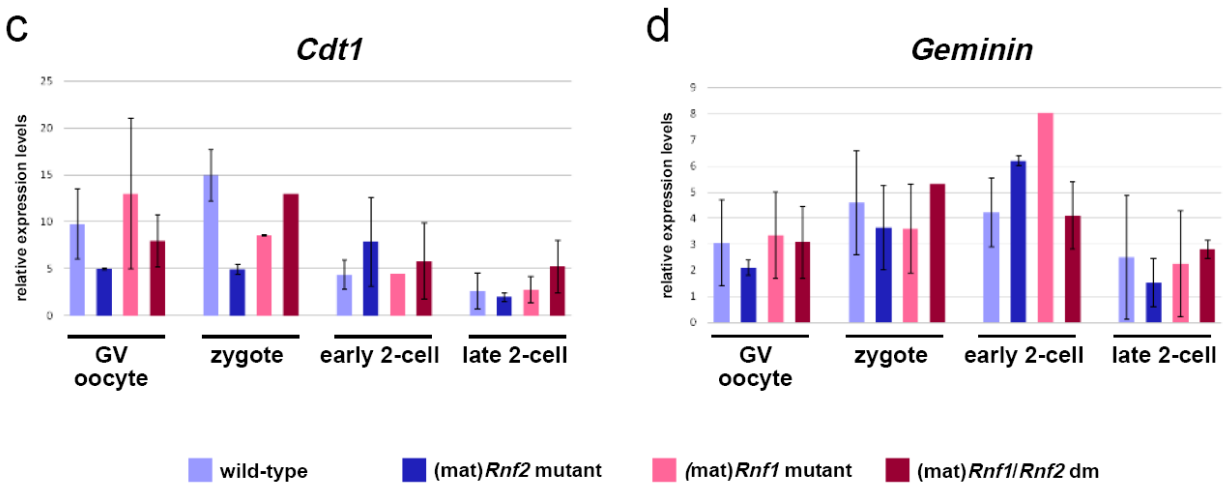


Figure 4 | Replication initiation inhibitor Geminin and replication initiation factor Cdt1 patterns in control and mat*Rnf1/Rnf2* dm 2-cell embryos. Control (upper panel) and mat*Rnf1/Rnf2* dm (lower panel) 2-cell embryos stained for **a,** Cdt1 or **b,** Geminin at different time points, as indicated on the left hand side. DNA was stained with DAPI. Real-time PCR analysis of **c,** *Cdt1* and **d,** *Geminin* transcripts in wild-type (light blue), (mat)*Rnf2* (dark blue), (mat)*Rnf1* (pink) and (mat)*Rnf1/Rnf2* dm (red) GV oocyets, zygotes, early 2-cell and late 2-cell embryos. Transcript levels were normalized to *LnmB1* control.

A novel γ-H2AX pattern during the 2-cell stage correlating with cell cycle phases

To address whether the prolonged S-phase in mat*Rnf1/Rnf2* dm embryos would be due to replication fork stalling, we stained embryos at different cell cycle phases during the 2-cell stage with anti-serine 139-phospho H2AX (γH2AX) antibody, a well known marker of DNA damage and also known to accumulate in response to replication stress due to for stalling (Rao *et al.*, 2007; Rogakou *et al.*, 1999; Rogakou *et al.*, 1998; Ward and Chen, 2001). We observed an interesting pattern of γH2AX correlating with the cell cycle phase in control embryos. Early 2-cell embryos around G1/early S-phase exhibited only a few foci of γH2AX. The number of γH2AX foci increased drastically by mid/late S-phase, while in G2-phase were reduced again to only a few foci and disappeared completely by the end of G2. Mitotic chromatin was heavily labeled with γH2AX, as reported before (Ziegler-Birling *et al.*, 2009). The increase of γH2AX labeling correlating with S-phase progression suggested that normal DNA replication in the embryo, without any exogenous DNA damage may be accompanied by activation of an intra-S-phase checkpoint.

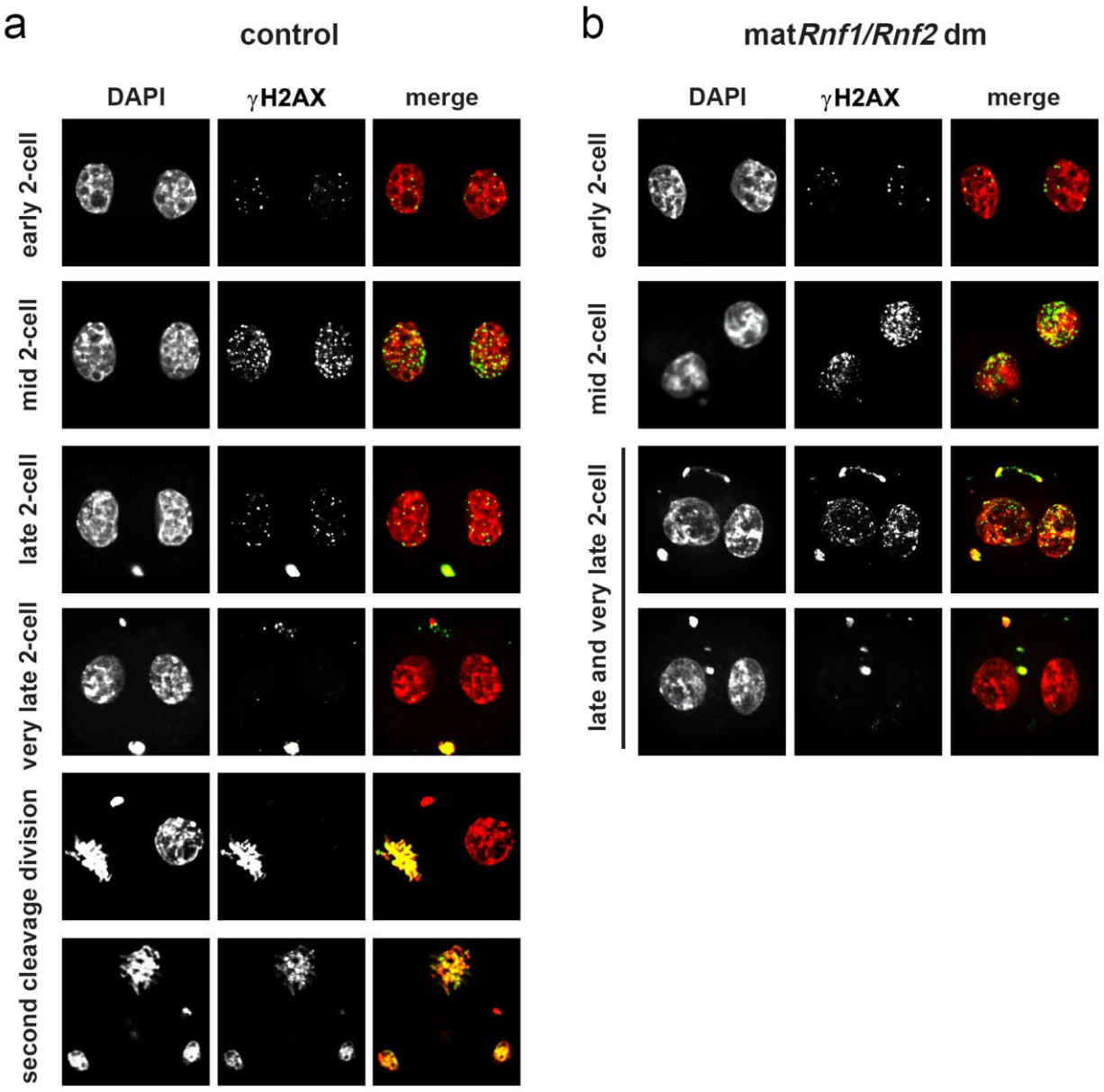


Figure 5 | γ-H2AX patterns in control and mat*Rnf1/Rnf2* dm 2-cell embryos. a, Control and **b,** mat*Rnf1/Rnf2* dm 2-cell embryos stained for γ-H2AX at different time points, as indicated on the left hand side. DNA was stained with DAPI.

Mat*Rnf1/Rnf2* dm embryos do not accumulate more γH2AX than S-phase controls, however unlike S-phase controls, they activate a checkpoint

Mat*Rnf1/Rnf2* dm embryos showed γH2AX patterns similar to control embryos at G1/early S- and mid S-phases (Fig 3b). However, during G2-phase almost 80% of mat*Rnf1/Rnf2* dm embryos still showed an S-phase-like γH2AX pattern (19 out of 25 embryos), while only 20% (6 out of 25 embryos) showed G2-like reduction of γH2AX foci. These results also support the notion that majority of mat*Rnf1/Rnf2* dm embryos do not finish S-phase by the time control embryos are in G2. However, it also shows that mat*Rnf1/Rnf2* dm embryos do not accumulate more γH2AX then a control embryo during normal S-phase.

To test whether elevated γH2AX levels during a normal S-phase or during "G2-phase" in mat*Rnf1/Rnf2* dm embryos would lead to checkpoint activation, we stained embryos for active forms of checkpoint components, such as ATR/ATM (Phospho-(Ser/Thr) ATM/ATR Substrate) (Fig 6a), Chk1 (Phospho-Ser345) (Fig 6b) and Chk2 kinases (Phospho-Thr68) (Fig 6c). Surprisingly, although γH2AX was high in mid 2-cell (S-phase) control embryos, this did not trigger detectable checkpoint activation (top left row in each panel). As controls for checkpoint activation, we used γ-irradiated control embryos or control embryos treated with HU (second and third left rows in each panel). γ-irradiation triggered a strong checkpoint response in control embryos, while HU treatment resulted in a weaker activation of all three checkpoint proteins examined. γH2AX levels increased compared to non-treated embryos at the same cell cycle phase, the number of foci correlating with the strength of checkpoint activation. As expected, control embryos possessing only few γH2AX foci during late 2-cell stage (G2-phase) did not activate checkpoint components (top right row in each panel). Interestingly however, (16 out of 18) mat*Rnf1/Rnf2* dm embryos (+ *Zp3*-cre) at the late 2-cell stage had activated a checkpoint response, despite the fact that γH2AX levels visually did not exceed levels during a normal S-phase of a control embryo (second right row of each panel). These findings indicate that the prolonged S-phase in mat*Rnf1/Rnf2* dm embryos is in part due to reduced rate of DNA synthesis triggering an intra-S phase checkpoint response.

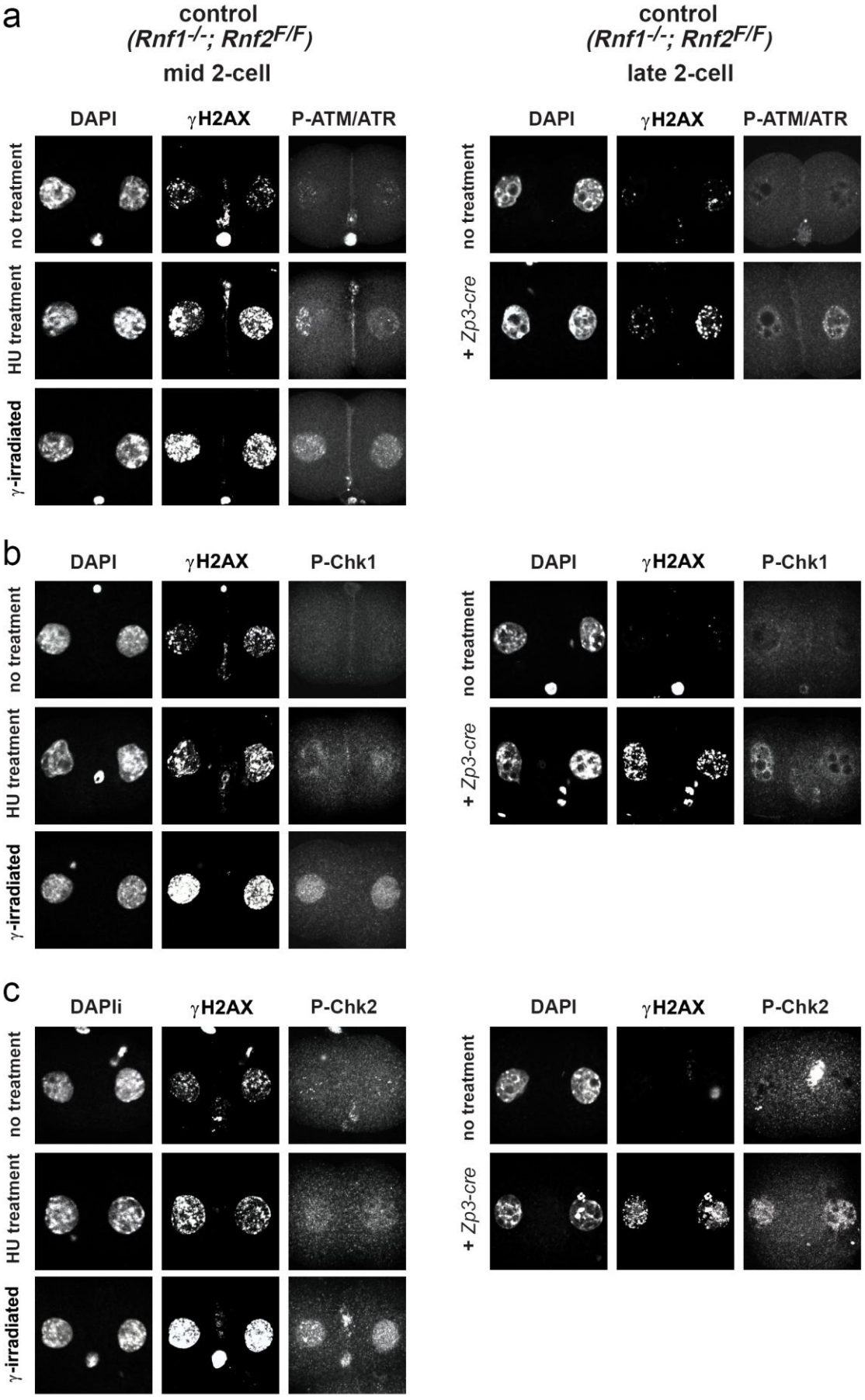

a
control
(Rnf1-/-; Rnf2F/F)
mid 2-cell
control
(Rnf1-/-; Rnf2F/F)
late 2-cell
DAPI
γH2AX
P-ATM/ATR
no treatment
HU treatment
γ-irradiated
+ Zp3-cre
b
P-Chk1
c
DAPIi
P-Chk2

Figure 6 | Analysis of checkpoint activation in 2-cell embryos in response to DNA damaging by γ–irradiation, replication fork stalling by HU treatment or maternal deficiency for *Rnf1/Rnf2*. Checkpoint activation was examined by staining embryos for phosphorylated (activated) forms of **a,** ATM/ATR kinase substrates, **b,** phosphorylated Chk1 and **c,** phosphorylated Chk2 kinases. γ-H2AX patterns were also examined. *Rnf1-/- RnfF/F* were stained at mid 2-cell (left pnels) or at late 2-cell (right panels). Treatment or genetic addition of *Zp3*-cre is indicated on the left hand side of images.

4.1.4. Discussion

Cell cycle characterization of mat*Rnf1/Rnf2* dm embryos revealed that maternal Polycomb deficiency lead to a prolonged S-phase at the 2-cell stage. We found that most mat*Rnf1/Rnf2* dm embryos remained in S-phase, only around 20% of embryos showed G2-like characteristics based on γH2AX patterns.

Cell cycle arrest could be due to misregulated maternal factors directly interfering with cell cycle progression. We examined the possibility whether cell cycle arrest could be due to up-regulated maternal cell cycle inhibitors, such as Cdkn1C or Cdkn2A, which are known Polycomb targets in other cell types (Jacobs *et al.*, 1999a; Mager *et al.*, 2003; Sharpless and DePinho, 1999; Yang *et al.*, 2009). Our results however, suggest that even though these Cdk inhibitors are up-regulated in *Rnf1/Rnf2* dm GV oocytes, they are not the likely cause for the 2-cell block phenotype.

Prolonged S-phase in mat*Rnf1/Rnf2* dm 2-cell embryos correlated with reduced DNA synthesis rates, suggesting that a checkpoint may be activated. Indeed, delayed mat*Rnf1/Rnf2* dm embryos with an S-phase-like γH2AX pattern showed nuclear enrichment of activated checkpoint kinases ATM/ATR, Chk1 and Chk2. However, literature suggests that checkpoint activation in early embryos results in cell cycle delay, but not full arrest (Shimura *et al.*, 2002a; Yukawa *et al.*, 2007). Therefore checkpoint activation does not explain why these embryos do not progress further. In line with this, the few mat*Rnf1/Rnf2* dm 2-cell embryos that show G2-like reduced γH2AX foci did not activate a checkpoint response, yet also did not divide further.

PRC1 has been reported to directly regulate protein levels of the cell cycle regulator Geminin, by ubiquitinating it and therefore targeting it for proteosome-mediated degradation (Ohtsubo *et al.*, 2008). Geminin plays a key role in inhibiting replication licensing: it associates with Cdt1, a component of the pre-replication complex (pre-RC), to prevent reinitiation of DNA replication (Saxena and Dutta, 2005). Based the observation of Ohtsubo et al., in mat*Rnf1/Rnf2* dm embryos loss of PRC1 in theory may lead to accumulation of Geminin. Although overexpression of Geminin was shown to arrest cells in G1 (Shreeram *et al.*, 2002), which is not what we observe in mat*Rnf1/Rnf2* dm embryos, we still decided to

investigate Geminin and Cdt1 levels in double mutant embryos. Surprisingly, we found that Geminin was absent in S-G2-phase mat*Rnf1/Rnf2* dm 2-cell embryos. Although we cannot link absence of Geminin to PRC1 deficiency, it may have implications for the prolonged S-phase phenotype of these embryos. Loss of Geminin in *Drosophila* (Mihaylov *et al.*, 2002; Quinn *et al.*, 2001) or in human cells (Melixetian *et al.*, 2004; Tachibana *et al.*, 2005; Zhu and Dutta, 2006b) causes partial over-replication of the genome. Re-replication results in stalled replication forks and DNA damage that triggers the ATR-Chk1-Cdc25 DNA damage signaling pathway (Ullah *et al.*, 2009). Moreover two studies have simultaneously shown that in mouse embryonic deficiency for Geminin also results in genome re-replication and DNA damage checkpoint activation starting at the 8-cell stage (Gonzalez *et al.*, 2006; Hara *et al.*, 2006). Maternal stores of Geminin before the 8-cell stage presumably mask any earlier effect in *Geminin-/-* embryos. Nevertheless, these studies demonstrate that Geminin-mediated replication control functions in early embryos. Therefore the extended S-phase in mat*Rnf1/Rnf2* dm 2-cell embryos that is accompanied by checkpoint activation may be due to re-firing of replication origins caused by the absence of Geminin. The presence of weak nuclear Cdt1 in some mat*Rnf1/Rnf2* dm 2-cell embryos further supports the notion of de-novo replication licensing. Further quantification of total DNA content of arrested mat*Rnf1/Rnf2* dm embryos is needed to clarify this issue.

Although reduced DNA synthesis rates and genome re-replication at first may seem contradicting, it can well be that re-initiation of replication origins cause replication fork stalling and checkpoint activation. The checkpoint will then actively reduce DNA synthesis, prolonging the replicative phase.

We characterized a novel γH2AX pattern during the 2-cell stage of wild type embryos. Previous studies show either no γH2AX, even in response to DNA damage (Adiga *et al.*, 2007b; Yukawa *et al.*, 2007) or very few foci of γH2AX in non-damaged 2-cell embryos (Ziegler-Birling *et al.*, 2009). These differences in γH2AX patterns may be attributed to different staining protocols, different antibodies used or simply that neither study examined the entire course of the second cell-cycle. Of note, with our staining protocol the anti-γH2AX antibody (Upstate 05-636) is extremely sensitive. Although the observed increase in γH2AX foci number during S-phase did not trigger detectable checkpoint activation in our experimental conditions, it is intriguing to speculate that weak checkpoint activation may take place during normal cell cycle progression in the embryo to regulate the length of the cycle. In *Drosophila*, the maternal to zygotic transition (MZT), which occurs by the 13th mitosis and is equivalent to the ZGA in mouse, requires a functional DNA damage checkpoint. Removal of maternal *ATR* homolog *Mei-41* or *Chk1* homolog *Grapes*, results in developmental arrest at the 13th mitosis with a failure to activate the zygotic genome (Fogarty *et al.*, 1994; Su *et al.*, 1999). In wild type fly embryos, lengthening of the 11th and 12th cycles that precede MZT was observed, while this was not the case in *Mei-41* or *Grapes* mutants, suggesting that a checkpoint functions to slow cleavage speed and allow time for the initiation of the MZT. Involvement of checkpoint activation in murine ZGA by depleting maternal checkpoint components has not been examined so far.

Cell cycle regulation is taken over in the mouse embryo by embryonic gene products during ZGA at the late 2-cell stage. Proper ZGA is essential for progression beyond the 2-cell stage as demonstrated by inhibiting ZGA with the transcription inhibitor α-amanitin (Warner and Versteegh, 1974) or by a number of maternal effect genes, whose deletion results in incomplete ZGA and subsequent developmental arrest (Bultman *et al.*, 2006; Tong *et al.*, 2000; Wu *et al.*, 2003). Maternal Polycomb deficiency may also effect ZGA directly, by failing to repress target genes during the activation of the embryonic genome, or indirectly, through the action of misregulated maternal factors inherited from the *Rnf1/Rnf2* dm oocyte. Characterizing ZGA in mat*Rnf1/Rnf2* dm embryos will provide further clues as to why these embryos cease development at the 2-cell stage.

5. General discussion and outlook

5.1. Polycomb proteins Rnf1 and Rnf2 are required during oogenesis for early embryonic development

It is well established that early mammalian development is driven by factors that are prepared but dormant in the oocyte, inherited by the embryo, where they exert the program known as maternal to embryonic transition. However, the nature of this program as well as the mechanisms involved in establishing it still hold many unknowns. Polycomb proteins are evolutionarily conserved chromatin–modifying factors that maintain cellular identity during many rounds of cell division by transcriptionally repressing developmental regulator genes that are inappropriate for the given cell lineage. Previous studies from our lab have shown that Polycomb proteins are present in the mouse oocyte and are inherited by the embryo. Therefore in this study we aimed at dissecting the role of Polycomb proteins, with focus on PRC1 members Rnf1 and Rnf2, during oogenesis and early embryonic development.

We demonstrate that maternal deficiency for *Rnf1* and *Rnf2*, the two ubiquitin ligase members of the PRC1 complex, compromises embryonic development beyond the 2-cell stage. We observe defects in chromosome alignment during oocyte meiotic maturation and a developmental delay during the first two cell cycles of embryogenesis before development halts. We characterized cell cycle defects in mat*Rnf1/Rnf2* dm embryos and found that majority of these embryos do not seem to enter the G2-phase of the second mitotic cell cycle. Although we do not understand the link to maternal Polycomb deficiency, we show that prolonged S-phase in these embryos may be due to re-initiation of replication origins and/or to activation of an intra-S-phase checkpoint.

To gain better understanding of the mechanisms underlying of this phenotype, we performed expression profiling of fully grown oocytes, and revealed massive misregulation of transcription during oocyte growth in the absence of *Rnf1* and *Rnf2*. Genes up-regulated in *Rnf1/Rnf2* dm fully grown oocytes were enriched for key developmental regulator genes. Importantly, these developmental regulators represented several different lineages. Analyzing the fate of some of these up-regulated transcripts revealed that they are translationally repressed until fertilization. Upon fertilization, however, these differentiation specific transcripts are translated, resulting in the inappropriate presence of differentiation factors during the otherwise totipotent stage of early embryogenesis.

Although we only show this type of message regulation for three examples (we were only able to test translational control of three messages due to limited availability of antibodies against proteins encoded by misregulated transcripts), it is intriguing to speculate that majority of de-repressed transcripts do not get translated in growing or fully grown oocytes, as we do not observe any developmental or phenotypical defect at these stages. We propose that up-regulated messages in *Rnf1/Rnf2* dm oocytes only get recruited for translation during meiotic maturation or after fertilization, corresponding to the appearance of

developmental defects. Oocytes have a fine tuned translational regulation system that allows storage of maternal messages until the time they are needed for driving critical processes of early development. This storage system does not seem to be perturbed in *Rnf1/Rnf2* dm oocytes and allows the oocyte to cope even with the presence of transcripts that normally would not be present at this stage of development.

An exception to this translational repression may be oocyte specific messages that are normally expressed and translated in the growing or fully grown oocyte. Interestingly, although not a Polycomb target in other cell types, we observed up-regulation of *Ddx6* transcripts in double mutant oocytes. Ddx6 is a core member of P-bodies and the recently described P-body-like granules in mouse oocytes that were proposed to store dormant maternal messages until translational recruitment (Flemr *et al.*, 2010). Other members of this oocyte granule, like *Tnrc6* (GW182), *Edc4* and *Lsm14a* are normally expressed in double mutant oocytes. Elevated levels of Ddx6 in double mutants may provide means for accommodating storage of de-repressed messages. Specific sequences in the 3'UTRs of mRNAs control 3'-polyadenylation, which in turn regulates recruitment and stability of maternal messages (Dickson *et al.*, 1999; Hake and Richter, 1994; McGrew *et al.*, 1989; Pique *et al.*, 2008; Sheets *et al.*, 1994). It would be interesting to examine 3'UTR control elements in up-regulated transcripts and try to predict translational timing of these messages.

We are quite confident that the up-regulation of developmental regulators we observe are a direct consequence of the loss of PRC1 in oocytes, as these lineage determiners are confirmed Polycomb targets in ES cells by numerous studies (Boyer *et al.*, 2006; Endoh *et al.*, 2008; Ku *et al.*, 2008; Mohn *et al.*, 2008). A question that remains unanswered is whether the up-regulated and translated differentiation inducing factors are the cause of the 2-cell arrest phenotype in the embryo? We also find many other genes up-regulated in PRC1 deficient oocytes, which are not Polycomb targets in ES cells. These may be oocyte specific Polycomb targets or up-regulated due to secondary effects. Clear secondary effects include the number of genes we find down-regulated upon loss of PRC1. We cannot exclude that lack of certain messages are not the cause of the arising phenotype.

For example, we observed down-regulation of *ATRX* mRNA in double mutant oocytes and were able to show that this correlates with decreased ATRX protein levels in GV oocytes and during meiotic maturation. ATRX is a centromeric heterochromatin binding protein belonging to the chromatin remodeling SWI/SNF family of ATPases (Gibbons *et al.*, 1997; Picketts *et al.*, 1998). Depletion of *ATRX* in mouse oocytes was shown to cause meiotic maturation defects, namely chromosome condensation and alignment problems as well as spindle defects at the M-II stage (De La Fuente *et al.*, 2004b). This phenotype is reminiscent of the defects we observe during meiotic maturation of *Rnf1/Rnf2* dm oocytes, therefore down-regulation of ATRX transcripts may be in part responsible for the meiotic maturation phenotype. Moreover, ATRX was recently shown to act as an H3.3 histone variant chaperone at telomeres and specific sites in ES cells (Goldberg *et al.*, 2010; Wong *et al.*, 2010). Although ATRX

involvement has not been addressed in this process, H3.3 is specifically incorporated into the decondensing paternal genome following fertilization (Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005). Given we observe a delay in PN formation in mat*Rnf1/Rnf2* dm zygotes, decreased ATRX levels might be responsible for slowing H3.3 incorporation and PN formation.

Another point we have to consider is whether the 2-cell block phenotype is only due to misregulated messages which are stored in the cytoplasm, or does PRC1-deficient chromatin also have an effect? Maternal PN transfer experiments between control and mat*Rnf1/Rnf2* dm zygotes revealed that the developmental block is not only due to inappropriate cytoplasmic factors, but also to a defective chromatin setup inherited from the PRC1 deficient oocyte. Embryos reconstructed from control cytoplasm and *Rnf1/Rnf2* dm chromatin reach blastocyts stage with only 16% (6/38) efficiency, compared to the 77% (34/44) efficiency of control PN transferred embryos. Importantly, this experiment demonstrates that there is transgenerational inheritance of Polycomb-mediated epigenetic information from the oocyte to the embryo, which is functionally relevant for embryonic development. Although in 16% of cases wild type factors may re-program this defective chromatin to allow development to blastocyst, we did not test whether normal, viable mice could be produced from such embryos. It can well be, that “maternal PRC1-deficient chromatin embryos” encounter defects during post-implantation development. For example, we know from somatic cell nuclear transfer experiments, that rates for producing blastocyst stage embryos are much higher than the efficiency of producing viable offspring (Ogura *et al.*, 2000; Wakayama and Yanagimachi, 2001). It would be very interesting to further dissect the role of this PRC1-mediated transgenerational mark. We are planning to transplant PN transferred embryos back into surrogate mothers in the next round of PN transfer experiments. Unfortunately, due to technical difficulties to obtain sufficient numbers of such embryos, we are not able to investigate the role of maternally inherited H2AUb in more detail, for example its effect on embryonic genome activation.

Rnf1 and Rnf2 have been reported to co-purify with nuclear factors such as E2F6 and Bcor (Gearhart *et al.*, 2006; Ogawa *et al.*, 2002; Sanchez *et al.*, 2007). E2F6 mediates transcriptional silencing through H3K9 methylation while the Bcor complex is associated with an H3K36 and an H3K4 demethylase. Recently it was shown in mesenchymal stem cells that a mutantion in Bcor results in increase of H3K36 and H3K4 methylation at target genes, concurrent with de-repression of target gene expression (Fan *et al.*, 2009). To study possible contributions of deficiency in these complexes to the mat*Rnf1/Rnf2* dm embryonic phenotype, we assessed H3K9me2, H3K9me3 and H3K36me3 levels in *Rnf1*/*Rnf2* double deficient oocytes and early embryos. We failed to observe any global difference compared to controls (data not shown), supporting the notion that the phenotype likely results from a loss of PRC1 function during oogenesis.

In certain scenarios PRC1 is known to be targeted to chromatin independently of PRC2 (Kalantry and Magnuson, 2006; Pasini *et al.*, 2007; Puschendorf *et al.*, 2008; Schoeftner *et al.*, 2006; Terranova *et al.*, 2008), but in most cases PRC1 functions downstream of PRC2 in silencing target genes expression

(Boyer *et al.*, 2006; Cao *et al.*, 2005; Cao *et al.*, 2002; Wang *et al.*, 2004b). For instance in the zygote, PRC1 targeting is independent of PRC2 at PCH regions, but is PRC2 dependent at euchromatin (Puschendorf *et al.*, 2008). Conversely, we examined expression of PRC2 members (Ezh2, Eed, Suz12) and H3K27me3 presence in *Rnf1/Rnf2* dm growing oocytes and mat*Rnf1/Rnf2* dm embryos. In oocytes we did not detect any difference in level or localization of these proteins and marks compared to control oocytes. In mat*Rnf1/Rnf2* dm embryos PRC2 members show control-like patterns, while H3K27me3 levels vary a little between embryos, but still show labeling of both maternal and paternal PN in majority of zygotes examined. These data show that the phenotype resulting from PRC1 deficiency in double mutants is independent of PRC2. It would be interesting to compare *Rnf1/Rnf2* dm and PRC2 mutant oocyte gene expression profiles to determine the connection between these complexes for repression of target genes during oogenesis. On a side note, maternal *Ezh2* deficiency does not result in a maternal effect phenotype. However, the homologous *Ezh1* may act redundantly, just as *Rnf1* can rescue loss of *Rnf2* at this stage of development.

The key accomplishment of maternal to embryonic transition (MET) is to activate the embryonic gene expression program on the newly formed embryonic genome. Proper zygotic (or embryonic) genome activation (ZGA) is essential for progression beyond the 2-cell stage as demonstrated by inhibiting ZGA with the transcription inhibitor α-amanitin (Warner and Versteegh, 1974) or by a number of maternal effect genes, whose deletion results in incomplete ZGA and subsequent developmental arrest (Bultman *et al.*, 2006; Tong *et al.*, 2000; Wu *et al.*, 2003). Major ZGA in mouse is known to take place mainly during the prolonged G2-phase of the second cell cycle (Flach *et al.*, 1982). Therefore it is an important question whether mat*Rnf1/Rnf2* dm embryos manage to activate the embryonic genome and if they do, to what extent? Preliminary data of RNAPII stainings of mat*Rnf1/Rnf2* dm 2-cell embryos suggests that these embryos do activate transcription, although to a lesser extent then control 2-cell embryos. To answer this question more precisely, we are currently performing expression profiling of late G2 control and mat*Rnf1/Rnf2* dm 2-cell embryos. To distinguish between maternal transcripts and *de novo* embryonic transcripts, we will include control and mat*Rnf1/Rnf2* dm embryos treated with the RNAPII inhibitor α-amanitin - therefore embryos only containing maternal transcripts-, in our expression profiling analysis. Characterizing ZGA in mat*Rnf1/Rnf2* dm embryos will provide further clues as to why these embryos cease development at the 2-cell stage.

Taken together, our findings demonstrate an essential function of Polycomb during oogenesis in keeping the developmental potential of oocytes under close guard, which is a prerequisite for proper initiation of embryonic development.

5.2. Indications for germline function of Polycomb form other species

PcG proteins are conserved from plants to humans. During evolution, a number of Polycomb genes are thought to have undergone expansion through multiple duplication events. For example, in the plant kingdom, the moss and fern genomes mostly have single copies of genes encoding for PcG proteins, while seed plants have multiple paralogs for most (Hennig and Derkacheva, 2009). A very similar example exists in the animal kingdom, where *Drosophila* has single copies of most PcG genes (except for Psc and Esc), while mammals have five Pc, three Ph, two Psc, two dRing and two E(Z) homologs (Ohno *et al.*, 2008; Whitcomb *et al.*, 2007). Interestingly, classical targets of Polycomb proteins, such as *Hox* genes, have also undergone expansion from fly to mammals. In plants, which lack *Hox* gene clusters Polycomb targets include other key developmental regulators (Katz *et al.*, 2004; Schubert *et al.*, 2005). These findings have led to the conclusion that Polycomb function may be conserved throughout evolution.

In our study, we show an essential role for PcG proteins during mouse oogenesis. However, if we look around in other species, we find this is not the first evidence for Polycomb involvement in germline functions.

Homologs of PRC2 components are well described in plants (Hennig and Derkacheva, 2009). *Arabidopsis thaliana* has three E(Z) homologs (CLF, MEA and SWN), three Su(Z)12 homologs (EMF2, FIS2 and VRN2), one Esc homolog (FIE) and five NURF55 homologs (MIS1-5). These proteins form at least three PRC2-like complexes (EMF, VRN and FIS) with different subunit composition. By catalyzing trimethylation of H3K27, all three complexes have been implicated in silencing target genes during different developmental stages. For example, the EMF complex was shown to silence targets during sporophytic development (Makarevich *et al.*, 2006) with essential roles in suppressing precocious flowering through silencing flowering activators, such as the *FT* (*Flowering Locus T*) and *AGL19* (*Agamous-like 19*) genes (Jiang *et al.*, 2008; Schonrock *et al.*, 2006; Yoshida *et al.*, 2001). The VRN Polycomb complex on the other hand is required for flowering after vernalization (exposure to cold) by repressing the flowering-repressor *FLC* (*Flowering Locus C*) (Gendall *et al.*, 2001). The most exciting Polycomb complex in plants from our point of view is the FIS complex which is known to silence target genes during gametogenesis and early embryonic development. Maternal FIS function is needed for subsequent embryonic development, demonstrated by maternal *fis* mutants. These mutants are characterized by autonomous seed-like structure formation in absence of fertilization (Chaudhury *et al.*, 1997; Guitton *et al.*, 2004; Kohler *et al.*, 2003a; Ohad *et al.*, 1996). Even when fertilized, maternal *fis* mutants give rise to embryos that fail to complete development (Kohler *et al.*, 2003a; Kohler and Makarevich, 2006; Spillane *et al.*, 2000; Yadegari *et al.*, 2000). A direct target of the FIS complex is the PHERES1 (PHE1) transcription factor, which is silenced in the maternal germline as well as in the early embryo (Kohler *et al.*, 2003b; Kohler *et al.*, 2005).

Absence of H2AUb in plants and the fact that no PRC1 homologs could be found to date, could suggest that PRC2-mediated H3K27me3 may be responsible for silencing. However, this is probably not the case, because PRC1-analogs have been identified in plants that are required for PRC2-mediated silencing at certain target genes. These analogs include LHP1 (Like heterochromatin protein 1) and the RING finger containing AtRING1 proteins (Libault *et al.*, 2005; Nakahigashi *et al.*, 2005; Sanchez-Pulido *et al.*, 2008; Xu and Shen, 2008).

As in plants, PRC2 homologs have been identified and characterized in the nematode *C. elegans*. These include *Mes2*, the SET domain containing H3K27 HTMase, a homolog of the mammalian Ezh2 and *Mes6*, a homolog of mammalian *Eed* (Holdeman *et al.*, 1998; Korf *et al.*, 1998). Mes2, Mes6 and Mes3 (a protein with no known homology) were shown to function in a complex and to mediate di- and tri-methylation at H3K27. Mes genes were originally identified in genetic screens for maternal-effect sterile or "grandchildless" mutants (Capowski *et al.*, 1991). Mothers carrying *Mes* mutations produce viable offspring whose germline degenerates midway through larval development (Capowski *et al.*, 1991; Paulsen *et al.*, 1995). This effect is dependent on the number of X chromosomes: XX offspring are more severely effected than XO males (Garvin *et al.*, 1998). Indeed, in wild type animals, H3K27me3 is concentrated on the X chromosome, which is silenced in the germline (Fong *et al.*, 2002; Xu *et al.*, 2001). The working model is that the Mes2/Mes3/Mes6 complex operates in the maternal germline and during early embryogenesis epigenetically marks the X chromosome and therefore represses gene expression from it during germline development of the embryo (Xu *et al.*, 2001).

Recently PRC1 homologs have also been identified in the worm: *Mig32* and *Spat3*, homologs of *Bmi1* and *Rnf2* respectively (Karakuzu *et al.*, 2009). Consistent with being PRC1 analogs, mutants of either *Mig32* or *Spat3* show reduction or loss of H2AUb. *Mig32* and *Spat3* mutants display a similar somatic phenotype as *Mes* mutants and authors also show that at least for male tail development, worm PRC1-like proteins and Mes factors function in the same pathway. These results suggest that Mig32 and Spat3 may function in concert with the Mes2/Mes3/Mes6 complex, as PRC1 and PRC2 in fly and mammals. Surprisingly however, unlike *Mes* mutants, *Mig32* and *Spat3* mutants produce fertile offspring; therefore in the germline these PRC1-like proteins are not required for Mes2/Mes3/Mes6 function. It is possible that other redundant proteins may be responsible for H2A ubiqitination, or that H2AUb is simply not required for silencing in the germline (Karakuzu *et al.*, 2009).

Drosophila PRC1 and PRC2 components are perhaps even better described in literature than the mammalian homologs (Muller and Kassis, 2006; Ringrose and Paro, 2004). To our interest, genetic evidence indicates that the maternal contribution of the Eed homolog Esc is critical for early embryonic development (Margueron *et al.*, 2009; Ohno *et al.*, 2008; Struhl, 1981; Struhl and Brower, 1982). Embryos lacking maternal Esc display complete de-repression of all homeotic genes and conversion of all segments to the identity of the 14th parasegment. Zygotic *Esc* was able to rescue the maternal *Esc* defect when overexpressed shortly after fertilization (Simon *et al.*, 1995). These experiments show an essential

embryonic role of maternal Esc; however they do not address the function of Polycomb in the germline. In another study authors examine several different mutant alleles of *E(Z)* (Phillips and Shearn, 1990). While flies homozygous for *E(Z)* null alleles are lethal during larval development, weak mutant alleles, such as temperature-sensitive alleles cause adult homeotic phenotypes. Importantly, females homozygous for temperature-sensitive *E(Z)* alleles display a range of phenotypes from oogenesis defect to maternal-effect lethality, depending on the time and length of exposure to restrictive temperature (Phillips and Shearn, 1990).

Taken together, there are indications from other species for Polycomb functioning in the germline for proper embryonic development. In plants, there is a clear maternal effect phenotype due to the loss of the FIS complex in the germline. In the worm, the maternally provided PRC2-like complex is essential for germline development of the embryo. To our knowledge, Polycomb function in the germline has not been examined so far in other species.

5.3. Polycomb proteins Rnf1 and Rnf2 function during first linage specification events in pre-implantation embryos?

Maternal PRC1 function in the female germline is essential for early embryonic development. But is this the only stage during early embryonic development where PcG proteins have an essential role? We know that zygotic (embryonic) *Rnf2* mutants, that still have the maternal load of PRC1 proteins (*Rnf2m+z-*) are embryonic lethal around gastrulation (Voncken *et al.*, 2003). Importantly, these mutant embryos still have *Rnf1*, which may be able to compensate for the loss of *Rnf2* between the time when the embryo runs out of the maternal Polycomb supply and the time of gastrulation, where apparently Rnf1 redundancy is not sufficient to rescue development. A key process that takes place before implantation is the first lineage specification events in the embryo. The first two lineages, the ICM and the TE are established by the blastocyst stage; however, many studies have demonstrated that blastomeres are committed to either lineage already a few cleavages be*fore the blastocyst stage (Johnson and Ziomek, 1983; Rossant and Vijh, 1980; Suwinska et al., 2008; Ziomek and Johnson, 1982)*. A significant change occurs in the developmental potential of blastomeres around the 5th cleavage, so that cell fates become fixed by the 32-cell stage. Would Polycomb proteins that have been assigned key roles in regulating important developmental genes in ES cells, have a role in this change of developmental potential of blastomeres or during the establishment of the first lineages in the embryo?

To address this question, we produced embryos that have the maternal contribution of Rnf1, but will be zygotically *Rnf1/Rnf2* dm (*Rnf1m+z- Rnf2m-z-*). We achieved this by mating *Rnf1+/- Rnf2F/F Zp3-cre* females with *Rnf1-/- Rnf2F/F Prm1-cre* males. Half of the embryos from such crosses have the desired genotype, the other half of the litter has an embryonic *Rnf1+* allele. Unfortunately, this approach does not allow us to pool embryos, but restricts us to single embryo-analysis techniques. Nevertheless, preliminary

observations on the developmental potential of *Rnf1m+z- Rnf2m-z-* embryos revealed that they cease development somewhere between the 16-cell to morula (32- or 64-cell stage). More detailed analysis of these embryos is needed, but we can already draw two important conclusions. First, that the maternal presence of Rnf1 in *Rnf1m+z- Rnf2m-z-* embryos allows these embryos to overcome the 2-cell block. Second, that there is a developmental window around the time embryonic cells are undergoing important fate decisions where PRC1 plays an essential role. On a side note, we suspect that this role of PRC1 can be fulfilled by Rnf1, as *Rnf2m+z-* embryos only arrest at later stages of post-implantation embryonic development (Voncken *et al.*, 2003). Another possibility - as Rnf2 is considered a stronger H2A ubiquitin ligase and it is maternally contributed in wild-type embryos - is that the maternal load of Rnf2 in *Rnf2m+z-* embryos is sufficient to overcome this “around 32-cell block”. We are currently generating mice to obtain embryos that will have the maternal contribution of Rnf2 but will be zygotically dm for *Rnf1* and *Rnf2 (Rnf1m-z- Rnf2m+z-)* to address the roles of Rnf1 vs Rnf2 at the time of first lineage determination events.

Single-embryo protocols are currently being established in our lab, which will give us unique tools to address these roles of Polycomb proteins. It will be very interesting to dissect the role of PRC1 during this phase of development, where important fate decisions are made.

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