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Study of histone variants and chromatin dynamics in the preimplantation mouse embryo

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Abstract

Mammalian development starts at fertilization, when two highly specialized cells, the sperm and the oocyte, fuse and create the totipotent zygote. Through subsequent cell divisions and differentiation during development, the zygote gives rise to every cell type in the organism. In mouse, 3.5 days after fertilization, the blastocyst forms. Despite the DNA content of all cells being identical, the blastocyst already comprises two distinct cell types: the pluripotent inner cell mass and the multipotent trophectoderm. This suggests that the mechanisms additionally to the DNA sequence itself play a role in regulating cell fate specifications, pointing out towards a key role for epigenetic regulation of the earliest stages of development. How the zygote acquires totipotency from two fully differentiated cells, and how cell fate decisions are made later in development is a pivotal biological question.

In eukaryotes, the DNA is associated with histones into a nucleoprotein complex called chromatin. Chromatin structure can range from very loose to highly compacted, and can be permissive to ‘reading’ of the DNA or antagonize it. Importantly, chromatin can be extensively modified, with functional implications in various biological processes, such as transcription and DNA-damage repair.

My doctoral studies were focused around two main subjects. Firstly, I was interested in understanding how chromatin composition and biochemical posttranslational modifications of histones influence early mouse development. In particular, I focused on a histone variant, H2A.Z, and posttranslational modifications associated with transcriptionally active chromatin in the early mouse embryo. This study resulted in a publication which is presented in Part 2 of my thesis. The importance of histone variants in the transitions in genome organization during spermatogenesis is outlined in

Publication 2. Some unpublished results and a general discussion on the importance of H2A.Z in mouse embryogenesis are also included.

Early embryogenesis is a period of intense chromatin remodeling, both biochemically and physically. I became interested in the dynamic properties of embryonic chromatin at different developmental stages to understand if there is a functional link between changes in chromatin plasticity and cell potency. A publication documenting histone mobility for the first time throughout early embryogenesis with complementary nuclear ultrastructure in the developing embryo is presented in Part 3 of my thesis.

My doctoral thesis contributed to the understanding of the dynamic events affecting embryonic chromatin during epigenetic remodeling after fertilization. Findings obtained from the embryo will surely prove useful in future investigations on the impact of chromatin structure on cellular differentiation and reprogramming.

Avant-propos

Le développement des mammifères commence à la fécondation, lorsque deux cellules hautement spécialisées, le sperme et l'ovocyte, fusionnent et créent le zygote totipotent. Grâce à la division cellulaire et la différenciation ultérieure au cours du développement, le zygote donne naissance à tous les types de cellules dans l'organisme. Chez la souris, 3,5 jours après la fécondation, il y a formation du blastocyste. En dépit que la teneur en ADN de toutes les cellules soit identique, le blastocyste comprend déjà deux types de cellules distinctes: la masse cellulaire interne pluripotente et le trophectoderme multipotent. Ceci suggère que des mécanismes indépendants de la séquence d'ADN jouent un rôle dans la régulation du cahier de charges du destin cellulaire, pointant vers un rôle clé pour la régulation épigénétique des premières étapes du développement. Comment le zygote acquiert la totipotence à partir de deux cellules complètement différenciées, et comment les décisions du destin cellulaire sont faites plus tard dans le développement sont des questions biologiques essentielles.

Chez les eucaryotes, l'ADN est associé avec les histones dans un complexe de nucléoprotéine appelé chromatine. La structure de la chromatine peut varier de très faible à très compact, et peut être permissive ou antagoniste à la «lecture» de l'ADN. Fait important, la chromatine peut être largement modifiée, avec des conséquences fonctionnelles sur divers processus biologiques, tels que la transcription et la réparation de l'ADN endommagé.

Mes études de doctorat ont porté sur deux sujets principaux. Tout d'abord, je me suis intéressée à comprendre comment la composition de la chromatine et modifications post-traductionnelles biochimiques des histones influencent le développement précoce de la souris. En particulier, je me suis concentrée sur une variante d'histone, H2A.Z, et les modifications post-traductionnelles associées à la chromatine transcriptionellement active dans l'embryon de souris. Cette étude a donné lieu à une publication qui est présenté dans la deuxième partie de ma thèse. L'importance des variantes d'histones dans les transitions de l'organization du

génome au cours de la spermatogenèse est décrite dans la publication 2. Certains résultats non publiés et une discussion globale sur l'importance de H2A.Z dans l'embryogenèse de la souris sont également inclus.

L’embryogenèse précoce est une période d'intense remodelage de la chromatine, à la fois physiquement et biochimiquement. Je me suis intéressée aux propriétés dynamiques de la chromatine embryonnaire à différents stades du développement pour comprendre s'il y a un lien fonctionnel entre les changements dans la plasticité de la chromatine et la potence de la cellule. Une publication qui documente la mobilité des histones pour la première fois tout au long de l'embryogenèse précoce en association avec l’ultrastructure nucléaire au cours du développement embryonnaire est présentée dans la troisième partie de ma thèse.

Ma thèse de doctorat a contribuée à la compréhension des événements dynamiques affectant la chromatine embryonnaire pendant le remodelage épigénétique après la fécondation. Les résultats obtenus à partir de l'embryon vont sûrement s'avérer utile dans les enquêtes futures pour étudier l'impact de la structure de la chromatine sur la différenciation cellulaire et la reprogrammation.

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Abbreviations

2i	2 inhibitors
ac	acetyl
ATP	adenosine tri-phosphate
bp	base pairs
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CENP-A	entromere protein A
CHD1	chromodomain helicase DNA binding protein 1
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EDTA	ethylendiaminetetraacetic acid
EGA	embryonic genome activation
EPI	epiblast
FCS	fluorescence correlation spectroscopy
FDAP	fluorescence decay after photoactivation
FLIP	fluorescence loss in photobleaching
FRAP	flurescence recovery after photobleaching
FRET	Försters resonance energy transfer
GFP	green fluorescent protein
HAT	histone acetyltransferase
HDAC	histone deacetylase
HFD	histone-fold domain
HJURP	Holliday junction recognizing protein
HMT	histone methyl-transferase
HP1	Heterochromatin protein 1

IAP	Intracisternal A-particle
ICM	inner cell mass
iFRAP	inverse fluorescence recovery after photobleaching
INCENP	inner centromere protein
Kb	kilobase
kDa	kilo Dalton
LAD	lamina-associated domain
LIF	leukemia inhibitory factor
LSD	lysine(K)-specific demethylase
me	methyl
MGA	mid-preimplantation genome activation
min	minutes
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MuERV-L	murine endogenous retrovirus - leucine
NCP	nucleosome core particle
nt	nucleotide
p	phospho
PCR	polymerase chain reaction
PGC	primordial germ cell
piRNA	piwi-interacting ribonucleic acid
PrE	primitive endoderm
PRC	Polycomb-repressive complex
PTM	posttranslational modification
RD	replication-dependent
RI	replication-independent
RNA	ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II

RSF	remodeling and splicing factor
SCNT	somatic cell nuclear transfer
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interference ribonucleic acid
SMC	structural maintenance of chromosomes
TBS	Tris Buffered Saline
TE	trophectoderm
TF	transcription factor
Ub	ubiquityl
WB	western blot
XCI	X-chromosome inactivation
ZP	zona pellucida

Part 1. Introduction

I. Structure and function of chromatin

In a eukaryotic cell, genetic material is stored in a specialized organelle, the nucleus. Within the confined nuclear space, DNA is organized into a nucleoprotein complex called chromatin. In somatic cells, chromatin is composed of DNA and small, highly basic proteins, called histones. The wrapping of DNA around histones allows for the neutralization of negative charges of the DNA backbone, and the efficient condensation of genetic material, which (in human cells) if extended would be approximately 2 meters long. At the same time, organization of the DNA into chromatin interferes with the accessibility of regulatory DNA sequences, and chromatin is generally refractory to DNA-based processes that need reading of the genetic information.

1. Different levels of chromatin organization

The basic and repeating unit of chromatin is the nucleosome. The nucleosome comprises about 146 base pairs of DNA wrapped around a histone octamer in 1.7 left helical turn. The Histone octamer contains 2 copies of each core histone H2A, H2B, H3 and H4 (Luger, Mader et al. 1997) (Figure 1A, B). Core histones can be divided into two categories: replication-dependent (RD, or 'canonical') and replication-independent (RI, or 'variant'). Genes encoding for RD histones are organized into large co-regulated gene clusters (Marzluff, Gongidi et al. 2002), and are rapidly expressed during S-phase at high levels, coinciding with DNA-replication. RD histone mRNAs are the only known cellular non-polyadenylated mRNAs (Marzluff, Wagner et al. 2008). Canonical histones are namely H2A, H2B, H3 and H4, and they are incorporated into chromatin during DNA-replication through the action of specialized histone chaperones (Ellis 2013). Conversely, expression of RI histones, whose transcripts are polyadenylated and often contain introns, persists throughout the cell cycle. Histone variants include H2A.X, H2A.Z, H3.3 and others, and some have their own dedicated chaperones responsible

for their deposition and eviction onto and from chromatin (Weber and Henikoff 2014). Core histones consist of a highly conserved and structured central globular domain, called the histone fold domain (Figure 1C), and N- and C-terminal tails characterized by higher structural flexibility (Luger, Mader et al. 1997).

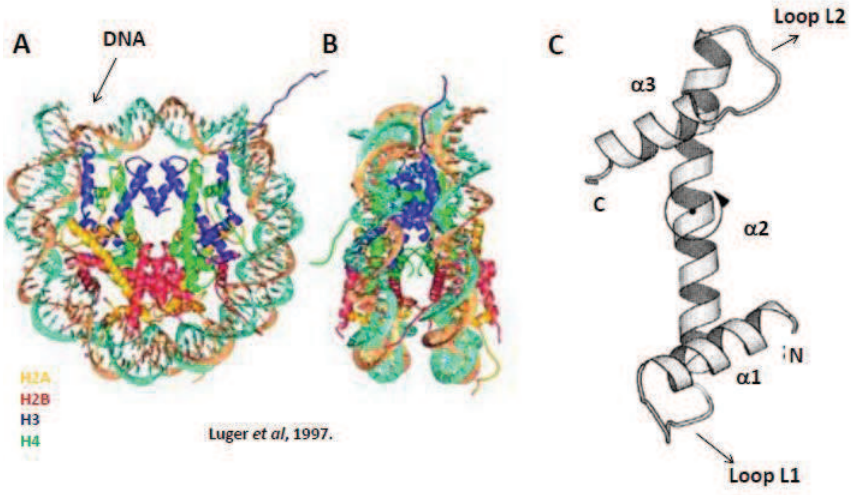

Figure 1. The nucleosome – basic unit of chromatin. (A and B) Crystal structure (at 2.8 Angstrom resolution) of the nucleosome core particle (NCP), containing 2 copies of each core histone H2A, H2B, H3 and H4 (color coded) around which 146 base-pairs of DNA double helix are wrapped. (B) Lateral view of the NCP. (C) The conserved histone fold domain. Three a-helices (a1, a2 and a3) are connected by relatively unstructured linker loops (L1 and L2). Adapted from (Ramakrishnan 1997).

The majority of DNA-histone interactions are between structured histone regions, while the more disordered histone tails protrude from the nucleosomes and can interact with neighbouring nucleosomes and other factors. Histone tails are also subject to extensive posttranslational modifications, discussed below.

a. 10-nm fiber chromatin fiber and higher-order chromatin structures

Nucleosomes are connected by short (10-80 nt) DNA segments, called linker DNA, and form nucleosomal arrays with a diameter of 10 nm. The existence of 10-nm fiber was observed by electron microscopy almost 40 years ago (Olins and Olins 1974) (Figure 2A, B). This so-called 'beads on a string' organization of nucleosomes is the first level of chromatin compaction (Kornberg 1974) and is generally permissive to transcription. Nucleosomal arrays can form short-range interactions with neighbouring nucleosomes to form chromatin fibers. Given the modularity of the nucleosomal composition and chemical modifications that the chromatin can be subject to, the possibilities to modify primary chromatin structure are virtually endless. Changes in nucleosome structure and stability can influence formation of higher-order structures.

Under physiological conditions, chromatin rarely exists in the simple and open mode of 10-nm fibres. Between the extended conformation of the 10-nm fiber and the highly compacted mitotic chromosomes there are several levels of chromatin organization that are less well understood. The secondary structure of chromatin includes internucleosomal contacts and comes about by folding of individual fibers into a defined fiber. Chromatin secondary structures are stabilized by linker histone H1 (or H5) and non-histone chromatin protein, such as HP1, Polycomb group proteins and others. In vitro experiments have shown that nucleosomal arrays can form helical structures of 30 nm in diameter, containing 6 to 11 nucleosomes per turn (Gerchman and Ramakrishnan 1987). This secondary structure, termed the 30-nm fiber, was proposed to be involved in chromatin compaction and transcriptional repression. Two models of 30-nm fibre structure have been proposed. The first one is the solenoid model, which proposed a single starting point of the 30-nm fiber, with a central axis of symmetry around which the fiber is formed (Finch and Klug 1976). Conversely, the zig-zag model predicts that the 30-nm fiber has 2 starting points and every other nucleosome interacts with each other

to stabilize the structure (Dorigo, Schalch et al. 2004)(reviewed in (Luger, Dechassa et al. 2012) (Figure 2C).

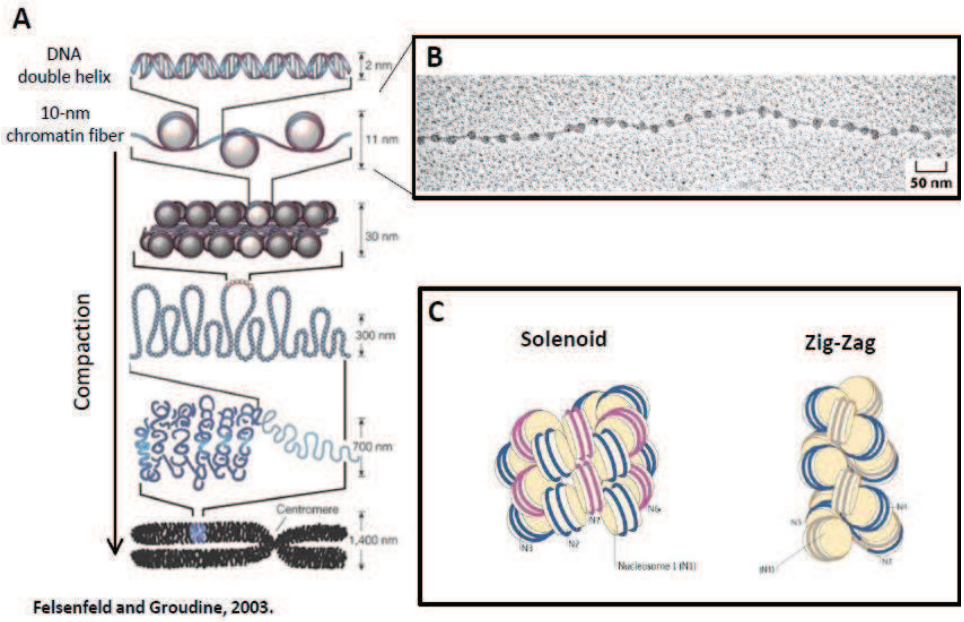

Figure 2. Levels of chromatin compaction. (A) Schematic representation of different levels of chromatin compaction, ranging from the extended 10-nm fiber, to the fully condensed mitotic chromosomes. (B) Electron micrograph of the so-called 'beads on a string' 10-nm fiber. From Alberts B *et al*, 2004. (C) Schematic of 2 proposed models of 30-nm fiber – the one-start solenoid model and the two-start zig-zag model. Adapted from (Luger, Dechassa et al. 2012).

Recent experiments designed to elucidate which of the models is predominant, including mesoscopic modeling, demonstrated that there is not one uniform type of helical fiber organization but rather conformational heterogeneity of nucleosome interactions (Grigoryev, Arya et al. 2009). It is now thought that the 30-nm fiber consists largely of zig-zag stacked nucleosomes interspersed with other structures (including solenoidal) with different levels of organization. Thus, it seems that the 30 nm fiber encompasses different chromatin structures which are not mutually exclusive. However, evidence for the 30-nm fibre existence in vivo remains elusive.

An alternative emerging concept is that chromatin in vivo is in a dynamically disordered state of a polymer melt, whereby linearly non-neighbouring nucleosomes can interact with each other (Sanyal, Bau et al. 2011).

Intramolecular interactions between secondary structures are thought to produce tertiary chromatin conformations, like such observed in mitotic chromosomes. The predominant view of how high condensation of chromatin in mitosis comes about was through sequential hierarchical coiling of the 30-nm fiber. This would presumably allow for the formation of non-random and constrained rod-like structures with reproducible dimensions. However, since the very existence of 30-nm fibers in vivo is questionable, a less-well defined organization of mitotic chromosomes is now proposed, in agreement with a study suggesting a disordered and random aspect of mitotic chromatin condensation (Nishino, Eltsov et al. 2012). In this model, the interactions of over-crowded and irregularly spaced nucleosomal arrays give rise to the physical arrangements of metaphase chromosomes similar to a 'molten globule' or 'melted polymer' state (Figure 3A). This model has one important shortcoming – it does not account for the rod-like structures observed in cytological preparations of mitotic cells. Most likely additional factors, such as condensin, influence the final physical properties of chromatin tertiary structures (Figure 3B).

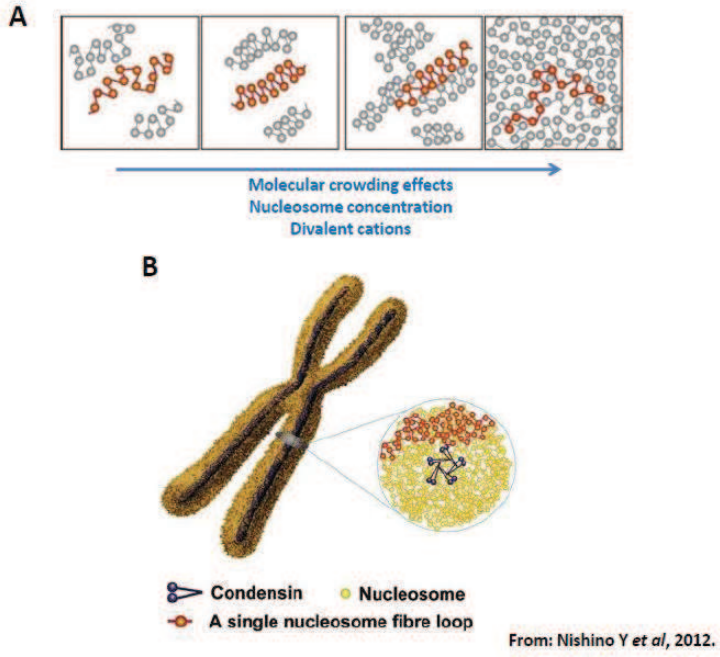

Figure 3. Polymer melt model of chromatin structure. (A) Individual 10-nm fibers fold into different types 30-nm structures. Nucleosomal concentration and crowding of 30-nm fibers causes interfiber interactions, which leads to the formation of disordered higher-order chromatin structures. Intramolecular contacts are further stabilized by the presence of divalent cations. (B) Mitotic chromosomes consist of disordered and diverse chromatin structures, which are stabilized by frequent inter and intranucleosomal protein-protein interactions and other factors, such as condensin.

b. Heterochromatin and euchromatin

Chromatin structure must be viewed through the prism of specific biological functions. It can range from very distinctive micro-scale domains, such as centromeres, to the nanoenvironment of active promoters. From a functional point of view, chromatin can be roughly divided into 2 categories: accessible and inaccessible. The former, usually referred to as euchromatin, is gene rich, transcriptionally permissive, replicates early and is characterized by a loose chromatin structure. On the other hand, heterochromatin is gene poor, tightly packaged, replicates late and is generally refractory to transcription and DNA damage repair.

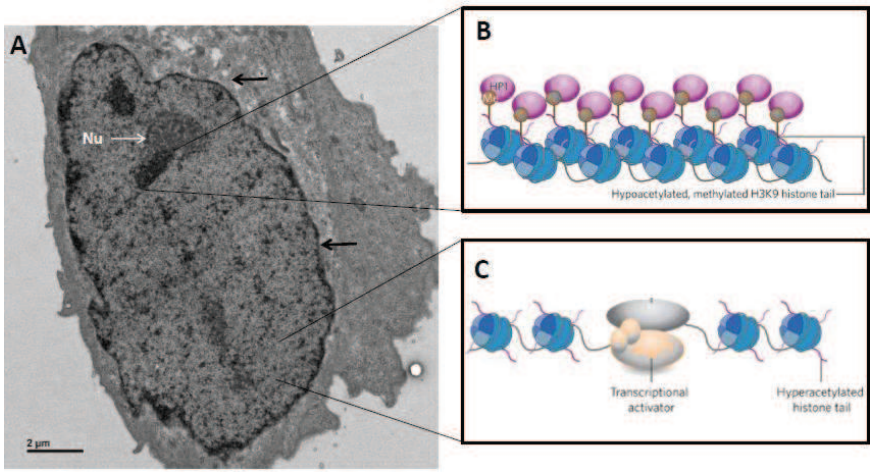

Figure 4. Heterochromatin and euchromatin. (A) Electron micrograph of a nucleus mouse embryonic fibroblast (acquired by Andre Eid). Regions of high and low electron density can be observed, corresponding to heterochromatin and euchromatin, respectively. Nuclear membrane is showed by an arrow and the nucleolus by the letters Nu. (B and C) Schematic depiction of genome organization in heterochromatin and euchromatin. (B) Heterochromatin is characterized by high nucleosome density and chromatin condensation and lack of histone acetylation. (C) Euchromatic regions display less chromatin condensation and associated with histone hyperacetylation, and are accessible to transcription factors. B and C are adapted from (Grewal and Elgin 2002).

The dichotomy between euchromatin and heterochromatin on a nuclear level was observed almost a century ago, in 1928, in cytological experiments by Emil Heitz. Euchromatin and heterochromatin are generally associated with distinct sets of histone posttranslational modifications and chromatin factors (Figure 4). During S-phase, euchromatic regions are replicated first, while heterochromatic regions are replicated only at the end of the S-phase (Rhind and Gilbert 2013). However, it is important to note that chromatin organization in a cell is a lot more 'fine tuned' and specific. For instance, heterochromatin can further be subdivided into facultative and constitutive heterochromatin. Facultative heterochromatin is formed in gene rich regions to ensure proper regulation of developmental genes. Facultative heterochromatin can become 'reactivated' depending on developmental and signaling cues. On the other hand, constitutive heterochromatin is formed on centric, pericentric, telomeric regions that harbor repetitive DNA elements and imprinted genes, which are silenced in all cells of the organism in a heritable manner. Regions of constitutive heterochromatin remain condensed throughout the cell cycle and often associate with distinct subnuclear compartments (Zhao, Bodnar et al. 2009; Towbin, Gonzalez-Sandoval et al. 2013). Furthermore, constitutive and facultative heterochromatin are characterized by distinct sets of histone PTMs and associated proteins. Constitutive heterochromatin is marked by H3K9me3, H4K20me3 and HP1 binding (Grewal and Elgin 2002)). Regions of facultative heterochromatin are associated with Polycomb-group proteins, H3K27me3 and specific histone variants, like macroH2A. So, although both chromatin types repress gene activity, they are quite specialized, with different subnuclear localization, biochemical properties and functions. Interestingly, a comprehensive study of chromatin components in fly cells subdivided genome organization into 5 principal chromatin types with distinctive chromatin characteristics (Filion, van Bemmel et al. 2010). While the main division between heterochromatin and euchromatin remains, the researchers were able to further distinguish specific types of transcriptionally repressive versus permissive chromatin. They also reveal that a large portion of the genome, about 48%, is associated with transcriptionally inert chromatin (so-called 'black' chromatin). Interestingly, 'black' chromatin was not enriched in canonical marks of constitutive heterochromatin (such as H3K9me3 or H4K20me3).

To make things even more complex, a temporal dimension needs to be taken into account. Changes into chromatin are introduced constantly, and genes can dynamically fluctuate between expression and repression during development and differentiation, and depending on environmental and/or intracellular signals.

Clearly, the structure of chromatin is set in place to ensure proper genome packaging and the transmission of genetic material to future generations, but at the same time needs to be flexible and reversible to allow for DNA-based processes, such as transcription and replication, to take place.

2. Studying chromatin dynamics in living cells

With the advance of optical and biophysical techniques, investigations into the dynamic properties of nuclear proteins were rendered possible. Microscopic techniques using fluorescently labeled molecules, termed F-techniques, have been particularly useful for such studies. A brief description of most commonly used F-techniques is outlined in table 1. Using FRAP (Fluorescence recovery after photobleaching) and FLIP (Fluorescence loss in photobleaching) (Figure 5), the kinetic properties of nuclear proteins involved in diverse processes, such as chromatin organization and rRNA processing, was determined in HeLa and BHK cells (Phair and Misteli 2000). The authors expressed GFP-tagged proteins (for instance HMG-17-GFP, fibrillarin-GFP) and photodestroyed flourescense in an area of the nucleus. Through measurments of fluorescence recovery in the bleached area over time, they could conclude on the dynamics of investigated proteins in living cells. In fact, rapid and complete recovery of fluorescence in bleached areas was observed for all proteins investigated, and ATP depletion did not affect protein recovery rates, suggesting energy-independent, diffusion-based processes and the absence of immobile protein fraction.

Table 1. Most commonly used F-techniques for measuring protein dynamics in living cells.

Technique	Description	Reference
FRAP Fluorescence recovery after photobleaching	Optical technique for visualizing and quantifying lateral mobility and dynamics of fluorescent molecules in living cells. Based on photodestruction of fluorescence in a defined area and monitoring of fluorescence repopulation of bleached area.	Sprague et al, Biophysical journal 2004
iFRAP Inverse FRAP	Involves photobleaching of whole cell apart from a small region of interest (ROI), and measures return of fluorescence to bleached area from the ROI.	Haustein and Schwille, 2007, Annu Rev Biophys Biomol Struct.
FLIP Fluorescence loss in photobleaching	Fluorescence is continuously bleached in an ROI, and loss of fluorescence is measured in a different area of the cell. Loss of fluorescence outside the ROI reflects protein exchange rates.	Lippencot-Schwartz et al, 2003, NatCellBiol
FDAP Fluorescence decay after photoactivation	Measures dynamics of subpopulation of molecules which can be visualized only after photoactivation (molecules tagged with photoactivatable fluorophores).	Patterson GH. 2008, Curr Protoc Cell Biol
FCS Fluorescence correlation spectroscopy	Single-molecule detection technique that measures and correlates fluctuations in fluorescence intensity within a very small detection volume. Used to measure binding kinetics and diffusion rates of fluorescent molecules in living cells.	Macháň R and Wohland T, FEBS Lett. 2014
FRET Förster-resonance energy transfer	Sensitive biophysical technique for quantifying distances between two fluorophores (donor and acceptor) based on efficiency of radiationless energy transfer from donor to acceptor.	Helms and Volkhard 2008 Principles of Computational Cell Biology

However, since the recovery was slower compared with GFP or reported dynamics of free solutes in the nucleus, it was hypothesized that protein-protein interactions within the nucleus cause this reduction in kinetics of the proteins analysed. These observations raised an interesting question: How do proteins reside within a subnuclear compartment and at the same time move throughout the nucleus in an unrestricted manner? The authors proposed that the compartments themselves are in constant turnover, and that proteins that occupy them roam (more or less) freely throughout the nucleoplasm in search for appropriate interactors.

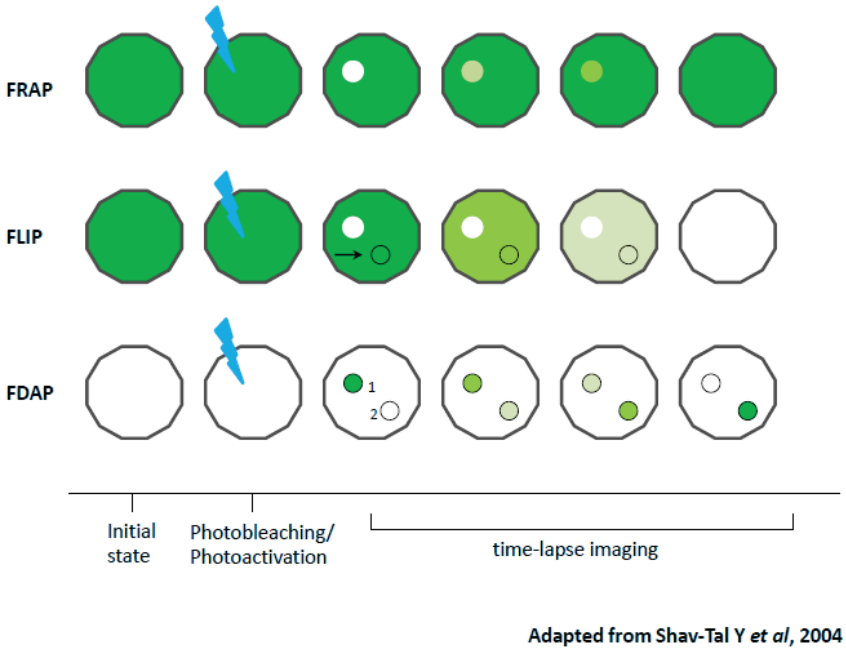

Figure 5. Illustration of the experimental setup for 3 commonly used F-techniques, FRAP, FLIP and FDAP. All techniques are based on measuring changes in fluorescence levels in a defined region over time. In FRAP and FLIP, flourescence is photodestroyed in a given area while in FDAP, laser power is applied to photoconvert a molecule from a non-fluorescent to fluorescent state. Regions of interest are marked by a circle and the laser photodestruction /activation by ( ).

The histone octamer is in the centre of the nucleosome, with 146 base pairs of DNA wrapped around it and it provides the structural basis for chromatin. Thus, the mobility of histone proteins in the octamer is generally very low, with recovery rates of hours (Kanda, Sullivan et al. 1998), (Kimura and Cook 2001). Even so, differential mobilities can be observed if one compares H2B-GFP and H3- or H4-GFP. In HeLa cells, the H3-H4 tetramer is very stable on chromatin and there is very little exchange of H3-H4 tetramers, as observed by FRAP experiments (Kimura and Cook 2001), while a small pool of H2B-GFP on the nucleosome surface is more mobile and exchanges continually. Nonetheless histone-GFP recovery curves are often used as controls in FRAP (and other F-techniques) experiments, since they represent virtually immobile proteins, as anticipated by their function in chromatin organization and nucleosomal DNA wrapping. Conversely, many chromatin-associated proteins, including HP1

proteins, linker histone H1 and a number of transcription factors, show relatively low residence time on chromatin and exchange rapidly.

Early FRAP experiments showed that HP1α is very mobile in cell a nucleus (Cheutin, McNairn et al. 2003), which was suggested to be a mechanism of heterochromatin maintenance. A more recent study by P.Hammerich and colleagues combined FRAP with FCS to provide a more detailed characterization of HP1α/β/γ kinetics in living cells (Schmiedeberg, Weisshart et al. 2004). Initial FCS measurements revealed at least 2 populations of HP1α molecules in the nucleoplasm – a highly mobile fraction with uniform GFP signal, and a much less mobile one concentrated in bright GFP spots, which likely represent large and stable structures where a high fraction of fluorescent HP1s accumulate. Further investigation into these HP1 foci revealed 2 populations of HP1 molecules and a presence of a small but consistent immobile fraction (~5%), which was not observed in euchromatin. Interestingly, upon transcription inhibition or chromatin condensation, this very slow population of HP1 molecules can increase to ~20%. Interestingly, differences between different HP1 isoforms can be observed regarding their mobilities. During interphase, HP1γ is the fastest isoform, while in mitosis only HP1α is associated with chromatin, enriched at pericentromeres where it exhibits very slow mobility and contributes to heterochromatin maintenance. The presence of three distinct binding sites for HP1α/β was later confirmed in a comprehensive study using fluorescence fluctuation microscopy. One binding site is present everywhere on chromatin with low residence time, one enriched in heterochromatin and one found only in heterochromatin. Interestingly, the enrichment of HP1α/β in heterochromatin was correlated with the presence of H3K9me2/3 and the corresponding Suv39 methyltransferases (Muller, Erdel et al. 2009).

Most of the studies on chromatin mobility have been performed in fully differentiated or transformed cell lines, such as HeLa cells or 293HEK, presumably due to their extensive characterization, availability and ease of manipulation. These studies have provided invaluable insight into the behavior of nuclear factors and chromatin organization. However, a shortcoming in using these systems is that they provide little

information about the putative changes in chromatin dynamics during transitions in cellular states, like those observed during differentiation or lineage allocation in vivo.

This limitation was partially overcome by using pluripotent mouse ES cells, which have the ability to self-renew but also the potential to differentiate into various other cell types. Interestingly, the chromatin structure of ES cells was shown to be different from that of differentiated cells, and lacked the typical prominent DAPI dense heterochromatin foci enriched in H3K9me3 and HP1α (Meshorer, Yellajoshula et al. 2006). When the behaviour of architectural chromatin proteins was investigated by FRAP in ES cells, it was observed that most core histones, linker histone H1 and HP1α in heterochromatin exhibit a highly dynamic and loose binding to chromatin. This behavior was attributed to pluripotency as it was not observed in differentiated cells which underwent lineage commitment. The authors confirmed the decreased binding of H3-GFP and H2B-GFP in biochemical essays and argue that loose association of core histones to chromatin contributes to the maintenance of pluripotency of ES cells. Recently, it was shown that H2A and H2A.Z mobility decreases upon ES cell differentiation (Subramanian, Mazumder et al. 2013), and that H2A.Z dynamics are at least partly controlled by its acidic patch. ES cells in which the linker H1 histone was tightly associated with chromatin failed to differentiate, suggesting the importance of dynamic exchange of linker histone in ES cell differentiation and lineage commitment. Interestingly, the histone variant H3.3 exhibited low mobility in both ES cells and neural progenitor cells (NPC) after differentiation. This finding was quite unexpected as H3.3 has long been associated with promoters of active genes and known to disrupt the stability of the nucleosome core particle (Jin and Felsenfeld 2007). A potential explanation for this observation is that H3.3 is indeed present on active genes, where it needs to be retained to mark their activity. This is indeed demonstrated by genome-wide mapping of H3.3 in ESCs, which revealed that H3.3 is enriched along the body of transcribed genes (Goldberg, Banaszynski et al. 2010). However, the genes in question are different in distinct cell types, such as ES cells and NPC cells.

Studying chromatin dynamics also entails investigating overall physical movements of chromatin fibres. Studies in budding yeast by the lab of Susan Gasser and others have

shown that chromatin fibres are constantly moving in the nucleus, and not only due to the temperature-dependent random Brownian motion (Dion and Gasser 2013). In fact, various factors, including ATP levels, protein-protein interactions and nucleoplasmic content affect chromatin fiber 'walking'. Interestingly, chromatin remodeling complexes play an important role in chromatin fiber dynamics. It was recently shown that homology search upon double-stand break repair increases the movements of chromatin fibers and this increase depends on the Ino80 remodeling complex (Seeber, Dion et al. 2013). Therefore, when investigating overall chromatin dynamics, one should take into account local association of proteins to chromatin, but also the three-dimensional displacements of chromatin structures themselves. With the advance of optical techniques, such as 3D-FRAP, accurate measurements of overall chromatin dynamics will be possible. Indeed, 3D-FRAP provides a unique possibility to measure not only lateral diffusion, but also recovery of fluorescence within a 3D volume (Braeckmans, Peeters et al. 2003).

3. Modulating chromatin organization and function

Chromatin structure is by no means static and 'locked' – it is constantly challenged by cellular events, such as passage of the replication fork or the transcriptional machinery as well as the result of DNA damage and repair. Indeed, ever growing body of evidence suggests that structural uniformity is not a predominant feature of chromatin. To ensure that chromatin retains its correct state but also the ability to change upon stimuli, several mechanisms have evolved. Chromatin structure is modulated by three main players, which are discussed below. Of note, the role of non-coding RNAs in chromatin regulation through recruitment of histone methyltransferases (Volpe, Kidner et al. 2002), HP1α (Maison, Bailly et al. 2011) or chromatin condensation (Verdel, Jia et al. 2004), (Moazed 2009) is rapidly emerging. Prominent examples of long non-coding RNAs important for facultative heterochromatin are Xist and its antisense counterpart Tsix, which directly influence transcriptional silencing of one of

the X-chromosomes in female cells (Avner and Heard 2001; da Rocha, Boeva et al. 2014).

a. Chromatin remodeling complexes

Chromatin remodelers use the energy of ATP hydrolysis to slide, destabilize or evict nucleosomes and thus change the local chromatin organization rendering it more or less accessible. It should be noted, however, that nucleosomes can also undergo spontaneous sliding, splitting and dissociation, without the need of remodelers (Miyagi, Ando et al. 2011). Although chromatin remodelers are divided into four families and differ in biological output and targeting mechanism, all of them use energy to introduce changes into chromatin (Figure 6). Furthermore, all remodelers contain a regulated DNA-dependent ATPase domain, recognize histone modifications (discussed in the next subsection) and prefer nucleosomes to DNA itself as a substrate. The 4 known families of chromatin remodelers are: SWI/SNF, ISWI, CHD and INO80 (Clapier and Cairns 2009). Some families, such as SWI/SNF, promote disruption and ejection of nucleosomes, while others, exemplified by the ISWI family, reassemble and organize chromatin (Clapier and Cairns 2009). Within individual families certain remodelers promote chromatin compaction while others loosen up the tight nucleosomal packaging. For instance, ISWI family members ACF and CHRAC promote chromatin assembly and repress transcription (Whitehouse and Tsukiyama 2006), while NURF assists RNAPII activation through randomizing nucleosomal spacing. These findings underscore the importance of domains other then the catalytic one as well as of proteins associated to the remodeler in mediating diverse functions on the chromatin. Often it is the non-catalytic domains, such as bromodomains or PHD domains which target or anchor the remodeling complexes to a particular locus. Many chromatin remodelers have particular functions. SWR1, a member of the INO80 remodelers, is unique in its ability to restructure the nucleosome by incorporating the H2A variant H2A.Z (discussed below) (Kobor, Venkatasubrahmanyam et al. 2004). Furthermore, during *Drosophila*

development, *Hox* gene expression is maintained by the action of NURF and its association with H3K4 trimethylation (Badenhorst, Voas et al. 2002). On the other hand, Mi2, a member of the CHD family of remodelers, is required for achieving the Polycomb-based repression of *Hox* genes (Kehle, Beuchle et al. 1998).

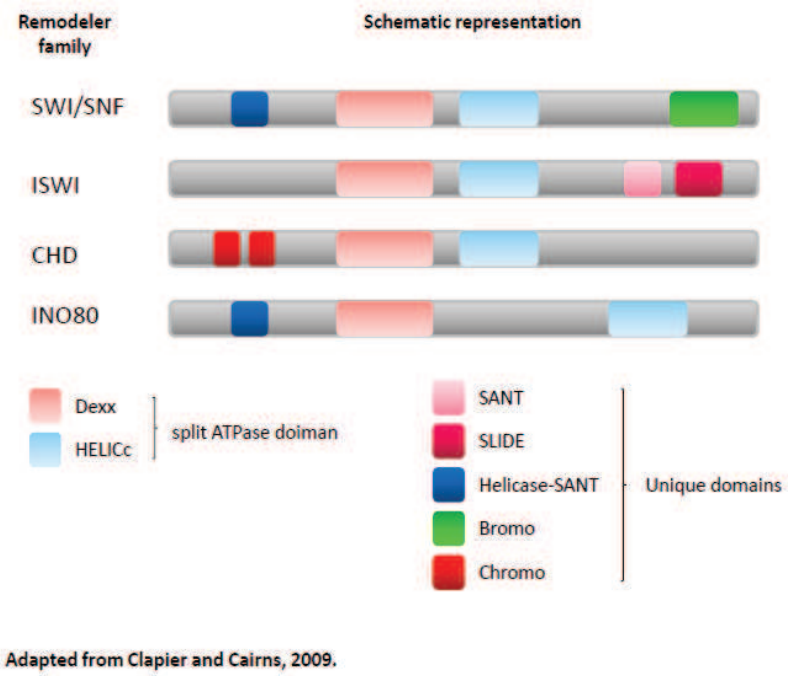

Figure 6. Schematic representation of 4 families of chromatin remodelers. Chromatin remodelers contain the conserved split ATPase domain and various specific domains. Members of INO80 families are characterized by an extended linker region between the Dexx and HELICc domains. Different remodeler families are associated with unique domains contributing to their specificity and function.

It is important to note that complex chromatin processes such as chromosome segregation and DNA repair, often require the concerted action of several distinct remodeling complexes. The vastness of cellular processes that require chromatin remodeling makes it logical for so many different types of remodelers and associated proteins to evolve. It is then not surprising that disruption or aberrant function of chromatin remodeling complexes can lead to a number of diseases, including cancer.

b. Post-translational modifications of histones

Histones and chromatin-associated proteins are subject to posttranslational modifications (PTMs). These modifications range from covalent attachment of simple chemical groups, such as the methyl group, to addition of big globular proteins such as ubiquitin or SUMO. There are more than 10 types of different histone PTMs, which are outlined in Table 2. While many types, such as phosphorylation and methylation have been known for a long time, with the development of sensitive proteomics methods many more histone PTMs are coming to light, with their functions still unknown. It is becoming increasingly clear that the number of PTMs as well as their target sites on histones have been greatly underestimated (Tan, Luo et al. 2011).

Table 2. Known histone PTMs and their functions. There are 13 different types of posttranslational modifications, and some of them can occur on the same residue or exist in several forms (e.g. mono-, di- and tri-methylation). Modified from (Kouzarides 2007).

PTM	Residues modified (+ annotation)	Ascribed Functions
Methylation (lysines)	**K**-me1, me2, me3	Transcription (+ and -), Repair
Methylation (arginines)	**R**–me1, me2a, me2s	Transcription
Acetylation	**K**-ac	Transcription (+), Repair, Compaction
Crotonylation	**K**-cr	Transcription (+), Compaction
Phosphorylation	**S**-ph, **T**-ph	Transcription, Repair, Compaction
ADP-ribosylation	**E**-ar	Transcription, Repair
Ubiquitylation	**K**-ub	Transcription (+ and -)
SUMOylation	**K**-su	Transcription (-)
Deimination	**R>Cit**	Transcription
Proline isomerization	**P**-cis > **P**-trans	Transcription
Formylation	**K**-fo	Unknown
Hydroxylation	**Y**-oh	Unknown
β-N-acetylglucosaminisation	**S**-O-GlcNAc, **T**-O-GlcNAc	Compaction

Histone PTMs can act in 2 distinct ways. First, they can directly affect chromatin structure by altering the charge of a residue, and therefore the affinity of histones to DNA or impair internucleosomal contacts. The best studied case for this is acetylation of H4K16. It was shown that H4K16ac disrupts higher order chromatin structure by neutralizing the positive charge of the lysine and thereby changing the affinity of the H4 tail to the acidic patch of the neighboring nucleosome (Dorigo, Schalch et al. 2003; Shogren-Knaak, Ishii et al. 2006). Not surprisingly, H4K16ac was found incompatible with formation of highly condensed mitotic chromosomes and this modification is removed from chromatin during G2/M phase of the cell cycle. More generally, histone hyperacetylation is associated with regions of open chromatin which are transcriptionally active. Another example is H3K122 acetylation. Lysine 122 on histone H3 is located within the histone fold domain, on the lateral surface of the histone octamer. The authors have shown that the H3K122 acetylation is sufficient for transcriptional stimulation, and they attribute this to direct effects of H3K122ac on weakening histone-DNA binding (Tropberger, Pott et al. 2013).

Secondly, histone modifications can act indirectly, by creating a docking site(s) for effector proteins which contain specific domains or modules that recognize modification or their combinations. For instance, bromodomain-containing proteins recognize acetylated histones, while PHD fingers and chromodomains dock onto methylated sites (Yun, Wu et al. 2011). These proteins are often enzymes and can then recruit other proteins, change the structure of chromatin or help maintain it in its original state.

A prominent example of such action is H3K9me3 (Figure 7). H3K9 trimethylation is catalysed by SUV3-9 histone methyltransferases. Once this modification is set, it is recognized by HP1α through its chromodomain. HP1a also contains a chromo-shadow domain, through which it can associate with SUV3-9 to promote more H3K9me3, but also with the SUV4-20 HMT, which catalyses H4K20me2/me3. This leads to signal amplification and the expansion of heterochromatic domains (Grewal and Elgin 2002).

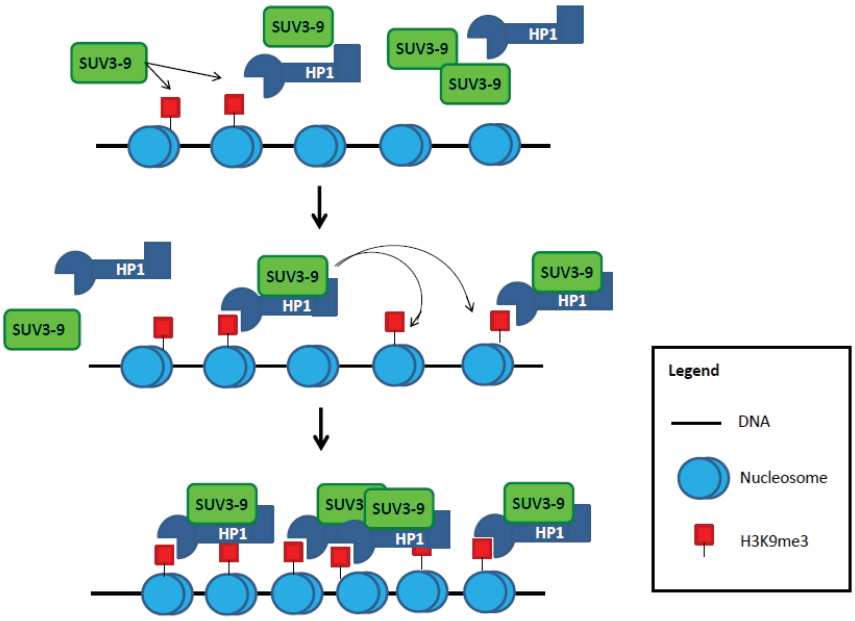

Figure 7. Deposition and spreading of H3K9me3 on chromatin. Suv3-9 represents the HMT responsible for H3K9me3 deposition. H3K9me3 is recognized by the chromodomain of HP1 proteins. HP1s can interact with Suv3-9 through their chromoshadow domain and recruit it to pre-existing H3K9me3. This feedback loop allows for spreading of H3K9me3 and expansion of heterochromatic domains.

Many, but not all, of the PTMs occur on the unstructured tails of histones H3, H4 and H2A, which protrude from the nucleosomes (Figure 8). This is particularly interesting since the tails of neighbouring nucleosomes can interact with each other and help organize chromatin into higher-order structures. Thus, changing the properties of histone tails can have profound effects on overall chromatin structure. However, new histone modifications located close to the positions of the nucleosome that are in direct contact with the DNA are being uncovered and their impact on nucleosome stability is a subject of intense research. Because these residues have the ability to form physical contacts with the DNA backbone, they can potentially directly regulate nucleosomal stability.

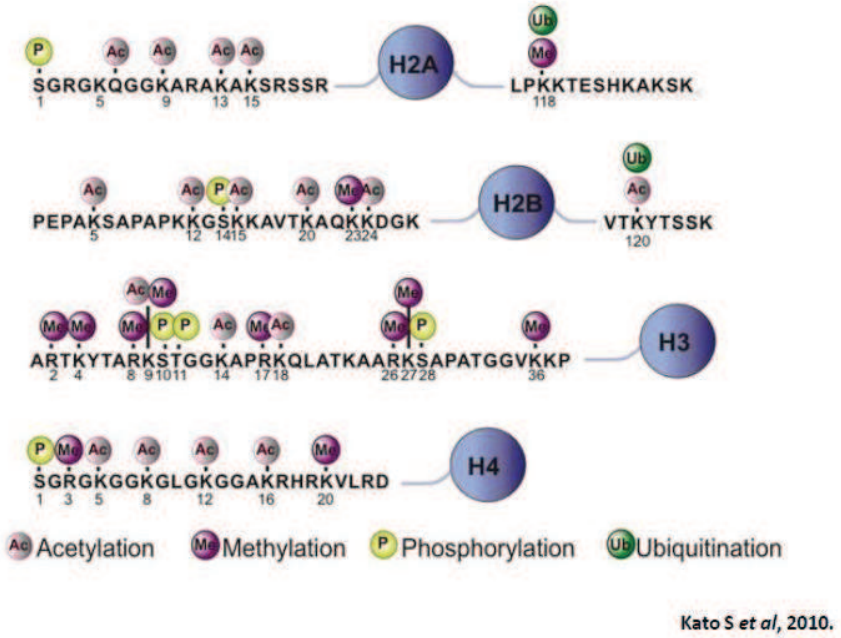

Figure 8. Most characterized PTMs of core histones. Globular histone fold domain is represented by the round shape, while N- and C-terminal aminoacids are annotated. Most histone PTMs occur on the N-terminal tail, and fewer on C-terminal part and in the globular domain (not shown).

Addition of small chemical groups

Acetylation

Histone acetylation entails the addition of an acetyl-group to specific lysines on histones. Histone tails, which are lysine-rich, are often acetylated at several neighboring residues and their hyperacerylation contributes to the overall modulation of DNA-histone interactions.

The enzymes catalyzing histone acetylation are called histone acetyltransferases (HATs) while the reversal of this PTM is achieved by histone deacetylatese (HDACs). Both types of enzymes are often associated with multiprotein complexes, such as the transcriptional coactivator SAGA, which contains the HAT GCN5 (Timmers and Tora 2005).

Almost exclusively, histone acetylation is linked to active transcription and chromatin 'openness'. Due to the net increase in negative charge, histone acetylation weakens histone-DNA electrostatic interactions and promotes looser chromatin structure. Histone acetylation is enriched at active promoters and euchromatic regions in general. In fact, histone H4 lysine 16 acetylation is one of the few histone marks shown to directly antagonize higher order chromatin folding (Shogren-Knaak, Ishii et al. 2006). Interestingly, newly synthesized histones are hyperacetylated before their incorporation, but also during the transition from nucleosomal to protamine packaging of the sperm genome (Pivot-Pajot, Caron et al. 2003). Like methylation, histone acetylation can also act indirectly, by creating docking sites for effector proteins containing acetyl-recognizing folds called bromodomains (Jacobson, Ladurner et al. 2000).

Phosphorylation

Phosphorylation is the chemical addition of a negatively charged phosphate group to serine/threonine/tyrosine aminoacid residues. This modification is catalysed by protein kinases and is readily reversible through the action of phosphatases. Phosphorylation is one of the most studied protein PTMsin cells, and it can regulate protein function and localization very fast. On histones, 2 prominent examples of phosphorylation have been described. Firstly, serine 10 on histone H3 is known to be phosphoylated in mitosis by aurora B kinase (Johansen and Johansen 2006). This PTM promotes chromatin condensation but also antagonizes the neighboring H3K9 trimethylation, in a process termed phospho-methyl switch (Fischle, Tseng et al. 2005). A similar situation occurs between H3T3 phosphorylation and H3K4me3. It was shown that binding of TFIID to H3K4me3 is weakened in the presence of phosphorylated H3T3 during mitosis, concomitant with mitotic inhibition of transcription (Varier, Outchkourov et al. 2010).

Secondly, the histone variant H2A.X is known to be phosphorylated on its C-terminus within the aminoacid motif SQEY in response to DNA damage. Phosphorylated H2A.X, then called γH2A.Z is one of the first markers of DNA damage and serves as a signal and docking platform for repair enzymes, including Rad51 and Brca2 (Kang, Ferguson

et al. 2005). Furthermore, γH2A.X also recruits enzymes responsible for its own phosphorylation (ATM and ATR) which leads to the rapid amplification of the damage signal up to 1 megabase around a single double-stranded break and to an increased efficiency of DNA-damage repair.

The curious case of methylation

The covalent attachment of a methyl group to histone tails is a very interesting example of the fine-tuning of chromatin states as well as the importance of the context in which PTMs occur. Methylation can occur on lysine and arginine residues on histones, predominantly on H3 and H4. The complexity of this seemingly simple modification comes partly from the fact that lysines can be –mono, -di or –trimethylated, while arginines can be –mono and –dimethylated (symmetrically or asymmetrically). The enzymes responsible for setting methylation marks are termed histone methyltransferes (HMT). The catalytic domain of most HMTs, the SET domain, is highly conserved in eukaryotes. Many HMTs have been extensively characterized, including members of the Polycomb and Trithorax complexes, regulating methylation states of developmental genes, as well as *suppression of variegation* (SuVAR) HMTs important for silencing of constitutive heterochromatin (Elgin and Grewal 2003)

The mechanisms of methylation reversal were elusive for a long time, and it was thought that methylation was a very stable (if not irreversible) PTM. However, several histone demethylases have recently been identified (reviewed in (Shi and Tsukada 2013)), indicating that histone methylation can be enzymatically reversed. It is also possible that the combined action of enzymatic demethylation and histone exchange regulates methylation states at a given genomic region.

Interestingly, methylation can function both in transcriptional activation as well as heterochromatin formation, depending of the modified residue as well as effector proteins recognizing the modification. H3K4me3, H3K36me3 and H3K79me3 are modifications associated with open and transcriptionally active regions of the genome.

On the contrary, H3K9me3, H4K20me3 and H3K27me3 are most often found in heterochromatin. Thus, methylating histone tails provides a myriad of combinatorial possibilities to precisely regulate the chromatin status of a given region. While the presence of an active mark at a promoter usually excludes the presence of a repressive PTM, this is not always the case. Bivalent promoters, found at many developmentally regulated genes in ES cells, contain both H3K4me3 as well as H3K27me3 (Bernstein, Mikkelsen et al. 2006). The presence of histone PTMs with opposing roles on a single promoter is probably important for maintenance of pluripotency in ES cells, allowing for low-levels of transcription but also the competence of a promoter to become fully active or silent upon differentiation cues. During differentiation, transcription of a bivalent gene can thus be strongly activated by removal of H3K27me3, or completely silenced by demethylation of H3K4me3.

Addition of globular proteins

Ubiquitylation

Ubiquitin and SUMO are large (~7 kDa) globular proteins which can be enzymatically attached to histone tails.

Two ubiquitylation events with opposing roles have been mostly investigated. Ubiquitylation of histone H2B on lysine 120 in metazoans is present on almost 5% of H2B molecules in the nucleus, and is a mark of active transcription. This mark, catalyzed by the Bre1-Rad6 E2-E3 ubiquitin ligases(Jentsch, McGrath et al. 1987; Hwang, Venkatasubrahmanyam et al. 2003), is transient in nature, and its removal is achieved through the action of the histone-acetyltransferase deubiquitylation (DUB) module of SAGA. H2BK120ub functions to facilitate the smooth passage of the transcription machinery, in two separate ways. Firstly, H2BK120ub promotes the formation of H3K4me3 and H3K79me3, both important marks of active transcription, possibly by creating a docking platform or ‘bridge’ for COMPASS and Dot1 HMT.

Secondly, it facilitates the work of the FACT remodeler in reestablishing chromatin structure after the passage of the RNAPII (Braun and Madhani 2012). How these distinct types of H2BK120ub action are achieved is still not fully understood. The possibility that the ubiquityl moiety (76 aminoacids) physically interacts with different HMTs and FACT provides an attractive explanation, which is yet to be experimentally confirmed.

Conversely, H2A monoubiquitylation on lysine 119 (in mammals) plays a role in chromatin compaction. H2A and its variant H2A.Z are monoubiquitylated by several E3 ligases, including the members of the Polycomb repressive complex 1 (PRC1), Ring1B and Ring1A. This histone PTM is located on many sites of facultative heterochromatin, including silenced developmental genes and the inactive X chromosome in females. As is the case with H2B, H2AK119ub is a transient mark, and it can be removed by the PR-DUB complex, making H2AK119ub a tightly regulated mark under the control of Polycomb group proteins (Osley 2006; Scheuermann, de Ayala Alonso et al. 2010). Monoubiquitylation of H2AK119 generally acts as a repressive mark. It was shown that it can cause RNAPII pausing whilst not affecting its initial recruitment to promoters (Stock, Giadrossi et al. 2007). Furthermore, H2AK119ub provides a recognition site for its catalyser, PRC1 complex and it can also recruit the PRC2 complex, which methylates H3K27 and helps to tighten the overall chromatin structure (Margueron, Justin et al. 2009).

Thus it seems that H2AK119ub and H2BK120ub function in a mechanistically similar way, providing docking sites for effector-proteins and changing chromatin accessibility, but with opposite biological outcomes.

c. Histone variants

Histone variants can replace canonical histones in chromatin and can assume different roles in the cell. They differ from their canonical counterparts in various ways. Their primary sequence can be very similar- as in the case of the replacement variant H3.3 compared to the canonical H3.1 and H3.2- or extremely divergent –as in

macroH2A compared to H2A- from the canonical histones (figure 9). Their genes are located outside of the histone clusters and often contain introns. Furthermore, they are synthesized and incorporated into chromatin throughout the cell cycle.

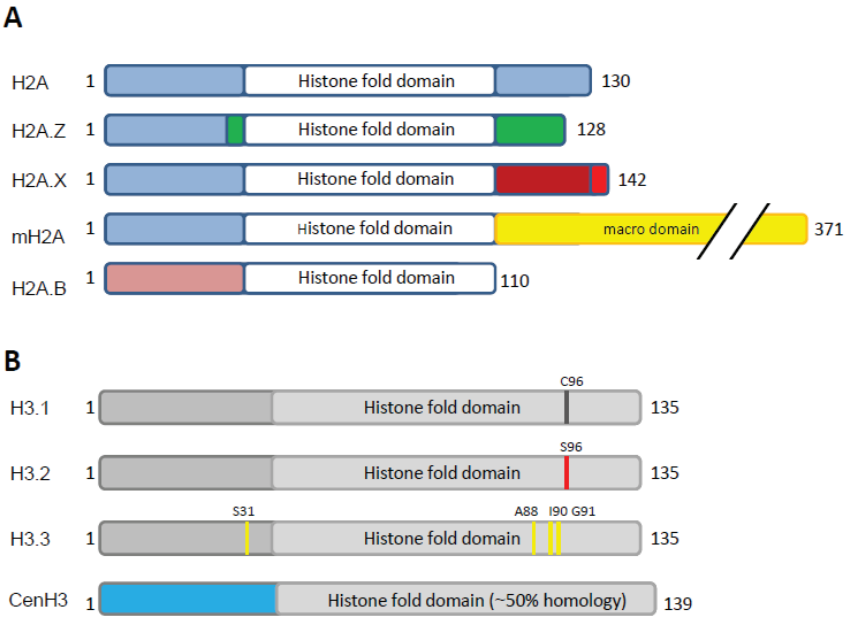

Figure 9. Schematic representation of different H2A and H3 variants. Upper panel (A) shows the 5 most studied somatic H2A variants. Regions of divergence between H2A variants are marked in different color compared to H2A. Lower panel (B) represents the main types of histone H3 variants in mammals. The aminoacids that differ in each of the H3 variants are annotated and numbered.

Considering their conservation, histone variants can be roughly divided into two subgroups. The highly conserved ones like H2A.Z and CenH3 have evolved to perform essential functions in cells (DNA damage response, heterochromatin boundaries, formation of the centromere) and cannot be replaced by their canonical counterparts. Others, like H2A.Lap1, tH2B (now (TS)H2B.1) and H2A.Bbd (now H2A.B) are evolving rapidly and are seemingly undergoing Darwinian selection. These histone variants are evolving quickly to fulfill specific roles in certain cells or tissues, and are often specific to the germline. Because of the diversity and the increasing number of histone variants identified to date in different organisms, a new unifying nomenclature has recently been proposed (Talbert, Ahmad et al. 2012), to which I will adhere throughout the text.
Because this constitutes a major interest of my thesis, histone variants and their roles in early mouse development are discussed at length in a separate chapter below.

4. Heritability of chromatin marks

In the post-genomic era, it became clear that the vastness of cellular processes and phenotypes cannot be explained only by the information encoded in the DNA. Concomintanly, a myriad of chromatin modifications, as well as their combination, were discovered and have become the focus of intense research in trying to undestand how different functional outputs come about from simple nucleotide sequences. Factors contributing to phenotypic changes, not caused by changes in DNA sequence, including histone modifications, regulatory RNAs, histone variants and nuclear localization, were referred to epigenetic ('above' genetic).

From its first definitions (proposed by Conrad Waddington), what is considered epigenetic has changed substantially. Currently, epigenetics is defined as the study of heritable changes in gene activity that are not caused by changes in DNA sequence. While modulation of chromatin structure can indeed result in altered gene expression and phenotypic changes, histone modifications are not always epigenetic in nature. Many histone PTMs are transient in their temporal character (such as histone acetylation necessary for their incorporation during S-phase, or H3K36me3 after the passage of the transcriptional machinery), and are thus not transmitted to the next generation. On the other hand, some genomic regions, such as pericentromeres, are stably silenced in a heritable manner. Indeed, even after replication and dilution of the original chromatin marks, positive feedback loops like that described above for H3K9me3 ensure that the information of transcriptional silencing of pericentromeres is transmitted to daughter cells.

Subnuclear localization of certain parts of the genome was also considered as epigenetic. A specialized form of chromatin domains are found at the nuclear lamina (NL), and are called lamina-associated domains (LADs). The Nuclear lamina provides the interface between the inner nuclear membrane, the nuclear pore complex and

nearby chromatin, and is usually a transcriptionally refractory region. LADs contact with NL is linked to transcriptional repression mediated through the G9a methyltransferase and H3K9me2. Strikingly, however, after cell division and on a single cell level, the positions of the LADs within the nucleus are not inherited but instead randomly rearranged. (Kind, Pagie et al. 2013)

Heritability of chromatin states can also be assessed on different timescales. It is clear that somatic cells retain and propagate their epigenetic states to daughter cells. However, in mammals, genome-wide erasure and re-setting of chromatin marks takes place in the germline and after fertilization. Thus, transgenerational epigenetic inheritance in mammals, especially through chromatin, is not well established.

II. Gametes, fertilization and mouse preimplantation development

Germ cells are arguably the most particular cell type in an adult organism. These cells have the potential to initiate and support a new developmental program. In mammals, fertilization of the oocyte by the sperm results in the creation of the zygote, whose subsequent divisions will give rise to an entire organism. Cellular and molecular events taking place during the formation of the germline and later on, during early development are therefore of crucial importance for the continuation of the species.

1. Oogenesis – general overview

Female gametogenesis, or oogenesis, is a very highly regulated process, starting from the primordial germ cells (PGCs) and ending in fully mature, developmentally competent oocytes after reprogramming and subsequent differentiation.

Mouse PGCs originate from the extraembryonic mesoderm at day 7.5 of embryogenesis and migrate to the genital ridges at day 10. Around day 13.5, the PGCs colonize the gonads. They then undergo mitotic proliferation with incomplete cytokinesis creating germ cell cysts or nests. These germ cells are now called oogonia. PGC formation and the regulation of gene expression in PGCs is influenced by various growth factors, including members of the TGFb family (BMP2, BMP4, BMP8b) (Sanchez and Smitz 2012). Further, specific transcription factors and chromatin-associated proteins such as BLIMP1 and PRDM14 are necessary for PGC specification (Ohinata, Payer et al. 2005; Yamaji, Seki et al. 2008), while OCT4 and NANOG are essential for their survival(Kehler, Tolkunova et al. 2004; Yamaguchi, Kurimoto et al. 2009).

Between the creation of germ nests and follicle formation, mitotic divisions stop and meiosis commences, giving rise to primary oocytes. The primary oocytes synthesize and accumulate large amounts of RNA and proteins important for their maturation and growth, as well as for early embryonic development after fertilization. The meiotic programme in the mouse is regulated by retinoic acid and Stra8 signaling. Primary oocytes arrest at prophase I of the first meiotic division, and around this time germ cell nests break down to initiate follicle formation. Oocytes become surrounded by pre-granulosa cells and give rise to primordial follicles. Oocytes that were not surrounded by somatic cells at this time undergo apoptosis and many are lost. In the mouse, this occurs immediately after birth. Importantly, primordial follicles contain the entire reservoir of female germ cells available during the reproductive life (Sanchez and Smitz 2012). This is in striking contrast with male gametogenesis which occurs almost continuously during the life of a male (Figure 10).

Primordial follicles become active in cohorts to initiate folliculogenesis. The combinatorial effect of different signaling pathways, including Tsc/mTORC1 and PTEN/PI3K and Anti-Mullerian hormone (AMH) control the resting versus activation of primordial follicles (Durlinger, Kramer et al. 1999; Adhikari and Liu 2010). The latter will subsequently give rise to primary follicles, which initiate expression of the maternal effect genes essential for embryonic development, and their expression continues to antral stages of gametogenesis. Once the follicles have reached the preantral stages, they become responsive to LH (luteinizing hormone) and FSH (follicle stimulating hormone). During follicular development, oocytes synthesize and secrete an accelular glycoprotein matrix called Zona pellucida (ZP). ZP is composed of 3 or 4 glycoproteins in mice and humans, respectively – ZP1-3(4). This egg coat plays a critical role during fertilization, mediating acrosomal reaction and sperm-egg recognition (Gupta, Bhandari et al. 2012).

It is important to mention that during all stages of folliculogenesis, the communication between the oocyte and granulosa cells surrounding is essential. This is exemplified by the need for several gap-junction proteins, such as connexin 37 and 43.

The lack of these proteins results in early folliculogenesis arrest and compromised meiotic competence of the oocyte (Norris, Freudzon et al. 2008).

Once antral development begins, granulosa cells differentiate into two compartments – mural cells and cumulus cells. This signals the oocyte to become meiotically competent. Signaling by LH and FSH induces ovulation – meiosis I is resumed followed by nuclear envelope dissociation (GVBD – germinal vesicle breakdown), and leading to the extrusion of the first polar body. Meiosis II commences immediately, but arrests at metaphase II, which will be resumed only upon fertilization (Figure 10 A). Therefore, mature oocytes only complete meiosis upon fertilization, and the second polar body is excluded concomitantly.

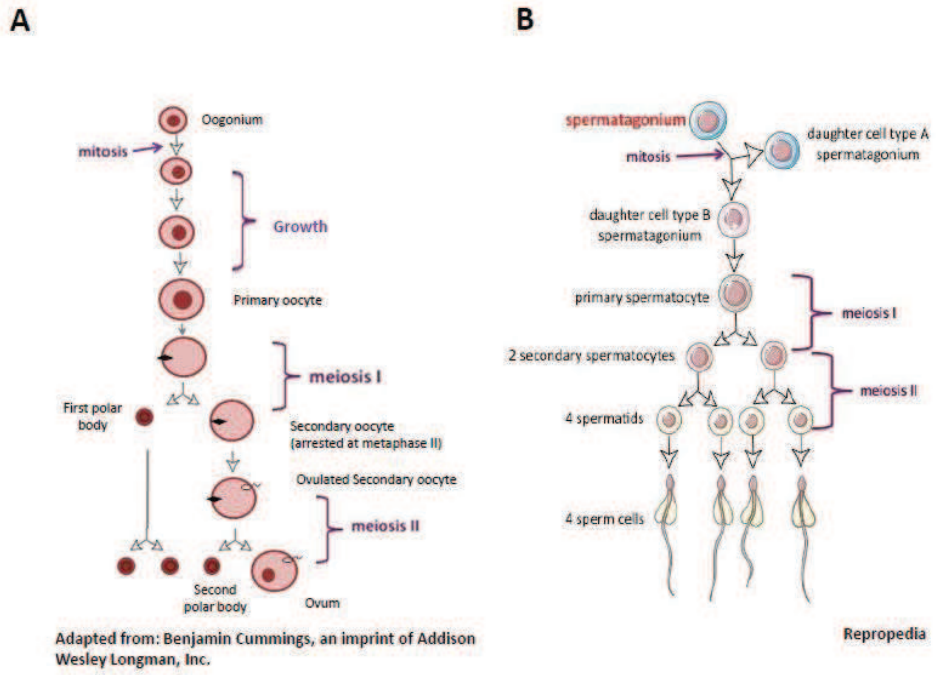

Figure 10. Schematic representation of meiotic events during female and male gametogenesis. Oogenesis (A) and spermatogenesis (B) start from diploid stem cells which will eventually give rise to mature germ cells. Primary spermatogonia and spermatocytes undergo two meiotic divisions and maturation process before haploid gametes are formed. (A) Oogenesis is only completed upon fertilization, when meiosis II is resumed and only one mature oocyte is created. (B) Spermatogenesis occurs continuously throughout the life of an animal and 4 haploid sperm cells are generated from each spermatocyte.

a. Regulation of gene expression during oogenesis

During maturation, oocytes expand both in size and volume. Oocytes synthesize and store numerous RNA and proteins necessary for their development and growth, as well as the ones needed to support the earliest stages of embryogenesis. Transcription is highest during the earliest stages of folliculogenesis which coincide with active proliferation of follicular cells, but ceases during antral stages. Oocytes are thought to contain about 6 ng of RNA, most of which are ribosomal RNAs, which is approximately 200 times more than the average somatic cell (Sternlicht and Schultz 1981).

Gene expression in the oocyte is regulated at various levels, and the fate of the transcripts generated varies. While most transcripts are polyadenylated, their regulation also depends on the conserved 5' and 3' UTRs, which play a role in translation initiation, RNA masking/sequestering or deadenylation and degradation. Indeed, some transcripts are synthesized for immediate translation, while others are stored in ribonuclear particles in the ooplasm where they associate with masking factors preventing their translation.

Between the transcriptional arrest and full maturity of the oocyte, there is a decrease in the overall RNA content of about 30% (Su, Sugiura et al. 2007). Furthermore, posttranscriptional and posttranslational regulation of RNAs and proteins, respectively, takes place during the antral stages of oogenesis. Indeed, gene expression is partly controled by endogenous siRNAs, miRNAs and piRNAs, which can silence mRNAs through different mechanisms. The importance of siRNA pathway is illustrated by the misregulation of a significant proportion of mRNAs during oocyte maturation in mice lacking the Dicer protein (Murchison, Stein et al. 2007).

2. Spermatogenesis

Spermatogenesis is a complex process that results in the generation of mature spermatozoa, which will exit the organism and fertilize the oocyte to start a new developmental program. Spermatogenesis takes place within the seminiferous tubules of the testis, which contain both germ cells and surrounding somatic cells (Sertoli cells).

Male PGCs migrate and proliferate, and eventually colonise genital ridges at E10.5. They remain quiescent until birth, when they start to proliferate again and form spermatogonia (or spermatogonial stem cells). Spermatogonia then mitotically divide and give rise to primary spermatocytes. The primary spermatocytes go through the first meiotic division and generate secondary spermatocytes. Secondary spermatocytes then divide once more during meiosis II to form haploid round spermatids which will in turn mature into fertilization-competent spermatozoa (Figure 10 B). Spermatogenesis can roughly be divided into 3 main phases – the mitotic proliferation phase, the meiotic and the postmeiotic phase (spermiogenesis) (Rathke, Baarends et al. 2014).

The proliferation phase includes cycles of self-renewal as well as lineage commitment of spermatogonial stem cells. This ensures that the pool of stem cells remains constant while retaining the ability to produce spermatozoa throughout adult life. Mitotic proliferation of spermatogonia is under tight control of paracrine signaling from the surrounding Sertoli cells, ensuring that the balance between self-renewal and commitment remains constant (Rossi and Dolci 2013). Thus, in males, generation of mature germ cells is a continuous process and occurs throughout the life of an individual.

During the meiotic phase, homologous chromosomes come together in pairs and form synapses enabling the exchange of genetic material in a process called meiotic recombination. Chromosomal regions which remain unpaired are transcriptionally silenced during this time, a process known as meiotic silencing of unpaired chromosomes (Kelly and Aramayo 2007). This process is important for preventing

erroneous crossing-over events which can trigger apoptosis. Sex chromosomes are particularly affected due to their lack of homology, and the inactivation on sex chromosomes entails several proteins such as BRCA1 and γH2A.X. Different chromatin players, including *de novo* DNA methylation, histone methylation/demethylation and even the RNAi pathway (Kota and Feil 2010) in different species were shown to play important roles during the meiotic stages of spermatogenesis.

Post-meiotic germ cells can be divided into round, elongated and maturing spermatids, and finally mature sperm cells. These cells undergo global morphological and cytological transformations, including changes in cell shape and size, the formation of acrosome, eviction of majority of cytoplasm as well as the development of a flagellum and gain of motility.

3. Chromatin organization during gametogenesis

a. Foliculogenesis

The maturation and acquisition of developmental competency in oocytes are strongly correlated with global changes in nuclear architecture and chromatin organization. From a chromatin perspective, two types of oocytes can be found in the antral compartment of the ovary: those with a non-surrounded nucleolus (NSN) with dispersed Hoechst staining, and those with a surrounded nucleolus (SN) characterized by the presence of a sharp rim-like chromatin around the nucleolus (Mattson and Albertini 1990). Initially, all oocytes in late dictyate are in the NSN configuration but the ratio of NSN versus SN oocytes changes with age. It was shown that the association of preovulatory oocytes with cumulus cells promotes the NSN to SN transition after gonadotropine stimulation. While both types of oocytes are capable of germinal vesicle breakdown, when matured and fertilized in vitro, only SN-derived embryos can develop to blastocyst, while NSN-embryos arrest at the 2-cell stage (Inoue, Nakajima et al. 2008; Zuccotti, Merico et al.

2012). NSN oocytes remain transcriptionally active, while SN oocytes exhibit global transcriptional repression. Interestingly, in SN oocytes, most of the centric and pericentric heterochromatin forms a ring around the nucleolar surface, presumably facilitated by the high homogeneity of heterochromatin composition and organization.

The chromatin composition also changes during oocyte maturation. Namely, histone H3.3 accumulates in the germinal vesicle, and is incorporated into maternal chromatin, but can be detected in the cytoplasm as well. Additionally, quantitative measurements of H3.3 protein levels in developing oocytes revealed that H3.3 becomes more abundant during oogenesis, contributing to ~30% of all H3 variants (unpublished). It has been suggested that H3.3 might also play a role in the reprogramming process of the primordial germ cells (PGCs), as inferred from the localization of HIRA in the PGCs during this process (Hajkova, Ancelin et al. 2008).

b. Spermiogenesis

From a chromatin perspective, the process of spermiogenesis in mammals entails very dramatic changes in DNA packaging, illustrated most clearly by the fact that most of the nucleosomal content of the maturing sperm is exchanged by protamines (Figure 11) (Gaucher, Reynoird et al. 2010).

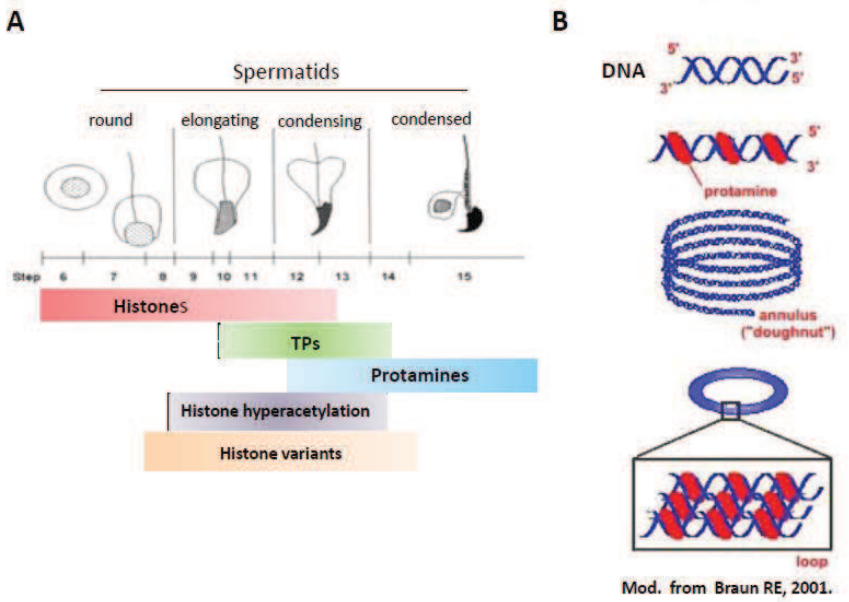

Figure 11. Changes in chromatin composition and genome packaging during spremiogenesis. (A) Sperm genome is packaged mostly by protamines. In elongating spermatids, histones get extensively acetylated and various histone variants become incoprorated, leading to the overall destabilization of nucleosomes. Transition proteins (TPs) are expressed during this time and replace most of the histones. TPs are eventually displaced from sperm DNA by protamines. The figure is adapted from (Gaucher, Reynoird et al. 2010). (B) Histone-to-protamine transition in genome packaging ensures high condensation of sperm genome, in the toroidal ring structure, necessary for sperm maturation and fertilization competence.

Actually, mouse and human sperm retain only about 1 and 10% of histones, respectively (Wykes and Krawetz 2003; Hammoud, Nix et al. 2009; Erkek, Hisano et al. 2013)). This ensures the tight condensation of the sperm DNA, necessary for fertilization competence. Furthermore, the complement of histone variants present in maturing spermatids is unparalleled in any other cell type. The incredible variety of chromatin components and modifications, as well as plasticity of spermatids to accommodate changes in DNA organization is detailed in Publication 2.

The intricate details and complexity of gametogenesis should not come as a surprise, as ensuring unperturbed and timely formation of mature germ cells is essential for the propagation of genetic material through generations and for the preservation of the species. Undoubtedly, future investigations into the molecular mechanisms of male and female gametogenesis will deepen our understanding of fertility and reproduction.

4. Mouse preimplantation development

a. Fertilization and formation of the zygote

Mammalian development starts at fertilization, a process during which male and female gametes (spermatozoon and oocyte, respectively) fuse to form a zygote. The zygote is a totipotent cell from which all other cells of the new organism will originate. Fertilization is a highly regulated process neccesary for the continuation of the species. In mammals, it takes place in the oviduct of the female. At ovulation, mature oocytes are surrounded by cummulus cells and are arrested at metaphase II of meiosis II. Oocytes are picked up by oviductal fimbriae and transferred to the oviduct, specifically in the swollen ampulla region of the oviduct, where fertilization will take place. Conversely, upon leaving the male reproductive tract, spermatozoa are not yet competent to fertilize the oocyte, but have to go through a process of capacitation and acrosome reaction. Numerous factors, including glucose, bicarbonates and intracellular calcium levels were implicated in regulating capacitation (Okabe 2013). After capacitation, spermatozoa undergo hyperactivation and vigorously move through the female reproductive tract to fertilize the oocyte. The final step before fertilization is the acrosome reaction. The acrosome is an organelle at the apical tip of the sperm head that contains various lytic enzymes and zona-binding proteins. The exocytosis of acrosomal contents is thought to enable the sperm penetration of the zona pellucida. However, the exact timing and mechanism that induce the acrosomal reaction are still unclear. In vitro experiments have indicated that most of the fertilizing sperm are undergo acrosome reaction prior to reaching the zona pellucida (Jin, Fujiwara et al. 2011) and that they retain fertilizing ability for a time period after the acrosome reaction (Kuzan, Fleming et al. 1984).

At fusion, the spermatozoon activates the egg, inducing Ca^{2+} oscilations and completion of meiosis II, which results in the extrusion of the second polar body (Miyazaki and Ito 2006). The activation of the egg leads to the zona reaction – a process of exocitosis of

peripheral cortical granules containing enzymes (namely, ovastacin) which cleaves ZP2 protein and decreases the zona afinity to spermatozoa after fertilization (Burkart, Xiong et al. 2012). The zona reaction is important for the prevention of polyspermy. Proteins such as CD9, fertilin and Izumo1 were shown to be important for the process of egg-sperm fusion (Okabe 2013).

The formation of a zygote initiates a new developmental program and represents the culmination of cellular potency, as all other cells will be generated by subsequent divisions of the zygote. The early developmental stages are characterised by rapid cell divisions without an increase in cell volume, termed cleavages (Figure 12 A). In fact, preimplantation embryos mostly cycle between rounds of DNA synthesis and mitosis, with quite short gap phases (Martin, Beaujean et al. 2006).

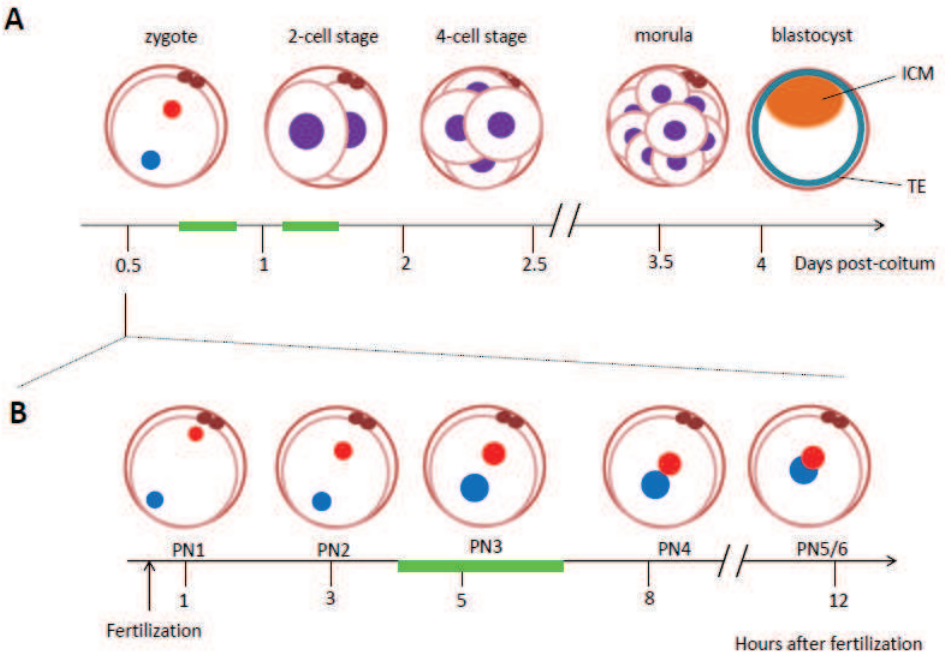

Figure 12. The stages of mouse preimplantation development. (A) Schematic represenation of the stages of mouse preimplantation embryogenesis. Male and female pronuclei in the zygote are represented by blue and red circles, respectively. Green rectangle symbolises the S-phase. This figure is adapted from (Martin, Beaujean et al. 2006). (B) Schematic represenation of pronuclear stages in the mouse zygotes. Positions of male (blue) and female (red) pronuclei relative to each other at different PN stages are shown. Timing of different pronuclear stages (relative to fertilization) is indicated. Green rectangle depicts S-phase, as above. Adapted from (Adenot, Mercier et al. 1997).

After gamete fusion, chromosomes from both parents remain physically separated during the first cell cycle, and form so-called female and male pronuclei (PN). The male and female PN can be cytologically distinguished by their size (the male PN is bigger than the female one) and they acquire (or retain) differential chromatin modifications. The zygotic stage can be divided into 6 pronuclear stages, according to the position of the PNs relative to each other in the cytosol (Adenot, Mercier et al. 1997) (figure 12 B). Despite remaining separated, both pronuclei undergo decondensation and extensive chromatin remodelling, with perhaps the male PN being more prominently affected. Upon zygote formation the parental genomes undergo global epigenetic reprogramming, comprising rapid and dramatic changes in chromatin structure and organization. These changes are believed to be a pre-requisite for the acquisition of totipotency. The chromosomes originating from different parents come together only before the 1st cleavage on the metaphase plate, in a process called syngamy. While genetic material from the mother and the father are then mixed and distributed equally between the two blastomere nuclei at the 2-cell stage, they can still be distinguished by their chromatin marks, as discussed below.

b. 2-cell and 4-cell stage – start of embryonic transcription

After the first cell division, a 2-cell stage embryo is formed, with blastomeres positioning themselves along the longer diameter of the zona pellucida, most likely to ensure physical stability (Fujimori 2010). For various reasons, it is one of the most particular stages of early development. Both blastomeres at the 2-cell stage are strictly speaking totipotent (Tarkowski and Wroblewska 1967), and the cell cycle lenght is unusually long, about 24 hours (Lehtonen 1980). Given that important developmental processes take place during 2-cell stage, including global chromatin rearrangments (discussed in a separate chapter) and embryonic genome activation (EGA), this length is prehaps not surprising.

The 2-cell stage in the mouse represents a period of maternal-to-embryonic control of development. This means that most (>90%) of the maternally provided RNAs and protein, inherited from the oocyte, become degraded and the embryo starts robustly transcribing its own genome during EGA (Schultz 1993). Importantly, the function of EGA is not only to replenish degraded RNAs and proteins, but to reprogramme gene expression to generate novel transcripts which are normally not expressed by the oocyte (Latham, Solter et al. 1991). It should be noted that embryonic transcription occurs also in the zygote (with a bias towards the male pronucleus), but on a smaller scale (Bouniol-Baly, Nguyen et al. 1997), (Aoki, Worrad et al. 1997). However, it seems that transcription and translation might be uncoupled in the zygote. In other mammalian species, including humans, EGA occurs later, around 8-cell stage (Telford, Watson et al. 1990). A striking feature of EGA in the mouse is the switch in the requirement for TATA-box containing promoters. While about 70% of transcripts in the oocyte are derived from TATA-containing promoters, only about 25% 2-cell specific transcripts initiate from these promoters (Davis and Schultz 2000). Furthermore, efficient transcription at the 2-cell stage involves the requirement of enhancer elements. It was suggested that enhancer engagement at the 2-cell stage is necessary to aleviate the chromatin-mediated gene repression which occurs concomitantly with EGA (Wiekowski, Miranda et al. 1993). Interestingly, it was also found that inhibition of DNA replication at 2-cell stage affects expression of endogenous genes. The molecular mechanisms of how replication represses transcription are not clear, but it was proposed that the replication machinery could somehow displace productive transcription complexes formed on promoters (Aoki, Worrad et al. 1997).

It seems that EGA is a relatively opportunistic process arising from global chromatin remodeling. One could envisage that the requirement for the establishment of a repressive chromatin structure at this point would serve the purpose of minimizing the expression of repetitive elements. Indeed, about 15% of 2-cell transcripts are derived from retrotransposons (Ko, Kitchen et al. 2000; Ma, Svoboda et al. 2001; Fadloun, Le Gras et al. 2013). Interestingly, a study on global patterns of gene expression showed that, out of 217 genes analysed, 45% are subject to repression between the 2-cell and the 4-cell stage (Ma, Svoboda et al. 2001), supporting a role for the establishment of

chromatin structure as a barrier for productive transcription (Wolffe and Hayes 1999) at this time.

The 4-cell stage represents the shortest cell cycle during mouse preimplantation development (only about 11 hours), but is nonetheless an interesting developmental period. Unlike 2-cell stage, individual blastomeres of 4-cell stage embryos no longer have the ability to generate an entire embryo without the need of carrier cells (Tarkowski and Wroblewska 1967). Thus, the mouse embryo loses its totipotency sometime between 2-cell and 4-cell stage. Interestingly, depending on the division planes of the 2-cell stage blastomeres, 4-cell embryos can either form a tetrahedral structure or all blastomeres can be positioned on the same plane. It was shown that the orientation of the cleavage plane from the 2- to the 4-cell stage can be predictive of their fate and potency later in development (Piotrowska-Nitsche, Perea-Gomez et al. 2005). Thus, while generally assumed to be identical, differences between blastomeres of early embryos exist already at the 4-cell stage. Certain chromatin modifications, namely H3R (2,17,26)me2, were shown to be assymetrically distributed in 4-cell stage blastomeres (Torres-Padilla, Parfitt et al. 2007), a finding that will be discussed later in my thesis.

c. Later stages of preimplantation development: 8-cell stage, morula and blastocyst

One cleavage later, the 8-cell stage embryo is formed, which will undergo morphological changes, necessary for subsequent lineage allocation and blastocyst formation. Between the 4-cell and the 8-cell stage, more than 4000 genes increase in expression levels, in a process termed mid-preimplantation gene activation (MGA) (Hamatani, Carter et al. 2004). Interestingly, about 10% of those genes are responsible for the transition of 2/4-cell embryos to later stages of development. Furthermore, several genes involved in the formation of cellular junctions and in ion gradient establishment are expressed during MGA. These results suggest that MGA marks the activation of genes responsible for the morphological changes following 8-cell stage cleavage (Figure 13).

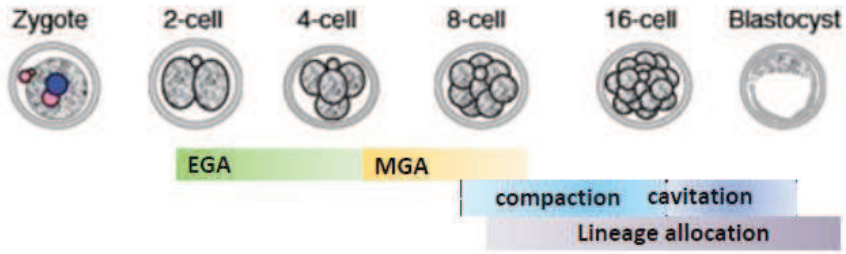

Figure 13. Characteristic molecular events during mouse preimplantation development. Mouse embryos start transcribing their genome at the 2-cell stage, in a process called embryonic genome activation (EGA). A second wave of embryonic transcription takes place from the 4-cell to 8-cell stage, termed mid-preimplantation gene activation (MGA). Later, two morphogenetic processes precede blastocyst formation. First, compaction occurs through creating cell-cell contacts through E-cadherin and adherens junctions. Secondly, caviatation takes place forming a central ion-filled cavity in the blastocyst, called blastocoel. During this time, first cell differentiation event during development occurs.

Two morphogenetic events, compaction and cavitation, precede blastocyst formation and create asymmetries in the embryo, which in turn influence cell fate decisions (Saiz and Plusa 2013). During compaction, 8-cell embryos develop intracellular junctions and apical-basal polarity (Johnson and Ziomek 1981) and individual blastomeres can no longer be clearly discerned, creating a morula. When the embryo reaches the size of approximately 30 cells, cavitation takes place and a blastocyst (comprising an ion-filled cavity called blastocoel) is formed. The early blastocyst consists of 2 cell types: trophectoderm (TE) and inner cell mass (ICM).

Compaction of the 8-cell stage embryo is Ca^{2+}-dependent and is mediated by the cytoskeletal changes and formation of E-cadherin-dependent adherens junctions (Ducibella and Anderson 1975), (Vestweber and Kemler 1984). Formation of tight junctions and desmosomes follows, and is necessary for the expansion of the blastocyst cavity. During this time, blastomeres develop apical polarity which later influences the cell position in the morula (Reeve and Ziomek 1981), (Vinot, Le et al. 2005).

In the mouse, the expansion of the blastocyst cavity during preimplantation development facilitates hatching from the zona pellucida. Also, this causes the expansion of the whole embryo, enlarging the surface area of the blastocyst, in turn increasing the efficiency of interaction with the uterine wall of the mother at implantation (Marikawa and Alarcon 2009)

Four essential steps lead to the formation and maintenance of the blastocoel:

1) Formation of the initial small cavity from (microlumen), originating from vacuoles formed in the outer cells and released by exocitosis (Motosugi, Bauer et al. 2005).

2) Generation of an osmotic gradient beween the cavity and the environment , established by differential Na^+ concentration (active transport of Na^+ into the microlumen through Na^+/H^+ transmembrane exhangers)(Barcroft, Moseley et al. 2004); (Kawagishi, Tahara et al. 2004)

3) Water influx into the cavity, driven by osmotic gradient and aquaporins

4) Paracellular sealing in the TE to ensure Na^+ retention and subsequent blastoyst expansion, through the formation of tight junctions beween TE cells.

Faliure at any of these steps compromises proper blastocyst formation and subsequent embryonic development.

The first cell fate decision that mouse blastomeres have to make during development is to become either extraembryonic TE cells or ICM cells, and is necessary to form a functional blastocyst capable of implantation.

In the second cell fate decision, the ICM cells choose between two different fates, and become either pluripotent epiblast (EPI) cells, which will give rise to the embryo proper, or extraembryonic primitive endoderm (PrE) cells. (Morris and Zernicka-Goetz, 2012). Thus, a mature blastocyst contains 2 extraembryonic and 1 embryonic lineages. The TE contributes to the fetal portion of the placenta, while PrE is essential for embryonic patterning and developmental circulation (Schrode et al 2013). Epiblast cells will

differentiate into all the tissues of the developing embryo, and are the source of pluripotent embryonic stem (ES) cells.

Different determinants, including cell position, signaling input and transcriptional regulation influence cell fate choices in the early mouse embryo. Pioneering studies from the 1960s by Tarkowski and colleagues showed that the relative positions of cells within the morula at least partly influence cell fate decisions (Tarkowski and Wroblewska 1967). It was later shown that the acquisition of cell polarity affects cell fate perhaps even before the emergence of inner and outer cells, and that signaling pathways involving aPKC during this time play an instructive role (Johnson and Ziomek 1981), (Plusa, Hadjantonakis et al. 2005). Importantly, the expression levels of lineage-specific transcription factors in inner and outer blastomeres at 8-16 cell stage embryo, and later on, are also different and important for lineage segregation to TE versus ICM, as well as EPI versus PrE (Guo, Huss et al. 2010). Indeed, expression of *Cdx2* in TE-precursor blastomeres suppresses expression of ICM-specific genes *Oct4* and *Nanog* (Niwa, Toyooka et al. 2005) Ralston and Rossant 2008). Furthermore, it was shown that the Hippo pathway, through the transcription factor TEAD4 and its coactivators, functions upstream of *Cdx2* restricting its activity to TE-precursor cells (Nishioka, Yamamoto et al. 2008; Ralston, Cox et al. 2010).

For the second lineage decision (EPI vs PrE), it was initially proposed that the relative position of the ICM to the blastocoels influences this cell fate choice (Enders, Given et al. 1978). This was later challenged by the observation of salt - and – pepper distribution of EPI and PrE precursors (Nanog or Gata6 positive nuclei, respectively) within the blastocyst (Chazaud, Yamanaka et al. 2006), which lead to a three-phase model (Plusa, Piliszek et al. 2008). Firstly, early ICM comprises double-positive (NANOG and GATA6) cells, after which one of the transcription factor is shut-down creating the salt-and-pepper distribution. Finally, cell movements lead to the final spatial segregation of PrE and epiblast lineages, at the stage of expanded blastocyst (see also (Schrode, Saiz et al. 2014).

5. In vitro systems for studying cell potency

a. Embryonic stem cells

ES cells are a paradigm for in vitro systems for investigating mechanism regulating pluripotency in vitro. They provide a powerful tool for studies of chromatin organization and transcriptional regulation of pluripotency outside of the embryo, and are suitable for biochemical screens due to their abundance and general ease of manipulation.

Mouse ES cells can be derived by microsurgery of the preimplantation epiblast (Evans and Kaufman 1981), (Martin 1981), (Brook and Gardner 1997), but not from post-implantation. However, post-implantation epiblast can also give rise to a specialized cell line called Epiblast stem cells (EpiSC). ES cells can be considered as the immortalization of the naïve epiblast (Nichols and Smith 2009) existing in the artificial environment of cell culture. Under appropriate conditions, ES cells retain some of the characteristics of the pluripotent epiblast – they can be maintained in culture virtually indefinitely, as they have the ability to self-renew but also upon injection to the blastocyst, they can differentiate into any cell type of the embryo. Other similarities between the epiblast and its in vitro counterparts exist: activation of pluripotency network and X-chromosome reactivation in both systems in female cells. Initially, successful derivation of ES cells from blastocysts was enhanced by culturing them in the presence of serum upon a feeder layer of mouse embryonic fibroblasts (MEFs), which produce and secrete cytokine Leukemia inhibitory factor (LIF). It was later shown that LIF activates STAT3, which in turn promotes ES cell viability and suppresses differentiation, in combination with either serum or (BMP). In fact, the suppression of phosphorylated Erk1 MAP kinase also suppresses the propensity of ES cells to differentiate (Silva, Nichols et al. 2009). This type of ES cell culture conditions (serum + LIF) is considered standard. In such conditions, the majority of ES cells in a population exist in the so-called ‘primed’ pluripotency, but some occasionally exit the self-renewal pathway and spontaneously differentiate.

ES cell self-renewal ability was for a long time thought to depend on various transcriptional networks, predominantly by the cytokine-mediated activation of STAT3 (Niwa, Burdon et al. 1998). Such views began to change with the report from Austin Smith and colleagues, indicating that by blocking MAPK (Erk1/2) signalling, the differentiation inputs that are mediated through this pathway are inhibited and ES cells retain their ability to self-renew even in the absence of external signaling (Ying, Wray et al. 2008). It was proposed that LIF and/or BMP signaling works downstream of the phosphor-ERK in blocking ES cell lineage commitment. Furthermore, the combined inhibition of MAPK and GSK signaling almost completely abolishes ES cell differentiation while supporting robust ES cell propagation. These culture conditions, named 2i or 3i (2 or 3 chemical inhibitors of differentiation pathways) promote ground state of pluripotency and self-renewal of ES cells in culture. Furthermore, ES cells established from *Stat3*-null blastocysts in the 3i condition express normal levels of pluripotency factors such as OCT4 and NANOG, and can differentiate into embryoid bodies with multiple cell lineages. This indicates that upon chemical inhibition of differentiation signals, STAT3 is dispensable for the generation and maintenance of ES cells.

Interestingly, when applied to the early embryo from the 8-cell stage onwards, blocking Erk signaling does not prevent normal blastocyst formation, but it suppresses PrE development (Nichols, Silva et al. 2009). The ICM retains its normal size and cell number, but all the cells are NANOG-positive and epigenetic silencing of the paternal X chromosome in female embryos is erased. These findings suggest that the entire ICM can acquire pluripotency and it can do so without the paracrine support of the PrE.

Recently, a new population of cells within ES and iPS cell colonies was identified, characterized by elevated expression of transcripts found in 2-cell stage embryos (Macfarlan, Gifford et al. 2012). These transiently and apparently stochastically arising cells, termed 2-cell-like (2-CL) cells, lack the expression of typical ICM markers at the protein level, such as OCT4, and have the ability to contribute to embryonic and extraembyonic tissues. Thus, 2-CL cells have totipotent-like features, similar to totipotent blastomeres of 2-cell stage embryos. Interestingly, one of the most elevated

transcript in both 2-cell stage embryos and 2-CL cells corresponds to the MuERV-L LTR retrovirus, which is about 300-fold activated in 2-CL cells compared to ES cells. It is possible to generate reporter ES cell lines for the identification of 2-CL cells, in which a fluorescent protein are under the control of the MuERV-L regulatory sequences. The study also showed that cells can fluctuate between the ES state and 2-CL state and that these transitions in cell potency are at least partially controlled by histone-modifying enzymes (such as Kdm1a, Kap1, G9a and histone deacetylases). 2-CL cells are thus a putative novel in vitro system for totipotency, and could provide a powerful tool for investigating mechanisms governing totipotency.

b. Inducted pluripotent stem cells – iPS cells

In vivo, fully differentiated cells are usually irreversibly committed to their lineage. However, experiments from the last 50 years revealed that even terminally differentiated cells can display remarkable plasticity. Different experimental techniques can be used to reprogram cell's fate into another cell identity. Somatic cell nuclear transfer to enucleated eggs, for instance, can induce reprogramming of donor nucleus to totipotency, while transcription factor overexpression can cause reprogramming to pluripotency. Acquisition of pluripotency through artificial reprogramming gives rise to induced pluripotent cells (iPS cells). The revival of the induced reprogramming to pluripotency started with the report by Takahashi and Yamanaka (Takahashi and Yamanaka 2006) in 2006 in which they established a simple protocol, consisting of overexpression of 4 transcription factors (*Oct4*, *Klf2*, *Sox2* and *cMyc* – OKSM), sufficient to generate iPSCs from fibroblasts. Indeed, this simple and straight-forward approach proved robust and functional for a variety of cell types for different organisms. Importantly, once reprogrammed, iPS cells are functionally indistinguishable from ES cells.

Reprogramming to pluripotency via the OKSM system entails several steps of action and failure at any of the intermediate stages leads to reprogramming arrest (for review, see (Papp and Plath 2013). The activation of pluripotency genes is by no means a

simple task and it includes profound changes in chromatin organization and composition, as well as DNA methylation. It is therefore not surprising that modulating levels of different chromatin players, such as histone methyltransferases or deacetylases, can influence the overall efficiency of iPSC derivation.

The ability to reprogram terminally differentiated cells by simply overexpressing 4 transcription factors reveals how thin a line separates different cellular states and stresses the underlying plasticity of cells. Nonetheless, the reprogramming process leading to the establishement of iPS cells remains relatively inefficient and slow, especially compared to the naturally occurring reprogramming to pluripotency, taking place in the early embryo. Molecular details of individual steps leading to the final acquisition of plutipotency in iPS cells are still being characterised (Cahan and Daley 2013; Papp and Plath 2013).

c. Pluripotency network

In vivo and in vitro, the acquisition and maintenance of pluripotency is regulated by a network of transcription factors and signaling pathways (Ng and Surani 2011). The core transcriptional regulatory network responsible for stem cell self-renewal and pluripotency is organized around 3 transcription factors: OCT4 – SOX2 – NANOG (reviewed in (Ng and Surani 2011; Festuccia, Osorno et al. 2013)). These TFs (and others, such as SMAD1) are downstream effectors of BMP, LIF and Wnt signaling pathways shown to be necessary for ES cell maintenance in culture, suggesting that extracellular signals communicate with the transcriptional network and their targets. Apart from regulating various transcription factors, OCT4 also regulates the chromatin landscape, microRNAs expression and non-coding RNA networks (Ng and Surani 2011). OCT4 is specifically required for cells allocated to the interior of the blastocyst to acquire pluripotent identitiy (Nichols, Zevnik et al. 1998), while SOX2 is required to prevent ES cell differentiation to trophectodermal lineages (Masui, Nakatake et al. 2007). Additionally, subsequent studies revealed that a number of pluripotency-associated TF can also regulate their own expression. This autoregulation presumably

ensures mouse ES cell homeostasis, keeping the levels of TFs in check. While OCT4 and SOX2 upregulation leads to increased differentiation, their downregulation in the embryo below basal levels causes extinction of pluripotency through chromatin condensation at key regulatory loci (Osorno, Tsakiridis et al. 2012). *Oct4* and *Nanog* genes are bound by 11 and 9 TFs, respectively, and are key integration points within the transcriptional regulartory network. The cohesin complex, known to hold two chromatin fibres together in mitosis, is also required for mouse ES cell pluripotency (Kagey, Newman et al. 2010), where it potentially stabilizes enhancer-promoter loops, together with the Mediator complex.

NANOG was shown to be essential for specification of pluripotent epiblast in vivo as well as needed for X-chromosome reactivation during the acquisition of ground-state pluripotency (Silva, Nichols et al. 2009) NANOG has the particular ability to modulate the activity of the pluripotency gene regulatory network and there is a strong correlation between *Nanog* expression and self-renewal capacity. Under standard culture conditions, NANOG levels (as well as STELLA and REX1) are heterogeneous between different cells, unlike OCT4 and SOX2 which are present at uniformly high levels. This heterogeneity can be reversed by altering culture conditions to inhibit Erk MAP-kinase, leading to the stabilization of ES cell self-renewal potential. Thus, NANOG might be the determining factor in discriminating between naïve and primed pluripotency.

III. Chromatin organization during reprogramming

After fertilization, global reogranisation of pericentric heterochromatin takes place. Centric and pericentric regions, containing minor and major satellite repeats, respectively, form ring-like structures around nucleolar precursor bodies (NPBs). The rest of the chromatin radiates away from the rings into the nucleoplasm, like a cart-wheel (Mason, Liu et al. 2012). This ring-like organization is still prominent at the early 2-cell stage. However, at the late 2-cell stage, concomitantly with EGA in mouse, centromeres and pericentromeres relocalise from NLBs to pro-chromocentres (Probst, Okamoto et al. 2010; Probst and Almouzni 2011). Remnants of heterochromatic ring structures can still be detected by the 8-cell stage, after nuclei start looking similar to somatic cell nuclei.

A number of events, including DNA demethylation, conversion of methylated DNA into hydroxymethylated DNA, eviction of histones and their differential modification, and RNA-regulated events have been implicated in epigenetic reprogramming during early development. The interplay between different epigenetic regulators is important in resetting of chromatin signatures in the oocyte and sperm and establishing chromatin domains *de novo* at the onset of development. In this context, incorporation of histone variants is now emerging as an additional layer of complexity during reprogramming events.

1. DNA-methylation during preimplantation development

In mammals, DNA methylation on promoters occurs predominantly in the CpG context and is generally associated with repression of a gene. Global erasure of DNA methylation at the onset of zygotic development is possibly the most studied change occurring during early reprogramming. Interestingly, one of the most remarkable features of DNA demethylation in the zygote is that it occurs differentially in both parental genomes: paternal DNA undergoes a rapid loss of methylation, while the maternal genome seems to be protected from demethylation (Figure 14). The global starting methylation level of both genomes is also different, with the sperm genome being significantly hypermethylated compared to the oocyte (Farthing, Ficz et al. 2008; Borgel, Guibert et al. 2010). Two distinct phases of paternal DNA demethylation – active and passive one – have been shown to contribute to the dynamics of DNA methylation (Wossidlo, Arand et al. 2010). Maternal DNA becomes demethylated apparently only passively, through replication. However, recent studies have revealed that the changes in DNA methylation in the early embryo are more dynamic and complex than originally thought (Borgel, Guibert et al. 2010; Smallwood, Tomizawa et al. 2011; Smith, Chan et al. 2012). Although the demethylation of paternal DNA is extensive, it is not complete and some regions (DMRs of imprinted genes, IAP elements and pericentromeric DNA) remain methylated to different extents.

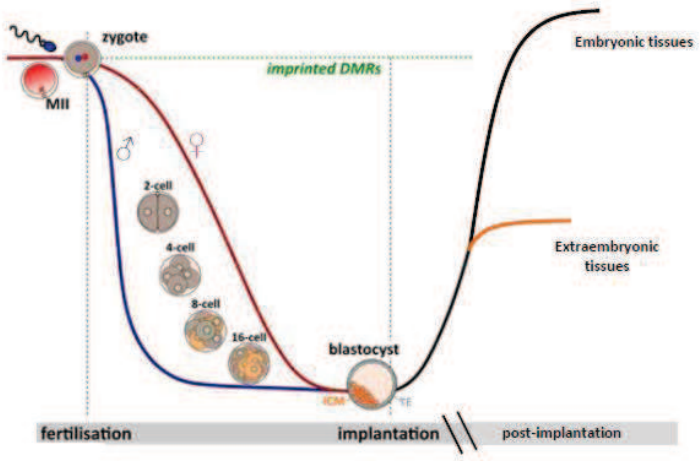

Figure 14. Levels of DNA-methylation after fertilization. Early developmental stages in mice are characterized by the sharp decrease in DNA-methylation levels, affecting both maternal and paternal DNA, and reaching lowest levels in the blastocyst. DNA-demethylation occurs genome-wide, but some regions of the genome (such as imprinted genes) retain their DNA-methylation. After implantation, DNA-methylation is re-established, particularly in embryonic tissues, and to some extent in extraembryonic tissues. Adapted from (Reik, Dean et al. 2001) and (Smallwood and Kelsey 2012).

Erasure of DNA methylation from the onset of development possibly facilitates chromatin dencondensation and transcription of genes necessary for developmental progression. Recently, it was shown that the loss of DNA methylation in the paternal pronucleus coincides with the accumulation of DNA hydroxymethylation, suggesting that what is observed as loss of DNA methylation is in fact the conversion of 5-methylcytosine into 5-hydroxymethyl-cytosine. The maternal genome, on the other hand, is largely protected from this conversion by H3K9me2-targeted accumulation of PGC7/Dppa3/Stella (Nakamura, Arai et al. 2007; Nakamura, Liu et al. 2012). These findings underline the importance of convergence between different epigenetic mechanisms during early reprogramming. Accumulating evidence is showing that sperm DNA is more methylated than the oocyte DNA, and presumably the rapid and global demethlylation of paternal DNA serves to equilibriate levels of DNA methylation between parental genomes prior to syngamy. Apart from specific loci (Borgel, Guibert et al. 2010; Smith, Chan et al. 2012), globally and during the following stages of preimplantation development, the DNA is passively demethylated until the morula stage,

when remethylation starts to occur globally. However, the significance of these changes or those of the conversion into hydroxymethylated DNA are not yet understood.

2. Histone posttranslational modifications at the onset of development

Apart from the so-called epigenetic asymmetry that results from the differences in global levels of DNA methylation between the two pronuclei in the zygote, the extent of histone methylation and acetylation also differs markedly between the two parental genomes. The maternal chromatin ‘inherits’ a myriad of histone modifications that accumulate during the phase of oocyte growth. As a result, the maternal chromatin is hypermethylated while the paternal one seems largely devoid of methylated histones. The paternal DNA is initially wrapped in hyperacetylated histones (Aoki, Worrad et al. 1997). This hyperacetylation has been suggested to promote decondensation of the forming male pronucleus, but it could also be the result of the genome-wide incorporation of histones into chromatin, which are pre-acetylated as a requisite for their incorporation (Kaufman 1996). A striking characteristic of zygotic chromatin (and generally chromatin of early mouse embryos) is the lack of marks of constitutive heterochromatin (namely H3K9me3 and H4K20me3). (Figure 15, top panel) Indeed, the male pronucleus does not contain almost any of the histone marks usually associated with constitutive heterochromatin, H3K9me3, H3K64me3 or H4K20me3.

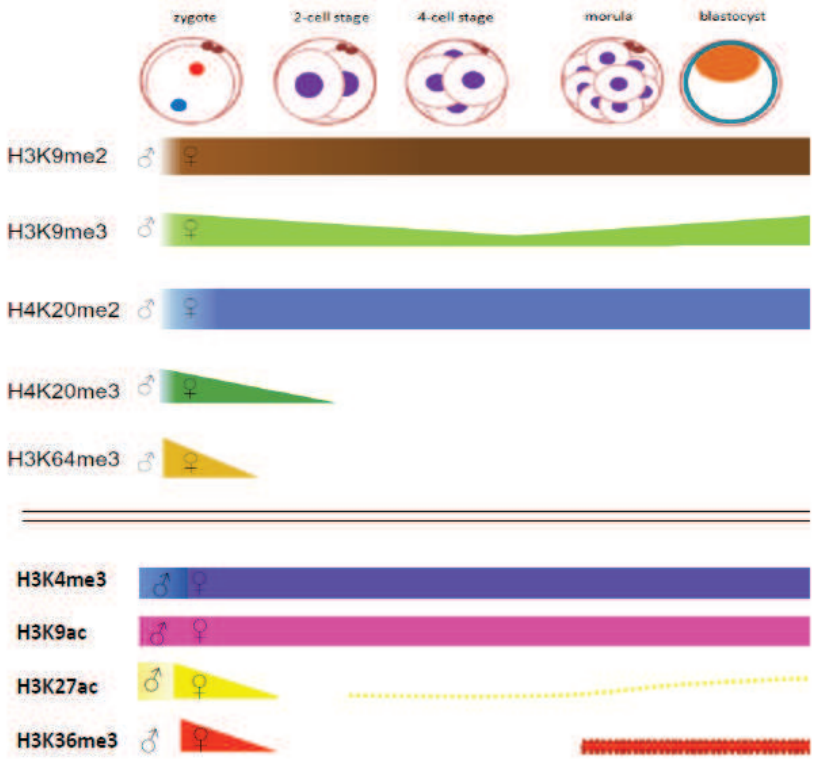

Figure 15. Global levels of histone modifications during murine preimplantation development. The bars depict the schematic representation of global PTM levels along development, as determined by immunofluorescence. In the zygote, differential accumulation of a histone PTM in the female (♀) or the male (♂) pronucleus is illustrated. Top panel: levels of histone PTMs present on constitutive heterochromatin (in somatic cells). Bottom panel: levels of histone PTMs associated with active transcription. The dotted line for H3K27ac indicates that endogenous levels have not been determined. This figure is adapted from (Fadloun, Eid et al. 2013).

Levels of H3K9me3 further decrease as development progresses, and the marks 'come back' only at late stages of preimplantation development, morula and blastocyst. Furthermore, the histone methyltransferases responsible for establishment of H3K9me3 (which is conventinally upstream of H4K20me3) show different expression levels in the zygote and later in development (Burton, Muller et al. 2013). Studies from our lab and others have implicated Polycomb group proteins and H3.3 lysine 27 methylation in the establishment of pericentric heterochromain in the zygote (Puschendorf, Terranova et al. 2008), (Santenard 2010). Trimethylation of H3 at lysine 27 is therefore one of the few heterochromatic marks on the male pronucleus, and during the earliest cleavage stages of development (Santos, Peters et al. 2005; Puschendorf, Terranova et al. 2008), but it

is only detected on the male chromatin concomitant with the first DNA replication phase in the zygote (Santos, Peters et al. 2005), (Santenard 2010). It thus seems that in the absence of somatic-cell constitutive heterochromatin, marks usually associated with facultative heterochromatin ensure chromatin organization and silencing. Furthermore, transcription from pericentric repeats also influences normal heterochromatinisation of pericentromeres, and recently, correct subnuclear position of major satellite repeats was shown to be important for normal hc silencing and development (Jachowicz, Santenard et al. 2013).

While most of the attention was focused on understanding the dynamics of heterochromatin marks at the onset of development, it should be mentioned that euchomatin regions are also excensively remodelled and affected (Figure 15, bottom panel).

3. The components of the chromatin change as development proceeds

(This part of the interoduction is included in a book chapter: Histone variants in early development)

Within this very dynamic context of changes in covalent modifications of the DNA and of histones, the chromatin is also subject to major changes in its histone composition. In mice, immediately after fertilization, the protamines carried in the sperm are exchanged for maternally provided histones. Although a fraction of sperm DNA remains packaged in nucleosomes (Wykes and Krawetz 2003; Govin, Escoffier et al. 2007; Hammoud, Nix et al. 2009; Brykczynska, Hisano et al. 2010), the extent of a potential paternal epigenetic contribution to the new organism, if any, remains to be determined. One of the major processes that start with fertilization is the protamine exchange and the subsequent genome wide assembly of the paternal genome into a nucleosomal chromatin configuration. This process is rather quick and occurs prior to replication and before any detectable transcription in the newly formed zygote.

One of the key questions that arises to understand how the reprogramming process is regulated is therefore how is the newly formed embryonic chromatin assembled and what are its main components? A way to start addressing this question is to determine which histones and/or histone variants become incorporated in the chromatin of the embryo after fertilzation and to establish the temporal dynamics of this assembly.

Due to their divergence and importance, histone variants are becoming increasingly investigated. They are of particular interest with regards to reprogramming events in early development, when the abovementioned massive changes in chromatin composition take place and paternal chromatin needs to be assembled. Because the nucleosome, the building block of chromatin, can be regarded as a modular structure consisting of multiple subunits, it is clear that changing the modules (for example through exchanging canonical with variant histones) can alter its overall properties. This exchange has not only the potential to impart different modular configurations to the chromatin, but also to induce important changes in histone modifications through the incorporation of replacement histones that harbour a different repertoire of marks. These considerations anticipate that during reprogramming, histone variants could play important roles in the process itself. Indeed, over the last years, a number of reports have documented key roles for histone variants in reprogramming in mammals and in other model systems (Wenkert and Allis 1984; Ng and Gurdon 2008; Ingouff, Rademacher et al. 2010; Santenard 2010). Here, we summarize what is known about the function of different H2A and H3 variants during the earliest stages of development, since these have been the most studied ones (Figure 16). We also discuss the possible implications of histone variant incorporation on the efficiency of reprogramming and its consequences on development. We restrict our review to the changes that are documented during the early reprogramming phase of the early embryo and will direct the readers elsewhere for changes in chromatin remodelling in general occurring during the formation of the germline (Gaucher, Reynoird et al. 2010; Gill, Erkek et al. 2012). We will not review the different and specific deposition machineries for several histone variants that have been recently characterised. As most of the research on histone variants during early embryogenesis has been conducted in mice, unless otherwise specified, we will focus on the mouse model.

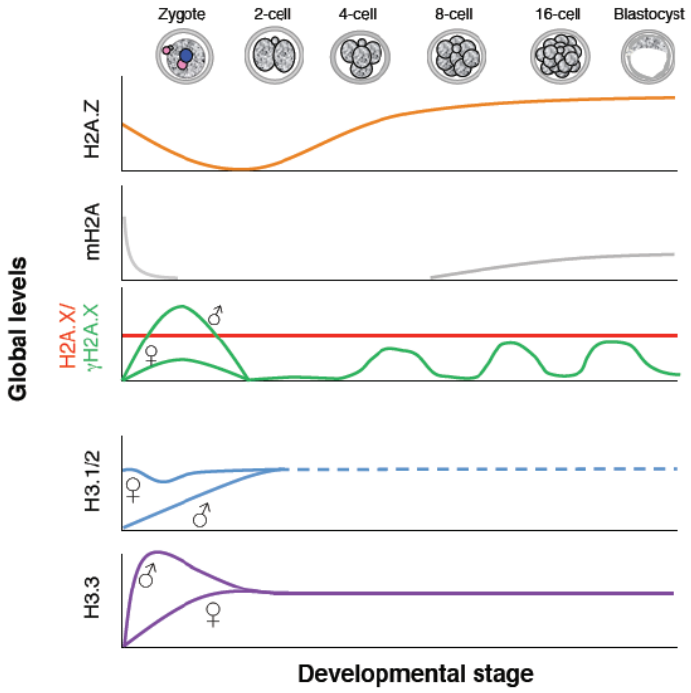

Figure 16. Global changes in the levels of histone variants during murine preimplantation development. The curves depict the schematic representation of global protein levels along development of the indicated histone variants or its modified forms (γH2A.X) as determined by immunofluorescence. In the zygote, differential accumulation of a histone variant in the female (♀) or the male (♂) pronucleus is illustrated by a double curve. The dotted line for H3.1/H3.2 indicates that their endogenous levels have not yet been determined at later stages.

a. Histone variants as regulators of epigenetic information during reprogramming

An exciting emerging concept relates to the question of whether the nucleosome depositing machinery can actually impart epigenetic memory by affecting the nucleosome turnover process. The most attractive candidates for this potential role are perhaps histone variants that could eventually be incorporated in the absence of transcription, such as H3.3 at the beginning of development. Indeed, a role for H3.3 in resetting the epigenetic signatures of heterochromatin after fertilization has been recently suggested (Santenard 2010). Mammalian cells posses 5 different H3 variants: the centromeric Cenp-A, (TS)H3.4 (formerly H3t), which is specifically expressed in the testis (Witt, Albig et al. 1996) and H3.1, H3.2 and H3.3, of which the two former are

incorporated exclusively concomitant with DNA synthesis. (reviewed in (Banaszynski, Allis et al. 2010)) and only H3.3 has the property of being incorporated outside of S-phase (Ahmad and Henikoff 2002; Ahmad and Henikoff 2002).

The first observation of differential histone variant incorporation in the mouse zygote was documented 7 years ago by Peter de Boer's lab, which used an antibody that recognizes H3.1/H3.2 specifically, and reported that H3.1/H3.2 was detected only in the maternal pronucleus immediately after fertilization (van der Heijden, Dieker et al. 2005). These observations were followed by our report documenting that H3.3 is deposited exclusively in the paternal chromatin concomitant with pronuclear formation using both an H3.3 specific antibody and a tagged exogenous H3.3 (Torres-Padilla, Bannister et al. 2006). Indeed, since the protamine-histone exchange occurs prior to S-phase, histone H3.3 is the major H3 variant present in paternal pronucleus of the zygote (Figure 17). It is important to note that there are only 4 and 5 aminoacid differences between H3.2 and H3.1 with H3.3, respectively, and therefore the quest for an H3.3 specific antibody has been long and painful. There is only 1 aminoacid difference between H3.1 and H3.2 and apart from mass-spectrometry analyses performed by the Allis lab (Hake, Garcia et al. 2006), there are no reports that characterise the endogenous H3.1 in comparison to the endogenous H3.2.

The findings in the mammalian zygote followed earlier observations in the *Drosophila* embryo, where it had been shown that HIRA-mediated incorporation of H3.3 is essential for chromatin assembly of the paternal chromatin (Loppin, Bonnefoy et al. 2005). Whether HIRA is the chaperone responsible for H3.3 incorporation in the mouse zygote has not been determined yet, but HIRA has been shown to localise to the paternal pronucleus transiently after fertilization (van der Heijden, Dieker et al. 2005) and our unpublished observations).

There is also remodelling of histone H3 variants after fertilization in other species, albeit with some differences to the mouse embryo or *Drosophila*. In *C. elegans*, H3.3 is actually carried from the autosomes of the sperm into the offspring and seems to disperse after fertilization (Ooi, Priess et al. 2006; Arico, Katz et al. 2011). As in the mouse, maternal H3.3 is incorporated into the paternal pronucleus in the zygote prior to

DNA synthesis or to embryonic transcription and it seems to be the major H3 variant until it is gradually replaced by H3.1 after the 8-cell stage (Ooi, Priess et al. 2006; Arico, Katz et al. 2011).

Although histone H3 variants seem to have evolved independently in animals and in plants, *Arabidopsis* H3.3 has a global genomic distribution that is similar to that of mammals (Goldberg, Banaszynski et al. 2010; Wollmann, Holec et al. 2012), indicating that, remarkably, H3.3 has acquired similar properties in the different phyla. The egg cell, which will form the zygote, expresses high levels of H3.3 and contains predominantly H3.3 (Ingouff, Hamamura et al. 2007). Interestingly though, there seems to be a mechanism of histone eviction after fertilization, whereby the H3.3 carried by the sperm is replaced by maternally provided H3.3 in the paternal chromatin (Ingouff, Hamamura et al. 2007; Ingouff, Rademacher et al. 2010).

The genome wide incorporation of H3.3 in the paternal chromatin after fertilization is therefore an amazingly conserved phenomenon. The major replacement that occurs in the male pronucleus probably has various downstream effects and raises a number of interesting questions, e.g. what is the consequence of histone turnover? is there a link between genome-wide replacement and the DNA demethylation that occurs predominantly in the male pronucleus? Could it be that this major turnover triggers DNA demethylation as a checkpoint ‘alert’ to protect DNA during this major replacement process?

As mentioned above, the histones inherited by the newly formed egg cell in *Arabidopsis* seem to be removed from the parental chromatin. Although the mechanism behind is unclear, these observations raise important considerations related to the potential epigenetic information that is transmitted through the histones in the gametes to the offspring. Partial removal of H3 variants from the gametes after fertilization was recently suggested to occur in the maternal chromatin of the mouse zygote as well (Akiyama, Suzuki et al. 2011). The lack of detection of epitope-tagged H3 in the zygote was suggestive of a similar eviction process to that observed in *Arabidopsis*. Although the significance of this observation remains to be determined, as is the mechanisms behind this phenomenon, the potential removal of maternal histones after fertilization would

have important consequences for maternal inheritance also in mammals. Other questions that remain to be addressed are: what is the fate of these histones once they are evicted? What happens to the DNA during this process? Akiyama and colleagues suggest a time window where the DNA is not wrapped around histones and therefore the regulation of checkpoints and DNA damage response are presumably important at this point.

It is important to remind the reader that the maternal chromatin 'loses' a number of chromatin marks in the ~10-12 hours that follow fertilization, including H3K64me3, H3K79me3, H4K20me3 and H3K36me3 (Arney, Bao et al. 2002; Santos, Peters et al. 2005; Burton and Torres-Padilla 2010; Akiyama, Suzuki et al. 2011; Boskovic, Bender et al. 2012) (Gill, Erkek et al. 2012). This suggests that the maternal genome is indeed subject to an important remodelling process, perhaps even more extensively than previously thought. A number of open questions arise from these latter considerations: what substitutes the histones that are evicted? are there specific loci remodelled and if so, which ones?. If there is a major remodelling process in the maternal genome that leaves an open DNA awaiting for newly synthesised histone variants to be incorporated, why does the embryo not trigger a DNA damage response under such conditions?

It is not yet clear to what extent, if any, the paternal histones are inherited to the offspring and contribute to the embryonic chromatin in the mouse. Although this has been suggested to be the case in in vitro fertilised human embryos (van der Heijden, van den Berg et al. 2009), this question is technically difficult to address because of the limits of detection for immunofluorescence in single embryos. Also, because of what we state above, it is unclear what percentage of histones and/or of the information carried in the form of histone modifications, persists in the maternal chromatin after fertilization. In our view, a potential epigenetic inheritance mediated through histone variants could also include the acquisition a particular 'signature' or a specific chromatin configuration in the gametes able to flag genomic regions for reprogramming after fertilization.

An extremely interesting case is indeed that of the pericentromeric chromatin. Constitutive heterochromatic regions tend to keep a distinctive organization during spermiogenesis, as both telomeres and centromeres retain features of their somatic

configuration, including histones and most likely some of their modifications (Wykes and Krawetz 2003; Govin, Escoffier et al. 2007). In particular, the testis-specific variants of H2A, H2AL1 and H2AL2 together with an H2B variant, TH2B package the pericentric chromatin to achieve a spermatid-specific DNA packaging structure that protects a ~60 bp DNA fragment (Govin, Escoffier et al. 2007; Boussouar, Rousseaux et al. 2008). Thus, the wrapping of pericentromeric DNA around a set of histone variants that are unique to the male germline could be potentially used as a 'flag' immediately after fertilization, in which H3.3 plays subsequently a major role in re-setting heterochromatin at these regions (Santenard 2010).

b. H3.3 and *de novo* establishment of heterochromatin

Whereas H3.3 had been traditionally associated with replacement sites of active transcription in somatic cells (Ahmad and Henikoff 2002), in the embryo H3.3 localises to heterochromatic sites (Santenard 2010; Akiyama, Suzuki et al. 2011). This suggests that the functions and/or properties of histone variants in embryos might be different to those in somatic cells.

As a first approximation to address how different histones contribute to the establishment of the newly formed embryonic chromatin after fertilization, the developmental dynamics of tagged H3.3 versions have been monitored using time-lapse or indirect immunofluorescence in various species (Loppin, Bonnefoy et al. 2005; Ooi, Priess et al. 2006; Ingouff, Rademacher et al. 2010; Santenard 2010; Akiyama, Suzuki et al. 2011). A thorough time-lapse analysis of H3 localization in the mouse zygote revealed the kinetics of incorporation of H3.3 and H3.1 (Santenard 2010), which confirmed that H3.3 is deposited in the paternal chromatin upon pronuclear formation. H3.1 was seen to first localise to both the maternal and the paternal pronuclei concomitant with the onset of replication (Santenard 2010). Subsequent immunofluorescence analysis using Flag-tagged H3 variants in fixed zygotes further suggested that H3.2 is more readily detectable than H3.1 during early embryonic development (Akiyama, Suzuki et al. 2011).

Although all these analyses have been very valuable in trying to determine the dynamics of incorporation of newly synthesised histones in the embryonic chromatin, they do have some important limitations. For example, localization in the nucleus does not always mean incorporation and a complementary, systematic analysis with triton extraction or similar procedures has to be implemented (Boskovic and Torres-Padilla, unpublished observations). An elegant combination of Snap-tagged technology and Triton extraction has been recently applied to fixed samples of cell culture to overcome such limitation and can be used to address dynamics if a time course is performed (Ray-Gallet, Woolfe et al. 2011).

In the mouse zygote, H3.3 was shown to be enriched in the pericentromeric chromatin of the male pronucleus, but not in the female pronucleus. It was postulated that H3.3 would favour a transcriptionally active environment for the pericentromeric repeats of the major satellites, which are transcribed during the first S-phase in the zygote (Probst, Okamoto et al. 2010; Santenard 2010). The transcription of these regions would be important for tethering of HP1β via an RNA-dependent mechanism, which together with the progressive methylation of H3K27 and Polycomb recruitment, would lead to silencing and condensation of pericentromeric chromatin (Santenard 2010). Probably one of the most interesting conclusions from this work is the fact that it demonstrated a role for a modifiable residue within a histone-variant-specific context during reprogramming. We had previously suggested that H3.3 could function in setting up 'transition' signatures by providing the possibility to infer changes to chromatin in the absence of DNA replication (Santenard and Torres-Padilla 2009). Indeed, it seems that H3.3 could fulfill at least two different roles within this context, as a mean of potentially transmitting epigenetic information but also to establish specialised chromatin signatures during development.

A function for H3.3 during reprogramming has been addressed using somatic cell nuclear transfer in *Xenopus* eggs and oocytes. The transplantation of a somatic cell nucleus to enucleated eggs leads to reprogramming of gene expression from the somatic programme to an embryonic one. This reversal includes both, downregulation of lineage-specific genes as well as upregulation of genes associated with pluripotency

such as *Oct4/Pou5f1*. The presence of H3.3 in the promoter of a gene seems to correlate with the extent of transcriptional memory exhibited by the lineage-specific genes upon reprogramming (Ng and Gurdon 2008). This led the authors to suggest that epigenetic memory – in this case mediated perhaps through H3.3- would help stabilise gene expression during development. It is possible that H3.3 can also play a role in promoting reprogramming of specific genes by promoting a more open chromatin configuration.

More recently, a role for H3.3 at later stages of development has been uncovered in *Xenopus*, where H3.3 is required for late gastrulation (Szenker, Lacoste et al. 2012). H3.3 seems to be required in a dose-dependent manner to maintain gene expression after gastrulation, perhaps indicating that some genes are more sensitive to H3.3 loss. Alternatively, it could reflect the affinity of the deposition machinery to different deposition sequences.

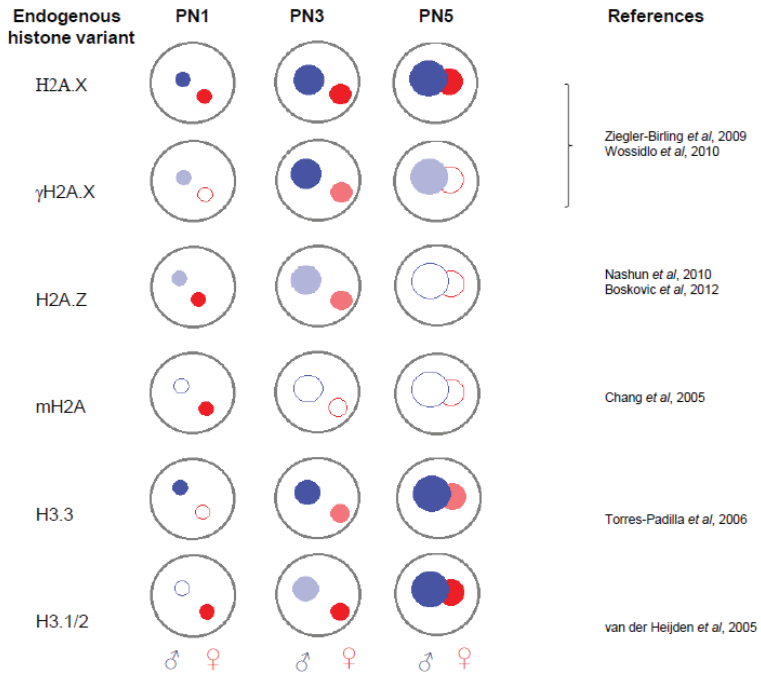

Figure 17. Profiles of endogenous histone variants during zygotic development. Localization and levels of endogenous histone variants obtained by immunostaining experiments in the zygote is shown. PN stands for pronuclear stage, 1 being the earliest after fertilization, 5 the latest. Male (♂) and female (♀) pronuclei are marked in blue and red, respectively. Levels of proteins in each pronucleus are colour-coded, from white (no protein) to dark blue/red (highest protein levels).

More generally, it could perhaps be suggested that H3.3 plays important roles in mediating epigenetic reprogramming during development and in experimental systems. In the future, it will be interesting to determine whether lack of H3.3 compromises the ability to form iPS cells and whether H3.3 would be required for reprogramming of chromatin signatures on specific genomic loci.

c. Variants of H2A: the case of macroH2A

One of the most interesting H2A variants with respect to reprogramming is macroH2A. MacroH2A is conserved in mammals and has three subtypes. Two of them, macroH2A1.1 and macroH2A1.2 are splicing variants of the same gene, while macroH2A2.2 is encoded by a separate gene. All three macroH2As have a very similar structure.

MacroH2A is the most divergent H2A variant, which consists of the N-terminal domain homologous to the canonical H2A and a large (30 kDa) C-terminus with no homology to histones, termed the macro domain (Figure 18). Despite its size, macroH2A is stably incorporated into nucleosomes wrapping the same length of DNA as the H2A-containing nucleosome, with the macro domain protruding from the nucleosome (Chakravarthy, Gundimella et al. 2005). This structural difference makes macroH2A-containing nucleosomes refractory to some ATP-dependent chromatin remodelers and the DNA wrapped around them is more resistant to nuclease digestion (Angelov, Molla et al. 2003).

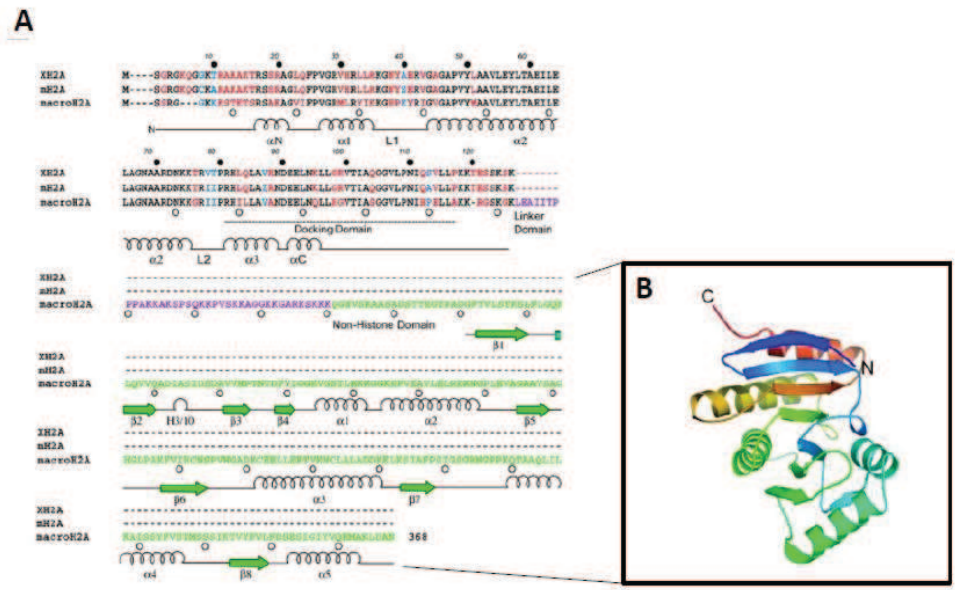

Figure 18. MacroH2A, a divergent H2A variant. (A) Multiple alignment of frog (XH2A), mouse (mH2A) and human macroH2A reveals overall similarities between histone core domains of all 3 H2A variants. MacroH2A contains a large C-terminal macro domain (in green) connected to the histone part via linker peptide. (B) Crystal structure of the macro domain of macroH2A. Figure adapted from Chakravarthy S *et al*, 2005.

Early studies of the biology of macroH2A showed that this histone variant is enriched in the inactive X-chromosome in female cells, forming the readily detectable macrochromatin body (MCB) in interphase nuclei (Costanzi and Pehrson 1998). This finding linked macroH2A incorporation into chromatin with gene repression. Further studies have confirmed the role of macroH2A in stable inactivation of X chromosome in somatic cells (Hernadez-Munoz, Lund et al. 2005), but have also shown that macroH2A serves as an important regulator of developmental genes in male pluripotent cells (Buschbeck, Uribesalgo et al. 2009). Indeed, increasing evidence suggests that the roles of macroH2A go beyond simple transcriptional repression and are in fact, context specific (Gamble and Kraus 2010).

The reprogramming process in mammals starts at fertilization, when both parental nuclei undergo dramatic changes in their chromatin structure. Profiling of macroH2A immediately before and during reprogramming yielded some very interesting findings. Although macroH2A is abundantly present in the chromatin of mature oocytes, upon

fertilization, a rapid removal of this histone variant from the maternal chromatin occurs (Chang, Ma et al. 2005). This seems to be an active process as it is completed by the late pronuclear stage 2 (PN2), before the onset of replication. Experiments with parthenogenetically generated embryos as well as embryos obtained through ICSI showed that macroH2A preferentially associates with maternal chromatin and it is removed from it during pronuclear formation and decondensation. MacroH2A remains undetectable from cleavage stage mouse blastomeres until the 8-cell stage, and macroH2A protein levels seem to increase from that point onwards (Costanzi, Stein et al. 2000; Chang, Ma et al. 2005). A subsequent study on the fate of macroH2A after somatic cell nuclear transfer reported that macroH2A is quickly removed from the heterochromatin of the transplanted nuclei (Ahmed, Dehghani et al.). However, the macroH2A 'stripping' ability was restricted to oocytes, requiring a step involving nuclear envelope breakdown. This suggested that elimination of macroH2A is an early step in the reprogramming upon nuclear transfer and presumably facilitates remodeling events taking place subsequently.

During early mouse embryogenesis, both X chromosomes in female cells are active until the 4-8-cell stage, when the inactivation initiates (Okamoto and Heard 2006). Imprinted X-inactivation occurs on the paternal chromosome before the blastocyst stage, after which the paternal X is reactivated exclusively in the epiblast cells. Imprinted X-inactivation persists in the trophectoderm. Analysis of the connection between macroH2A and X chromosome inactivation (XCI) during mouse development showed that macroH2A accumulates at the inactive X chromosome, forming a MCB, from the morula stage onwards (Costanzi, Stein et al. 2000; Nashun, Yukawa et al. 2010). Furthermore, at the early blastocyst stage, MCBs are almost exclusively present in the trophectoderm cells (Costanzi, Stein et al. 2000). The timing and localization of macroH2A in mouse blastomeres suggest that macroH2A accumulation on the paternal X-chromosome is an early event in its subsequent inactivation and occurs prior to changes in histone modifications and DNA methylation. This is in contrast with the random X inactivation process taking place in female ES cells. In the latter, macroH2A associates with the X chromosome only after the initiation and propagation of its inactivation have already taken place (Mermoud, Costanzi et al. 1999). It is therefore

possible to imagine that the timing of macroH2A incorporation into the chromatin of the inactive X chromosome distinguishes the imprinted and random X inactivation processes. Conversely, it is possible that the two types of X-inactivation differ in their stability and 'tightness', possibly illustrated by the difference in timing of macroH2A incorporation.

A recent study used the stability of X-inactivation to assess the extent and mechanisms of transcriptional reprogramming upon SCNT in Xenopus (Pasque, Gillich et al. 2011). By using donor nuclei from cells with different differentiation levels, the authors showed that the inactive X from post-implantation derived epiblast stem cells is more easily reactivated upon injection into enucleated *Xenopus* oocytes, as compared to the X chromosome of terminally differentiated or extraembryonic cells. Surprisingly, X-chromosome inactivation in differentiated cells was highly resistant to reactivation by the *Xenopus* oocyte. After somatic cell nuclear transfer, *Xist* coating was lost from all types of donor nuclei, and DNA methylation as well as PRC-mediated histone modification levels were comparable. However, epiblast-derived stem cells did not show macroH2A accumulation in the inactive X-chromosome, while other donor nuclei did. Thus, histone variant macroH2A incorporation was suggested to be a critical layer of protection against reactivation of X-chromosome upon nuclear transfer. Indeed, depletion of macroH2A increased the efficiency of reprogramming. These findings once more underlied the importance of interplay between different factors (DNA methylation, posttranslational histone modifications, non-coding RNAs and histone variant incorporation) to ensure a tight control of gene expression.

X-reactivation also occurs during PGC reprogramming from embryonic day (E) 11.5 to 13.5. During this time, XX cells progressively stop expressing *Xist,* which cannot be detected in most cells by E13.5. Interestingly, macroH2A does not localize to the MCB in PGCs, but in fact shows either a diffuse nuclear signal or accumulates in several foci in the nucleus. These foci, however, do not colocalise with *Xist* RNA (Nesterova, Mermoud et al. 2002). It therefore appears that there is no accumulation of macroH2A in the inactive X-chromosome of early PGCs, presumably facilitating or not interfering with the reprogramming process.

d. H2A.Z shows a dynamic localization during early reprogramming in embryos

The histone H2A variant that has possibly received most attention since its discovery is H2A.Z. H2A.Z is a highly conserved histone, even more so than H2A itself, which is already indicative of an important function of this variant (Iouzalen, Moreau et al. 1996). H2A.Z is essential in all metazoans tested thus far, but it is not essential in yeast. H2A.Z differs mostly from the 'regular' H2A in two regions. First is the L1 loop N-terminus from the histone fold, which was initially thought to prevent formation of heterotypic (H2A/H2A.Z) nucleosomes based on biophysical data (Suto, Clarkson et al. 2000). However, recent studies have identified the presence of these 'mixed' nucleosomes, both in vivo and in vitro, arguing against the previous biophysical data (Chakravarthy, Bao et al. 2004; Ishibashi, Dryhurst et al. 2009). More interestingly, the second divergent region is localized in the docking domain of H2A.Z. Once incorporated into the nucleosome, this docking domain allows H2A.Z to form an extended acidic patch, which is non-exchangable with that of H2A and is responsible for the specific biochemical and biophysical properties of H2A.Z-containing nucleosomes (Wang, Aristizabal et al. 2011; Wratting, Thistlethwaite et al. 2012).

After its discovery, H2A.Z was linked to gene activation, as it was found in the transcriptionally active macronucleus of *Tetrahymena thermophila* (Allis, Richman et al. 1986). However, subsequent research generated evidence for both positive and negative effects of H2A.Z on transcription, but also for a number of other functions. H2A.Z is thus somewhat of a controversial histone variant, localizing to both euchromatin and heterochromatin, and playing different roles in different systems. Some of the functions ascribed to H2A.Z include maintenance of heterochromatic boundaries, chromosome architecture, epigenetic memory, formation of pericentric heterochromatin and gene activation (Ma, Svoboda et al. 2001; Meneghini, Wu et al. 2003; Babiarz, Halley et al. 2006; Brickner, Cajigas et al. 2007; Zlatanova and Thakar 2008). Although some roles seem to be mutually exclusive with others, it could very well be that H2A.Z can both positively and negatively regulate gene expression, depending

on its binding partners, genomic and chromatin context as well as on its post-translational modifications.

As mentioned above, H2A.Z can be post-translationally modified, on both termini, adding to the complexity of this variant and the possibilities for regulation of chromatin function. N-terminal acetylation, which occurs on multiple lysines (5 in mouse and human), is the best studied H2A.Z modification (Pantazis and Bonner 1981; Ren and Gorovsky 2001; Zlatanova and Thakar 2008). H2A.Z acetylation is a mark of active chromatin and often works in concert with other core histone acetylations to destabilize the nucleosome, and to possibly enhance accessibility of DNA-binding proteins including transcription factors (Ishibashi, Dryhurst et al. 2009). Furthermore, H2A.Z acetylation was recently reported to be a key histone modification during gene deregulation in tumorigenesis (Valdes-Mora, Song et al. 2011). The C-terminal part of the protein was shown to be both ubiquitylated and SUMOylated (Sarcinella, Zuzarte et al. 2007; Kalocsay, Hiller et al. 2009). Monoubiquitylated H2A.Z comprises a small population of H2A.Z, which localises to the inactive X-chromosome, indicating a potential function in the formation and/or the maintenance of facultative heterochromatin (Sarcinella, Zuzarte et al. 2007). SUMOylation of H2A.Z, on the other hand, was shown to be important for nuclear localization of a damaged chromosome upon persistent DNA damage in *S. cerevisae*(Kalocsay, Hiller et al. 2009).

All these different properties make of H2A.Z a very versatile histone variant, with the possibility of modulating multiple chromatin-regulated processes. H2A.Z was the first histone variant shown to be essential in mammals. In 2001, the Tremethick group found that homozygous H2A.Z -/- mice are not viable. Detailed examination of heterozygous crosses concluded that mouse embryos lacking H2A.Z die at the time of implantation (Faast, Thonglairoam et al. 2001). While the trophectoderm cells of H2A.Z -/- blastocysts are able to attach to the surface of the culture dish and proliferate, the inner cell mass is completely degenerate and cannot divide further nor differentiate. Later, the same group reported based on immunofluorescence analysis, that in differentiating inner cell mass cells, H2A.Z is targeted first to pericentric heterochromatin and later to other parts of the nucleus (Rangasamy, Berven et al. 2003). They could not detect

H2A.Z in the inactive X-chromosome and hypothesized that it H2A.Z presence distinguishes constitutive from facultative heterochromatin. However, the possibility that H2A.Z ubiquitylation is rendering it undetectable at the inactive X-chromosome should be kept on mind, as it has been shown that ubiquitylation of H2A.Z results in epitope exclusion (Sarcinella, Zuzarte et al. 2007).

The finding that H2A.Z was essential in mice sparked an interest for the earliest developmental stages. A study documenting the mRNA levels of several histone variants in the earliest stages of mouse development revealed that H2A.Z mRNA is present in all cleavage stages, with lowest levels at the 2-cell stage and highest at the blastocyst (Kafer, Lehnert et al. 2010). However, since early development is characterized by large pools of maternal message as well as uncoupling of transcription and translation, the detection of H2A.Z mRNA alone was not sufficient to conclude on the presence and function of H2A.Z protein. A recent study has documented the distribution of H2A.Z protein in early murine development. Using in vitro fertilized embryos, Nashun and colleagues concluded that H2A.Z is detectable in the late oocytes, but H2A.Z becomes undetectable from embryonic chromatin rapidly after fertilization (Nashun, Yukawa et al. 2010). The authors also observed a similar lack of detection of the canonical H2A and macroH2A, while H2A.X was readily present and abundant at all cleavage stages. Through domain swapping and overexpression experiments, it was proposed that the presence of H2A and H2A.Z is deleterious for early development.

H2A.Z was also shown to be essential for the development of *Xenopus* and *Drosophila* embryos. In the frog, a single histidine residue specific for H2A.Z and the aforementioned extended acidic patch were shown to be necessary for proper development at gastrulation (Ridgway, Brown et al. 2004). Frogs without H2A.Z or expressing a mutated form of H2A.Z showed a notochord specific phenotype. This was the first indication that the H2A.Z residues present on the nucleosome surface could regulate the development of a particular germ layer, in this case, the mesoderm. In *Drosophila*, features of both H2A.Z and H2A.X (see below) are found into a single protein, H2A.Z (formerly H2AvD; see (Talbert, Ahmad et al. 2012). This histone variant

was shown to be essential in the fly, with null mutants arresting development during the third larval instar (van Daal and Elgin 1992). Through domain swap experiments, it was concluded that the region of H2A.Z (formerly H2AvD) essential for development resides in the C-terminal part of the protein, buried deep into the nucleosome core. This part of H2A.Z (formerly H2AvD) does not interact with the DNA, but is more likely to mediate protein-protein interactions (Clarkson, Wells et al. 1999). Alteration in the octamer interactions probably has therefore a direct effect on nucleosome stability and accessibility of DNA to factors acting upon it.

Apart from the dynamic behaviour of H2A.Z during the reprogramming process in early mammalian development, a comprehensive immunofluorescence-based study on chromatin signatures of PGCs undergoing reprogramming revealed that H2A.Z is also lost from the chromatin during this time (Hajkova, Ancelin et al. 2008). This was accompanied by a transient loss of detection of the linker histone H1 and of modified H3 on a large majority of the residues analysed (H3K9ac, H3K9me2/3, H3K27me3). Altogether this suggests that removal or H2A.Z from the chromatin occurs during the times of epigenetic reprogramming in vivo.

e. High endogenous levels of phosphorylated H2A.X are characteristic of early embryos

H2A.X is best known for its role in mediating the DNA damage response upon double-strand DNA breaks (reviewed in (Bassing and Alt 2004)). H2A.X becomes phosphorylated on its C-terminal SQE motif in response to damage by Ataxia-telangiectasia mutated (ATM) and other phosphoinositide 3-kinase-related protein kinases (PIKK). Deletion of H2A.X leads to genome instability and defefects in DNA repair, but H2A.X -/- mice are born at the expected mendelian rations (Zha, Sekiguchi et al. 2008).

H2A.X is uniformly present in preimplantation mouse embryos (Ziegler-Birling, Helmrich et al. 2009) and was suggested to be the preferred H2A variant during this time (Nashun, Yukawa et al. 2010). Interestingly, the levels of phosphorylated H2A.X,

H2A.X, appear to be intrinsically high in mouse embryos, even under conditions where no DNA damage has been induced, and oscillate throughout the cell cycle (Ziegler-Birling, Helmrich et al. 2009; Wossidlo, Arand et al. 2010). Moreover, levels of γH2A.X change dramatically during the earliest developmental stages(Ziegler-Birling, Helmrich et al. 2009). More recently, it has been shown that mouse ES cells and iPS cells have also high levels of γH2A.X. Most importantly, phosphorylation of H2A.X decreases upon differentiation and loss of H2A.X compromises ES cell self-renewal, which can be restored by the reintroduction of wild type H2A.X but not of a non-phosphorylatable H2A.XS139A mutant (Turinetto, Orlando et al. 2012). Thus, high levels of γH2A.X seem to be a feature of cells with higher degree of plasticity such as the early embryo or the pluripotent ES cells. It will be important to determine whether lack of H2A.X compromises the ability to form iPS cells.

A second important observation arose from the analysis of γH2A.X in early zygotes and 2-cell stage embryos (Ziegler-Birling, Helmrich et al. 2009; Wossidlo, Arand et al. 2010). While abundantly present in the zygote (particularly on paternal chromatin), γH2A.X signal dramatically decreases by the 2-cell stage. A detailed study of the timing and localization of γH2A.X was conducted to investigate the possible relationship of DNA repair and DNA demethylation, which is known to occur as one of the initial steps of reprogramming (Wossidlo, Arand et al. 2010). A strong γH2A.X signal was observed immediately after fertilization, followed by a decrease by the pronuclear stage (PN) 2. Prior to DNA replication, γH2A.X foci are present exclusively in the paternal pronucleus, and at the time of replication, both parental chromatins are marked with γH2A.X. Importantly, the signal detected in the male pronucleus is always stronger than that observed in the female one. Through a series of elegant experiments, the origins of γH2A.X foci during different pronuclear stages were traced. Foci appearing immediately after fertilization were attributed to DNA single-strand breaks occurring upon chromatin decondensation taking place upon fertilization (Wossidlo, Arand et al. 2010). Interestingly, γH2A.X foci which mark only the paternal pronucleus at PN2 represent nicks in DNA, which fully coincide with both the location and the timing of DNA demethylation. Finally, DNA breaks introduced through replication process occurring

between PN3 and PN4 produce γH2A.X foci that mark both the maternal and the paternal pronucleus. The presence of phosphorylated H2A.X at the onset of epigenetic reprogramming in mouse zygotes suggests that the DNA demethylation process might be mechanistically linked, at least partially, to pathways of DNA repair.

In *Xenopus*, a similar situation was previously reported (Dimitrov, Dasso et al. 1994). When incubated with egg extract, *Xenopus* sperm chromatin also undergoes rapid changes in composition and compaction. During the process of sperm chromatin remodelling and decondensation, γH2A.X is readily incorporated into the chromatin of the decondensing paternal pronucleus. Moreover, the level of efficiency of pronuclear formation correlates with levels of γH2A.X.

f. Barr body-deficient H2A: H2A.B

The most recently discovered H2A variant is H2A.Bbd, or H2A.B, according to the new unifying nomenclature. The original name stands for 'Barr body-deficient', as H2A.B was originally discovered to stain autosomes and the active X-chromosome, but was excluded from the inactive X of female cells (Chadwick and Willard 2001). This vertebrate-specific H2A variant was shown to colocalise with acetylated forms of histone H4, and was proposed to be a mark of transcriptionally active chromatin. H2A.B shares only about 48% of identity with H2A, mostly located within the histone fold helices, but it shows significant differences in the N-terminal tail and the docking domain, which is the interaction interphase with H3-H4 tetramer. In silico analyses revealed that H2A.B is a rapidly evolving histone variant (Eirin-Lopez, Ishibashi et al. 2008) and its behaviour is characteristic of sexually driven positive Darwinian selection, not unlike evolution of protamines (Ishibashi, Li et al. 2010). H2A.B is a much shorter protein (115 aminoacids) compared to other H2A variants. Despite its size, H2A.B was shown to associate with core histones and behaves like one itself. Nucleosome core particles (NCPs) reconstituted with H2A.B show similar salt-dependent properties to NCPs containing the canonical H2A (Bao, Konesky et al. 2004). However, conformational changes that occur in H2A.B-NCP upon increasing ionic strength are reminiscent to NCPs containing

acetylated histones. Structural and biophysical characterization of H2A.B-containing NCPs revealed that H2A.B octamers can protect only about 120-130 base pairs from MNase digestion and they bind less tightly to DNA ends (Bao, Konesky et al. 2004; Doyen, Montel et al. 2006). These findings, together with the coexistence of H2A.B and acetylated histone H4, support the view of H2A.B-containing nucleosomes as a part of transcriptionally permissive chromatin. Additionally, through the incorporation of H2A.B into nucleosomes, cells can employ a mechanism of modulating transcription, alternative to core histone acetylation.

H2A.B in the context of mammalian early development has not been thouroughly investigated. Based on mRNA analysis, one study reports that H2A.B is not expressed during cleavage stages of mouse development (Kafer, Lehnert et al. 2010). Recently, however, a role of H2A.B in male gametogenesis has begun to emerge. Ishibashi and colleagues (Ishibashi, Li et al. 2010) reported the presence of H2A.B in advanced stages of spermiogenesis at the time of histone-protamine exchange, when levels of H4 acetylation are highest. It is possible to imagine that H2A.B plays a role in facilitating the exchange of histones with protamines that is necessary for proper sperm DNA compaction. H2A.B was also detected in sperm samples, in the nucleosome-enriched fraction of chromatin, but the implications of this finding on the reprogramming and development remain to be investigated. It is becoming increasingly clear that H2A.B is more than a short H2A variant depleted from the inactive X. Subsequent research will undoubtedly provide insight into the biology of H2A.B and perhaps into its involvement in embryonic development.

Part 2. Roles of histone variant H2A.Z during early mouse development

Publication 1

I. Summary of publication 1

Histone variants modulate the structure of chromatin from its basic unit, the nucleosome. As I mentioned in the introduction, they have been shown to play important roles during epigenetic reprogramming. For instance, macroH2A incorporation renders chromatin tighter and more refractory to reprogramming cues, thus stabilizing cell fate (Pasque, Gillich et al. 2011). On the other hand, H3.3 incorporation into the male genome after fertilization, and its subsequent methylation on lysine 27, are necessary for formation of pericentric heterochromatin during mouse preimplantation development (Santenard 2010). H2A.Z, one of the most investigated histone variants was shown to be essential for mouse development, and its knock-out leads to developmental arrest at the time of implantation. This suggested possible roles of H2A.Z on chromatin during preimplantation stages of development. To better understand the role(s) of H2A.Z during early embryogenesis, I set out to investigate the levels of H2A.Z protein on mouse embryonic chromatin, as well as the localization patterns of several known euchromatic histone marks, using immunofluorescence. We observed that euchromatic marks are extensively modulated after fertilization, and that activating histone modifications do not necessarily correlate with transcriptional activity in the mouse and bovine embryos.

1. H2A.Z levels are dynamic during mouse preimplantation development

Firstly, spatiotemporal profiling of H2A.Z on embryonic chromatin was performed, by immunostaining experiments on freshly collected and fixed mouse embryos. I observed that H2A.Z levels are dynamic during early development, with low levels in the zygote and almost undetectable in 2-cell stage embryos, which increase at the 4-cell stage and persist until the blastocyst. When present on chromatin, H2A.Z showed a preferentially euchromatic localization and was excluded from DAPI dense regions.

2. H2A.Z is acetylated in mouse embryonic nuclei

H2A.Z is known to be posttranslationally modified on both N- and C-terminus. Because N-terminal acetylation has been shown to correlate with gene activation during differentiation but also cancer progression (Valdes-Mora, Song et al. 2011), I investigated whether H2A.Z was acetylated during mouse preimplantation development, and found that acetylation levels correlate well with overall H2A.Z levels. H2A.Z was not acetylated during mitosis, in line with its function in opening chromatin and promoting active transcription. Interestingly, at the 2-cell stage, when EGA occurs in mouse, H2A.Z acetylation was virtually undetectable. This finding prompted us to investigate the behavior of other known euchromatic marks in mouse embryonic nuclei.

3. Profiling of euchromatic marks during development

During epigenetic reprogramming in the zygote, extensive changes in chromatin composition and organization take place. Arguably, most investigated phenomenon during this time is the global organization and re-organization of heterochromatin on male and female pronucleus, respectively. As discussed in the introduction, embryonic heterochromatin is devoid of traditional marks of constitutive heterochromatin. Much less is known about the status of euchromatin. Our observation that H2A.Zac behaves

atypically at the beginning of embryogenesis prompted us to investigate other euchromatic histone PTMs. I determined the localization patterns of H3K4me3 (modification associated with active promoters), H3K9ac (PTM enriched on active promoters, and important for ES cell pluripotency and reprogrammability) and H3K36me3 (marker of elongating transcription). All three marks showed distinct behavior on embryonic chromatin. H3K9 acetylation and H3K4 trimethylation were present throughout development, but in the zygote H3K4me3 was preferentially localized on maternal chromatin, although the levels of H3K4me3 became equivalent in the two pronuclei towards the end of the first cell cycle (Torres-Padilla, Bannister et al. 2006). Conversely, H3K36me3 was undetectable on paternal chromatin in the zygote and remained so at the 2-cell stage. This was quite surprising as transcription occurs both on the male PN and in 2-cell stage nuclei. H3K36me3 reappears later in development, but in low levels and displaying a patchy nuclear localization.

4. Conservation of chromatin modifications at the time of EGA

Embryonic genome activation in bovine embryos takes place later than in the mouse, at the 8-cell stage. Given our observations above, we wondered if euchromatic marks present on chromatin at the time of EGA are conserved in evolution, possibly providing a common mechanism contributing to EGA regulation. Therefore, localization patterns of H2A.Z acetylation and H3K36me3, which were rather atypical in mouse nuclei, were investigated in bovine embryos. While H2A.Z acetylation could be detected throughout early bovine development, H3K36me3 displayed a similar patchy profile as in the mouse embryo. At the time of EGA, H3K36me3 could be observed in some, but not all nuclei, and in low abundance.

5. Conclusions

In this work, I characterized the spatio-temporal localization of H2A.Z and several known histone modifications associated with active chromatin during mouse

preimplantation development. We show that euchromatic PTMs are modulated during mouse embryogenesis and that histone marks dynamically change in development. Maternal chromatin is also affected during genome-wide epigenetic reprogramming, perhaps to an even higher extent than previously considered. Furthermore, canonical marks of transcriptional activation in cells do not necessarily correlate with embryonic transcription and should not be used as predictive during early development. Finally, by comparing histone modifications in mouse and bovine embryos, we concluded that species-specific differences exist in chromatin marking prior to embryonic genome activation and could potentially contribute to regulation of EGA.

II. Publication 1

III. Unpublished results

1. Effects of H2A.Z overexpression in the zygote

H2A.Z levels are very low immediatelly after fertilization, in the zygote and 2-cell stage mouse embryos. This suggests that H2A.Z is removed from chromatin during epigenetic reprogramming, alongside other histone variants, such as macroH2A. Given the repressive effects of macroH2A on reprogramming process, we wondered if H2A.Z is also refractory to acquisition of totipotency and therefore needs to be stripped from chromatin after fertilization. In other words, is loss of H2A.Z during early development necessary for subsequent developmental progression? Expanding on this question, we wondered if posttranslationally modified forms of H2A.Z mediate specific effects during early development. To address this question, we generated HA-tagged H2A.Z constructs, and synthesised the corresponding mRNA in vitro. We then microinjected HA.H2A.Z mRNA alongside with GFP mRNA as trancer into zygotes and followed development until the blastocyst (Figure 19). The same experimental approach was applied on non-modifiable H2A.Z forms, obtained by point-mutations of critical lysine residues on N and C terminus (figure 21 A).

Firstly, I verified if HA.H2A.Z mRNA is expressed after microinjection, by performing immunostaining experiments. Indeed, it was efficiently expressed as revealed by a clear HA signal in embryonic nuclei (not shown).

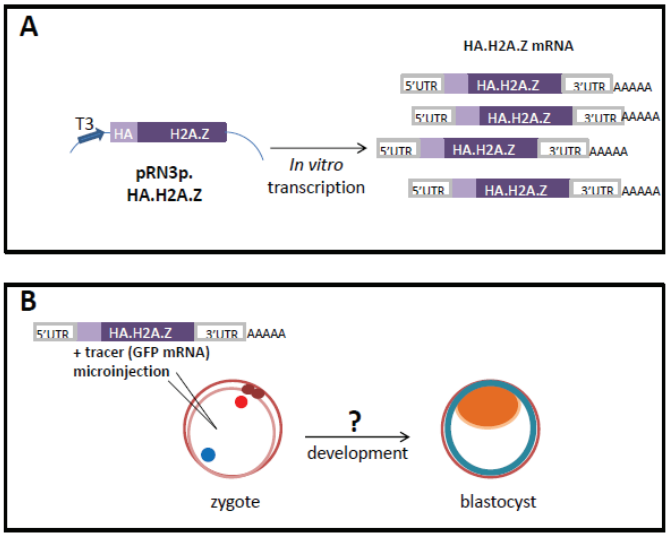

Figure 19. Experimental setup for investigating the effects of H2A.Z overexpression on embryonic development. (A) Coding sequence for mouse H2A.Z (*h2afz*) was cloned into pRN3P plasmid, with an N-terminal haemagglutinin (HA) tag. Viral T3 promoter is located upstream. mRNA was synthesized in vitro by mMessage Machine Kit using T3 polymerase. (B) HA.H2A.Z mRNA was injected into zygotes shortly after fertilization (~18 hours after hCG administration) together with mRNA coding for GFP, as a tracer. Injected embryos were placed in culture and development to blastocyst was monitored and scored.

Next, development to blastocyst was scored after microinjection with HA.H2A.Z and GFP mRNA. In parallel, non-injected embryos were cultured, as a negative control. About 50% of the H2A.Z-injected embryos (compared to control) reached the blastocyst stage after 3.5 days of culture (n= 41, Figure 20). A closer inspection of developmental progression revealed that a significant portion of injected embryos (about 35%) arrested between 8-cell stage and morula, while the remainder displayed abnormal morphology. Effects of non-modifiable H2A.Z overexpression are summarised in Figure 21. None of the mutant H2A.Z constructs had a dramatically different effect to expression of wild-type H2A.Z, suggesting that it is the expression of the H2A.Z histone variant itself that is mediating the developmental delay. Still, I cannot exclude the posibility that a milder effect is generated through overexpression of non-modifiable H2A.Z forms, that could be masked by the overall influence of H2A.Z itself embryonic development.

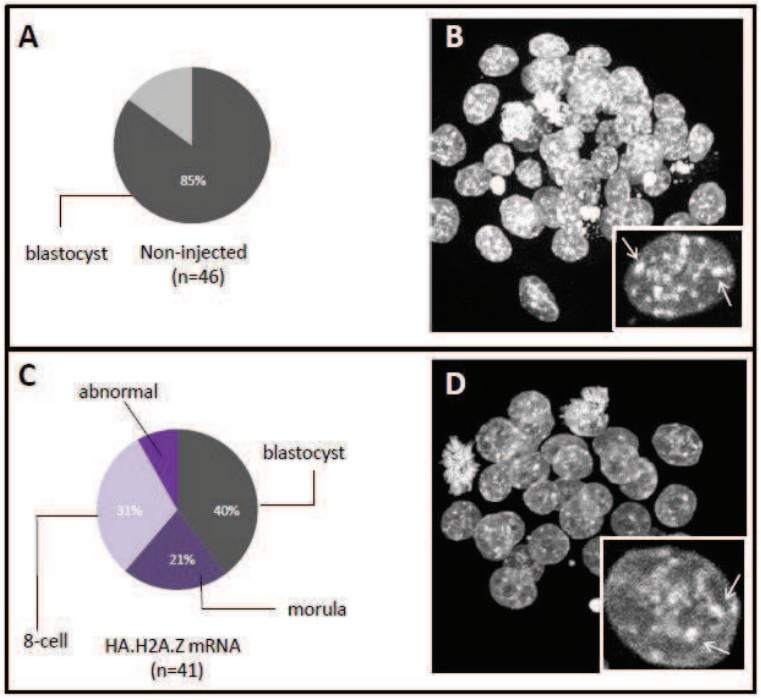

Figure 20. H2A.Z expression in the zygote delays developmental progression. (A) Development of non-injected embryos in culture is shown. 85% of embryos reached blastocyst stage by embryonic day 3.5. Number of embryos investigated is indicated. (B) DAPI-staining of a non-injected blastocyst, mounted onto a coverslip. White insert represents a magnification of a single nucleus of this blastocyst. Chromocenters are indicated by an arrow. (C) Developmental progression of HA.H2A.Z-injected embryos in culture. About 40% of embryos formed blastocysts by E3.5, while a significant proportion of H2A.Z-injected embryos were delayed or arrested at 8-cell and morula stage. (D) DAPI-staining of a H2A.Z-injected blastocyst, mounted onto a coverslip. White insert represents a magnification of a single nucleus of this blastocyst, as above. Nuclei of injected blastocysts contain fewer chromocentres (indicated by an arrow), which are smaller in size and intensity, compared to non-injected nuclei.

H2A.Z-injected embryos that reached the blastocyst stage were morphologically indistingushable from their non-injected counterparts. Trophectoderm and ICM were nomal in appearance, as well as the blastocoel. To begin to understand the effects of exogenous H2A.Z expression on the chromatin, control and experimental blastocysts were stained with DAPI for DNA visualisation. DAPI preferentially binds to AT-rich DNA sequences, enriched in satellite repeats in mouse cells, and thus allows for indirect identification of pericentric heterochromatin. Interestingly, H2A.Z-injected blastocysts displayed changed chromocentre morphology compared to control embryos. Qualitative observations in the microscope suggested that both the size and intensity of DAPI-rich regions were smaller.

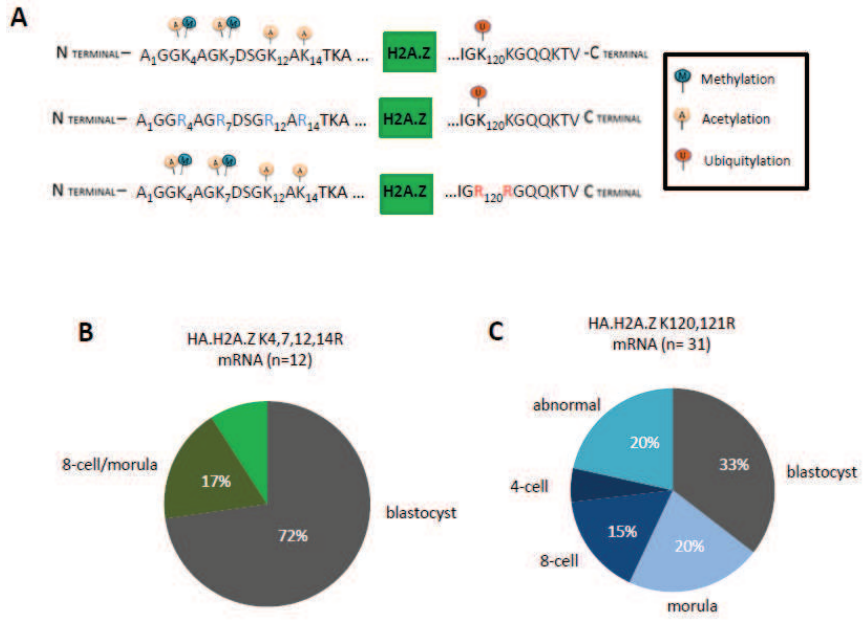

Figure 21. Effects of expression of H2A.Z point mutants on development. (A) Schematic representation of H2A.Z PTMs on N- and C-termini (top panel). Mutating N-terminal lysines 4,7,12 and 14 to arginine impairs H2A.Z acetylation (middle). H2A.Z unubiquitylatable form is obtained by mutating lysines 120 and 121 to arginine (bottom panel). (B) Developmental progression of zygotes injected with mRNA coding for unacetylatable H2A.Z. (C) Developmental progression of zygotes injected with mRNA coding for unubiquitylatable form of H2A.Z. (B and C) Experimental procedures were the same as described in figure 20. Number of injected zygotes is indicated. Development was scored at embryonic day 3.5.

These preliminary observations should be followed-up by a systematic quantification of chromocentre volume, intensity and localization in blastocysts imaged in 3D. Indeed, a detailed 3D reconstruction will allow us to generate a quantitative assessment of the effect of H2A.Z overexpression on chromatin organization in the embryo.

2. Other H2A.Z posttranslational modifications?

The number of known histone PTMs as well as their sites on histones is growing rapidly with technological advance and availability of experimental procedures (Tan, Luo et al. 2011). Indeed, it is becoming clear that histones are extensively chemically processed after translation and new roles for these modifications are emerging. As H2A.Z is reported to be acetylated, methylated and ubiquitylated, we decided to investigate

whether there are additional PTMs present on H2A.Z, with the aim of further investigation of its roles in chromatin regulation.

As a first step to identify putative phosphorylation sites within a protein, without a need for 2D protein gels or mass spectromentry, *in silico* methods can be used. We decided to screen H2A.Z aminoacid sequence to identify putative phosphorylation sites using two different phosphorylation predictors. The freely available NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) and Disphos (http://www.dabi.temple.edu/disphos/pred.html) provide two distinct types of freeware for phosphorylation screens. NetPhos is a neural network based predictor, trained on experimentally obtained datasets of phosphorylations sites. Additionally, Disphos looks for aminoacid stretches in a protein with intrinsic disorder, which could potentially be accessible to solvents (and kinases). Due to the difference in their modus operandi, NetPhos is generally less stringent (higher level of false-positive hits) while Disphos is very discerning (higher level of false-negatives)(Kobir, Shi et al. 2011).

When mouse H2A.Z-1 protein sequence was input into NetPhos 2.0 and Disphos, both softwares identified serine 9 as a putative phosphorylation site (Figure 22). Interestingly, this residue is surrounded by lysines at positions 7, 11 and 13, which are all known to be acetylated. Moreover, lysines 4 and 7 of H2A.Z are also reported to be methylated (personal communication). This observation was reminiscent of histone H3 N-terminal tail, subject to various posttranslational modifications that regulate different transcriptional states. The presence of a putative phosphorylation site close to methylatable residues is a hallmark of the phosphomethyl-switch occurring in H1 and H3. Indeed, the K9 and S10 residues in H3 are methylated and phosphorylated, respectively (Fischle, Tseng et al. 2005), when H3S10 becomes phosphoylated upon mitotic entry. This results in displacement of HP1 thereby antagonizing H3K9 methylation. Likewise, H1.4 is known to be phosphoylated on serine 27 by mitotic Aurora B kinase (Hergeth, Dundr et al. 2011). This phosphorylation prevents binding of HP1 to the neighbouring H1.4Kme2, reminiscent of the phospho-methyl switch on histone H3. Interestingly, H1.4 S27p and H1.4 S25p (Chu, Hsu et al. 2011) regulate H1.4 chromatin binding during mitosis.

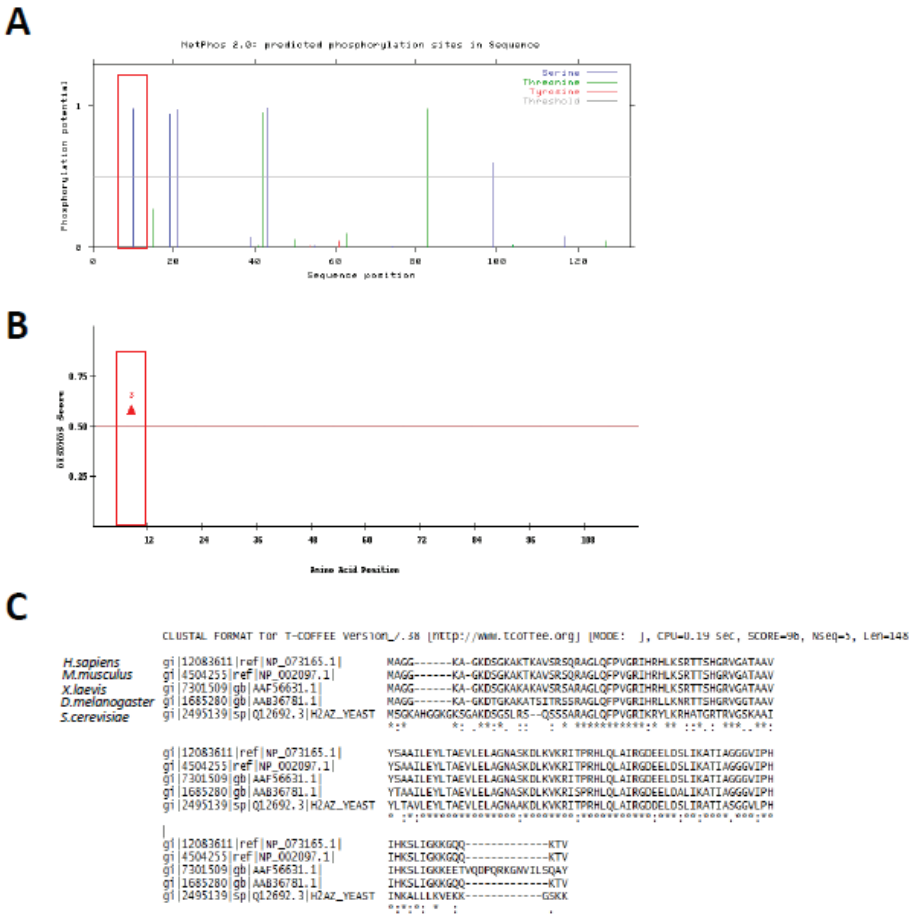

Figure 22. *In silico* identification of a putative phosphorylation site on H2A.Z. Primary protein sequence of mouse H2A.Z (H2A.Z-1) was input into phosphorylation predictors NetPhos2.0 and DISPHOS. (A) Neural phosphorylation predictor NetPhos 2.0 identified 7 residues (S9, S18, S20, T41, S42, T82, S98) on H2A.Z as potentially phosphorylated. (B) DISPHOS predicted serine 9 of H2A.Z as a putative phosphorylation site. (C) Multiple sequence alignment of human, mouse, frog, fly and yeast H2A.Z using T-COFFEE software revealed that phosphorylatable residue on position 9 of H2A.Z is conserved in evolution.

As previously mentioned, H2A.Z is a highly conserved histone variant, and I was interested in determining whether serine 9 is present from very early on in evolution, or was acquired later. Primary sequences from *S.cerevisiae* and several animal species were aligned using T-Coffee multiple alignment software and revealed that H2A.Z always contains a phosphorylatable residue at this position. In budding yeast, the N-terminal tail is more divergent and contains several other serine residues, many of

which are predicted to be phosphorylated. While the phosphopredictor softwares identify both S11 and S16 as putative phosphosites in yeast, the serine in **S**XSXR context is not predicted to be phosphorylated in mammals. Also, both S11 (yeast) and S9 (mammals) are predicted to be phosphorylated by the same kinase (PKC) suggesting that if they existe, they night be regulated in a similar manner.

Serine 11 (yeast) and serine 9 (mouse) are the most N-terminal phosphorylatable residues (apart from S1 in yeast, similar to canonical H2A) and they are surrounded by lysines and relatively chemically inert aminoacids (glycine, alanine). All the surrounding lysines can be modified (acetylated, methylated) reinforcing the idea of N-terminal PTMs as regulatory inputs for H2A.Z/Htz1 function. On the other hand, S17 in Htz1 is in a different aminoacid contest: S(16)XSXR (where X refers to other aminoacid). This is very similar to S(18)XSXR motif in mouse H2A.Z.

In order to investigate which of the residues are truly homologous between budding yeast and mouse, a structural comparison was attempted using published crystal and NMR structures of H2A.Z and Htz1, respectively. Unfortunately, the N-terminal tail of H2A.Z is not in the H2A.Z-containing nucleosome core particle crystal structure (N-terminal tails are reported not to crystallize very well due to their unstructured conformation) and the Htz1 structure is a Htz1-H2B fusion protein lacking the very N-terminal.

H2A.Z is relatively divergent from the canonical H2A. This prompted me to investigate whether serine 9 is also present in other H2A histones, including H2A and H2A.X. Interestingly, neither H2A nor H2A.X contain a phosphorylatable residue at this position, but instead have an alanine. On the other hand, macroH2A contains a serine at the same position, but the surrounding aminoacids are very divergent, suggesting that in case macroH2A is phorphorylated, this might imply a different function or regulatory mechanism. These results suggest that serine 9 is a H2A.Z-specific residue, with potential roles in modulation of modifications of H2A.Z N-terminal tail.

a. Characterization of H2A.Z phosphorylation

Since both tested in silico phosphopredictors suggested that H2A.Z S9 phosphorylation might exist, we reasoned that H2A.ZS9p could play a role in H2A.Z function. We thus raised a rabbit polyclonal antibody against the N-terminal tail of H2A.Z to recognise phosphorylated S9(sequence AGKDS(phospho)GKAKT corresponding to aminoacids 5-14 of mouse H2A.Z). The antiserum obtained was purified by affinity purification using Sulfolink beads coupled to the peptide used for antibody generation.

The phosphoH2A.Z (anti-H2A.ZS9p) antibody was then characterized in vitro, by western blot and dot blot. In western blots with whole cell lysates as well as high salt histone preparations, the purified H2A.ZS9p antibody recognized a protein corresponding to molecular weight of about 15kDa.

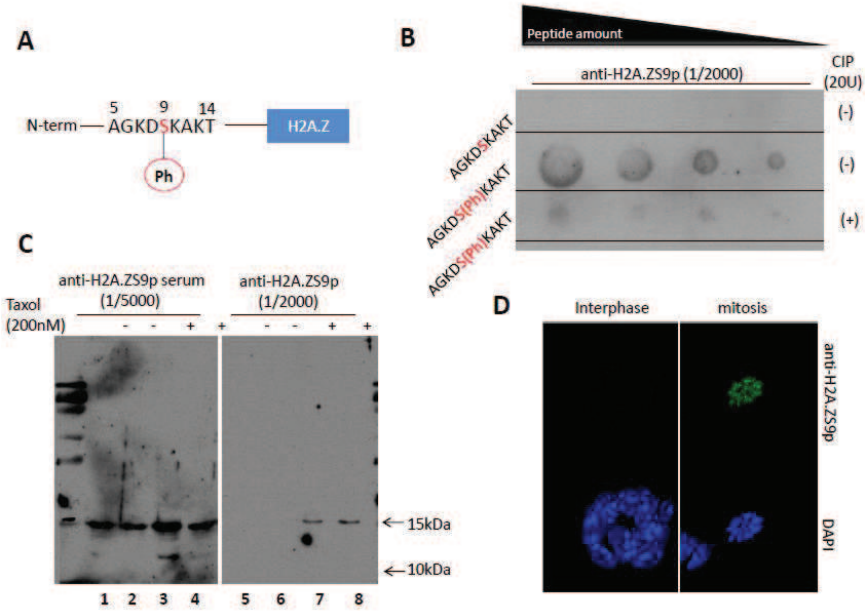

Figure 23. Characterization of anti-H2A.ZS9p antibody. (A) Schematic representation of H2A.Z and the peptide sequence used for antibody production. Serine residue, corresponding to serine 9 in H2A.Z, was phosphorylated in the peptide for antibody production. (B) Dot-blot experiment with non-phosphorylated (AGKDSKAKT) or phosphorylated (AGKDS(Ph)KAKT) peptide, using anti-H2A.ZS9p. Results reveal that antibody is specifically recognizing peptide, and the signal is lost after calf intestine phosphatase (CIP) treatment. (C) Western blot experiment using histone preparation from 3T3 mouse fibroblasts. Samples in lanes 3, 4, 7 and 8 come from cells treated with a citostatic drug Taxol which blocks mitotic progression at anaphase. Samples in lanes 1, 2, 5 and 6 were obtained from asynchronous cells. Anti-serum against H2A.ZS9p as well as the anti-H2A.ZS9p antibody recognize a band at 15 kDa, and the signal is enriched in mitotic cells. (D) Immunofluorescence using the anti-H2A.ZS9p antibody on asynchronous 3T3 cells revealed a strong enrichment of signal on mitotic chromatin.

This is a little higher compared to the predicted H2A.Z size (about 13.4 kDa). However, due to their basic nature histones often migrate differently on protein gels. I then used the commercially available H2A.Z antibody on the same extracts, which also recognized a band at 15 kDa, suggesting that H2A.Z migrates more slowly on high percentage polyacrylamide gels than predicted. Dot-blot experiments using different peptides H2A.Z peptides spanning aminoacids 5-14, and where S9 is phosphorylated or non-modified showed that the H2A.ZS9p antibody is specific for the S9 phosphorylated peptide. Importantly, the recognition by the H2A.ZS9p depends on peptide phosphorylation, since the signal was lost after phosphatase treatment (Figure 23 B) . Furthermore, the purified antibody did not recognize a peptide derived from the H3 N-terminal tail where S10 is phosphorylated.

Likewise, we used the H2A.ZS9p antibody in immunofluorescence in mouse NIH3T3 cells as well as E14 ES cells. Immunostaining experiments revealed a strong signal on mitotic chromatin, indicating that the antibody is indeed recognizing a chromatin protein and that the phosphorylation is enriched in mitosis. These results indicate that H2A.Z phosphorylation could be a potential novel marker of mitosis.

In summary, we have discovered a putative phosphorylation site specifically on H2A.Z, H2A.ZS9p, which is evolutionarily conserved. We were able to raise an antibody specific for this modification and show that it recognizes a chromatin protein of ~15kDa and that is enriched in mitosis. Although the above results strongly suggest that H2A.Z can indeed be phosphorylated in vivo, it cannot be excluded that we are a observing a technical artifact. To directly address whether H2A.Z is phosphoryalted at S9 in vivo, we decided to perform mass spectrometry to identify modified residues within endogenous H2A.Z. For this and because the results above suggest that H2A.ZS9p is highest in mitotic cells, we have synchronized 3T3 fibrobalsts in mitosis using Taxol (a citostatic drug) and prepared histones from mitotic cells. These histone preparations are currently being analysed by mass spectrometry to definitively reveal whether H2A.Z is indeed phosphorylated in vivo.

IV. Discussion

Global changes of chromatin organization take place after fertilization, including packaging of the sperm genome into maternally provided histones, the decondensation of the male and female pronuclei and the de novo establishment of pericentric heterochromatin. During very early mouse development, we and others have now reported the rapid disappearance of H2A.Z from embryonic chromatin (Nashun, Yukawa et al. 2010),(Boskovic, Bender et al. 2012). However, H2A.Z could be detected in the fully grown (GV) oocyte, suggesting that H2A.Z becomes rapidly removed from chromatin after fertilization. Although we cannot formally rule out epitope exclusion, the pattern of H2A.Z disappearance indicates that H2A.Z might be removed from chromatin through an active mechanism. Interestingly, Ino80 chromatin remodeler was shown to be responsible for eviction H2A.Z in yeast (Papamichos-Chronakis, Watanabe et al. 2011). Given that embryonic chromatin undergoes decondensation and remodeling in the zygote, Ino80 could potentially serve both purposes – participate in global changes in chromatin structure as well as evict H2A.Z. H2A.Z transcripts in detected in the zygote and the 2-cell stage (Kafer, Lehnert et al. 2010), while protein is only very weakly present on embryonic chromatin at these stages. This suggests that something is either blocking H2A.Z deposition or retention on chromatin, or that is very rapidly removed.

At later stages of mouse preimplantation development, H2A.Z shows a robust localization on chromatin. Interestingly, when present, it is excluded from DAPI-rich regions corresponding to pericentric heterochromatin. Studies in yeast (Meneghini, Wu et al. 2003) have shown that H2A.Z is important for boundary formation between different chromatin regions, such as eu- and heterochromatin. Given its localization around DAPI-rich regions, it is possible that in mouse embryonic nuclei H2A.Z plays a similar role, arguing for the conservation of H2A.Z nucleosomes in stabilizing boundary elements. Conspicuously, H2A.Z becomes detectable on embryonic chromatin after (or concomitantly with) reorganization of pericentric heterochromatin from NLBs to

chromocentres, suggesting that it could at that point play a potential role in preventing heterochromatin spreading. However, another study reported that in late blastocysts H2A.Z is weakly present in ICM nuclei, and is first expressed in trophectoderm, where it is targeted to pericentric heterochromatin (Rangasamy, Berven et al. 2003). The authors show that H2A.Z can biochemically directly interact with the centromeric protein INCENP. Furthermore, they report that H2A.Z is depleted from the inactive X-chromosome and other macroH2A foci in female TE cells. H2A.Z was subsequently shown to be ubiquitylated on its C-terminus and H2A.Zub is enriched on facultative heterochromatin (Sarcinella, Zuzarte et al. 2007). Because the antibody routinely used to detect H2A.Z that is commercially available does not recognize H2A.Z when H2A.Z is ubiquitylated, it is possible that the antibody recognizing C-terminal part of H2A.Z in the study by Rangasamy and colleagues, could not detect all H2A.Z.

1. H2A.Z on embryonic chromatin – timing is key

H2A.Z is the first histone variant shown to be essential in mammals (Faast, Thonglairoam et al. 2001). Zygotic H2A.Z knock-out led to embryonic lethality at perimplantation. A detailed dissection of H2A.Z requirement in development showed that H2A.Z-/- embryos can reach the blastocyst stage and that maternally loaded protein is depleted by that point. However, embryos fail to develop after hatching, a time when ICM undergoes reorganization and lineage segregation into epibalst and primitive endoderm. ICM cells from H2A.Z -/- blastocysts were unable to proliferate normally, suggesting a specific role of H2A.Z in differentiation and proliferation, but not in cell survival, as TE cells were still viable. The same study targeted H2A.Z transcript by dsRNA from the zygote, and reported that the effect of H2A.Z depletion was only evident in the late blastocyst, but not in earlier preimplantation stages.

Our experiments showed that H2A.Z is very weakly present during zygote and 2-cell stage, thus we opted for a converse approach to the one described above. We decided to re-introduce H2A.Z in the embryo when it is normally absent. This would allow us to understand if H2A.Z disappearance is functionally important for normal

developmental progression and to further characterize the effects of H2A.Z incorporation on embryonic chromatin. We observed that embryonic development is impaired after ectopic H2A.Z expression in the zygote. A significant portion of embryos injected with H2A.Z arrested at the time of compaction. However, if H2A.Z is specifically detrimental to the process of lineage allocation or the embryos simply can no longer cope with excess H2A.Z and thus arrest later in development (8-cell and morula) is unclear.

H2A.Z-packaged nucleosomes were shown to contribute to the formation of transcriptionally permissive local chromatin structures in *Drosophila* (Weber, Henikoff et al. 2010). While biophysical studies show that H2A.Z actually stabilizes the nucleosome when paired with canonical H3 (Park, Dyer et al. 2004), the situation is reversed when H2A.Z nucleosomes contain H3.3. Indeed, H2A.Z/H3.3 nucleosomes were shown to be particularly unstable, underlying the importance of binding partners on the overall biophysical properties of the nucleosome (Jin and Felsenfeld 2007). In our experiments, the H2A.Z-injected embryos that reach the blastocyst stage seem morphologically normal. On a chromatin level, however, they exhibit smaller and more dispersed (less bright) chromocentres. Two possible explanations for this effect can be proposed based on our knowledge of H2A.Z biology. Firstly, an attractive hypothesis could be that H2A.Z overexpression is in some way leading to impaired heterochromatinization, or that the boundaries between heterochromatin and euchromatin created by H2A.Z are spread into heterochromatin, potentially leading to aberrant repression. It would be interesting to investigate the transcriptional output of the H2A.Z injected embryos compared to the control ones to understand better how H2A.Z effects heterochromatin formation and gene silencing in the embryo. RNA-FISH experiments to detect major satellite activity could be a good starting point. Secondly, H2A.Z overexpression might affect clustering of chromocentres, and not necessarily their formation on individual chromosomes. This would be in line with biophysical studies from the Tremethick lab that show that H2A.Z-containing nucleosomes promote intrafiber interactions but antagonize interfiber contact and folding (Fan, Gordon et al. 2002). A detailed quantitation of the size and volumes of H2A.Z injected nuclei as well as chromocentre counting is the next step in discerning which of the two possibilities is predominant.

If H2A.Z in indeed antagonizing heterochromatin compaction, removing H2A.Z and macroH2A from chromatin after fertilization would have similar functional outputs. While macroH2A disappearance would facilitate chromatin reorganization and reprogramming, H2A.Z removal could allow for de novo heterochromatin formation and subsequent clustering. Thus, H2A.Z incorporation later on, when chromatin is globally already set in place, could promote stage-specific gene expression. A parallel can be drawn between macroH2A and ubiquitylated H2A.Z, which are both enriched on facultative heterochromatin. However, H2A.Zub constitutes a small fraction of H2A.Z pool (Sarcinella, Zuzarte et al. 2007), making its biological outputs very specific.

2. Marks of active chromatin follow unusual patterns in the mouse embryo

H3K36me3 is a canonical mark of transcriptional elongation. It is set in place by the NSD methyltransferases during elongation to ensure unidirectional transcriptional progression and prevent pol II backtracking (Wagner and Carpenter 2012). In the zygote, H3K36me3 is localized only on maternal chromatin, while paternal chromosomes never seem to acquire this mark until two cell divisions later. Maternal K36me3 could be a remnant of intense transcription taking place during oocyte maturation, but it is surprising that it cannot be detected on paternal chromatin as it was shown that the male genome is transcriptionally active in the zygote (Aoki, Worrad et al. 1997; Bouniol-Baly, Nguyen et al. 1997). Even more interestingly, by the 2-cell stage, H3K36me3 is undetectable on chromatin altogether, suggesting that H3K36 is demethyalted on the maternal chromatin very rapidly, much like H2A.Z. How this comes about is unclear. The demethylases responsible for H3K36me3 removal (KDM4A-E) (Black, Van Rechem et al. 2012) are expressed during early development (Burton, Muller et al. 2013), and as will later be discussed, histone exchange rates are very fast after fertilization. It is possible that both mechanisms contribute to the rapid disappearance of H3K36me3 by the 2-cell stage.

Apart from H2A.Z acetylation being a mark of open promoters and active transcription, acetylation of H2A.Z has also been described as the posttranslational modification important for eviction of wrongly incorporated H2A.Z in yeast (Papamichos-Chronakis, Watanabe et al. 2011). As H2A.Zac levels are quite correlated with overall H2A.Z levels in our staining experiments, it is difficult to discern whether it is H2A.Z acetylation that causes and/or promotes early H2A.Z eviction in the zygote. This remains an interesting possibility to address in the future.

An interesting question arises from our profiling of 'activating' histone marks. In the embryo, how reliable are histone modifications in predicting transcriptionally active or repressive chromatin? In other words, can we reliably correlate the presence of a histone PTM with a certain chromatin-regulated process? For instance, γH2A.X is very abundant in the zygote in the absence of DNA damage (Ziegler-Birling, Helmrich et al. 2009), (Wossidlo, Arand et al. 2010). We show that H3K36me3 and H2A.Zac are undetectable at the time of robust expression of embryonic genome. Pericentric heterochromatin is silenced in the absence of H3K9me3 and H4K20me3 (Puschendorf (Puschendorf, Terranova et al. 2008), (Santenard 2010). These and other examples indicate that we should be cautious when ascribing a functional role to a certain chromatin posttranslational modification in the mouse embryo. The characteristics of embryonic chromatin seem to be more an exception than a rule, and very few correlations between somatic cells and embryos can be taken for granted.

B. Publication 2

Part 3. Chromatin dynamics during early mouse embryogenesis

Publication 3

I. Summary of Publication 3

Extensive changes in chromatin organization and composition take place in the mouse embryo from fertilization to the formation of the blastocyst. At the same time, the embryo progresses from totipotency in the zygote and 2-cell stage, to pluripotency and differentiation during early blastocyst formation. These concomitant events are thought to be linked, but the evidence connecting changes in chromatin organization and cellular potency are scarce. This prompted us to ask whether chromatin dynamics are functionally linked to cell potency. To answer this question we measured histone exchange at the beginning of development and after lineage allocation in the blastocyst. We used an optical technique called FRAP (Fluorescence Recovery After Photobleaching) to determine the dynamic properties of core histones in the developing mouse embryo.

One of the most strinking observations was the unusually high core histone mobility at the 2-cell stage. This was followed by a global reduction of histone mobility at the 8-cell stage. Measurements of histone dynamics after lineage allocation revealed that pluripotent ICM cells exhibit higher histone kinetics compared to the cells in the multipotent TE. Importantly, lineage allocation from the 2-cell stage, induced by CARM1 overexpression, led to increased H3.1-GFP dynamics already at the 8-cell stage, suggesting a functional link between chromatin and cell plasticity. The high core histone mobility in the embryo prompted us to investigate the ultrastructural properties of embryonic nuclei. Thus, together with another Ph.D student in the laboratory, we characterized the chromatin ultrastructure of 2-cell and 8-cell stage mouse embryos by transmission electron microscopy. Finally, we utilized a novel in vitro system for totipotency, the 2-cell like ES cells, to investigate if histone mobility is a conserved property of totipotent cells.

1. Investigating histone mobility in the embryo by *in vivo* FRAP

Before experimentally addressing the question of histone mobility during development, we had to optimize the FRAP protocol, normally used in cells, to the developing embryo. It was imperative to establish experimental conditions that allowed for unperturbed developmental progression, as otherwise our results would have been less reliable. Thus, microscopy settings were optimised to obtain reproducible results without affecting normal development. Histone incorporation into chromatin was verified in 2 independent ways. Likewise, we tested different mRNA concentrations and injection timings to ensure that recovery curves do not depend on protein concentration, and that they indeed reflect protein dynamics at the embryonic stage analysed.

2. Canonical core histones are highly dynamic in 2-cell stage nuclei

Firstly, mobility of core histones was measured in embryonic nuclei. Zygotes were injected with mRNAs coding for H2A-GFP, H3.1-GFP, H3.2-GFP or H3.3-GFP, and FRAP experiments were performed at the 2-cell and 8-cell stage. The experimental region of interest within the nucleus was randomly chosen (apart from excluding the NLBs), so that obtained fluorescence recovery curves reflect overall chromatin dynamics. Canonical core histones H2A, H3.1 and H3.2 showed unusually high mobility in 2-cell stage nuclei, which was significantly reduced at the 8-cell stage. These results suggested that epigenetic reprogramming after fertilization is accompanied or facilitated by high core histone exchange. Interestingly, the histone variant H3.3, often associated with transcriptional activation and open promoter regions, was not highly mobile at either of the developmental stages tested. More detailed investigation of H3.3-GFP

dynamics at the 2-cell stage revealed that H3.3 is slightly more mobile in euchromatin compared to heterochromatin, but still substantially less mobile in comparison to the two canonical H3 histones.

3. H3.3-GFP mobility in different subnuclear compartments at the 2-cell stage

We could observe two populations of H3.3 in the early embryo – one uniformly present in the nucleosplasm and the other associated with pericentric rings surrounding the NLBs (and later chromocentres) (Figure 24). The ring-like localization of H3.3 in 2-cell stage embryo was not surprising given the reported roles of H3.3 (and particularly K27 methylation) in establishment of pericentric heterochromatin from the zygote onward. We wondered if low mobility of H3.3 at the 2-cell stage is a result of preferential inclusion of heterochromatic regions in our measurements.Therefore, we investigated H3.3-GFP mobility in euchromatin versus heterochromatin. We observed that H3.3-GFP is more dynamic in euchromatin compated to heterochromatic rings. However, even in euchromatin, H3.3 mobility was always substantially lower compared to H3.1 and H3.2 at the same embryonic stage. These results indicate that, even though there are compartmental differences contributing to global H3.3 mobility, this histone variant is tightly associated with embryonic chromatin already at the 2-cell stage.

4. Chromatin ultrastructure of early embryos, revealed by TEM

The unusually high histone mobility at the 2-cell stage that we reported raised questions about chromatin compaction and overall chromatin structure during development. We thus sought to investigate the ultrastructure properties of embryonic chromatin. For this, another student in the laboratory performed transmission electron microscopy on 2-cell and 8-cell stage embryos. Electron micrographs of 2-cell embryos revealed that nuclei are largely devoid of electron dense regions, suggesting loose chromatin conformation.

Notable exceptions were the NLBs, known to contain nucleophosmin and other electron dense components (Biggiogera, Burki et al. 1990). On the other hand, 8-cell stage nuclei already contained functional nucleoli and more electron density was observed throughout the nucleoplasm and close to the nuclear periphery. A global increase of electron density (ED) during development was confirmed by quantification of the proportions of ED regions in 2-cell and 8-cell stage nuclei. TEM results therefore indicate that embryonic chromatin undergoes progressive compaction as development progresses.

5. Pluripotent cells of the blastocyst exhibit higher chromatin mobility compared to TE cells

Blastocysts comprise 2 different cell types: pluripotent ICM cells and multipotent trophectoderm (TE) cells. To understand if lineage allocation affects chromatin mobility, we investigated H3.1-GFP mobility in both cell types of the blastocyst. Interestingly, the overall H3.1-GFP mobility was very low in the blastocyst, with a mobile fraction of approximately 5-8% in both lineages. However, pluripotent nuclei of the ICM exhibited higher H3.1-GFP mobility and kinetics compared to trophectoderm nuclei. This result indicated that cells with pluripotent features display higher chromatin dynamics after cell fate allocation, compared to more differentiated cells.

6. Lineage allocation to the ICM causes increased H3.1 mobility

The differences in H3.1-GFP recovery rates that we observed between the ICM and the TE led to the question if cell plasticity is linked to chromatin dynamics. To address this question experimentally, we took advantage of the finding that overexpression of the CARM1 methyltransferase at the 2-cell stage induces ICM fate to the progeny of the

injected cell. We first microinjected H3.1-GFP mRNA in zygotes, developed the embryos to the 2-cell stage, and performed a second microinjection with CARM1 mRNA together with RFP as lineage tracer in one of the 2-cell stage blastomeres (fig 3C). FRAP experiments for H3.1-GFP were performed at the 8-cell stage on CARM1 positive (destined to ICM) and negative blastomeres. CARM1-positive cells, destined to become ICM, displayed higher core histone mobility compared to their negative counterparts. Importantly, a catalytically inactive CARM1 mutant, which does not induce cell fate allocation to the ICM, did not cause increased H3.1-GFP mobility. These results indicate that cell potency and chromatin dynamics are functionally connected.

7. Transient modulation of histone marks did not significantly affect chromatin mobility in the embryo

Histone PTMs can directly or indirectly influence chromatin organization and function. We thus wondered: What is the contribution of histone modifications in the regulation of histone mobility? In other words, can we modulate histone recovery rates by changing the levels of histone modifications? To address this question, we treated embryos with chemical inhibitors of either a) histone deacetylases (TSA) or b) the histone methyltransferase G9a, at the 8-cell stage of development. Both treatments caused the expected effects on embryonic chromatin – histone acetylation was more abundant in upon TSA treatment, and H3K9 dimethylation was reduced when embryos were treated with the G9a inhibitor BIX01294. However, neither of the chemical inhibitors caused a dramatic change in histone recovery rates. Interestingly, inhibition of G9a resulted in a slight increase in core histone mobility, particularly affecting H2A-GFP mobility. While this change was not statistically significant, it points to the potential importance of histone methylation levels in regulating chromatin plasticity in the embryo.

8. 2-cell like ES cells recapitulate high histone mobility observed in early embryos

It was recently discovered that a small fraction of cells within ES cell populations can exhibit totipotent-like features, such as lack of pluripotency markers (e.g OCT4), expression of 2-cell stage specific genes, and contribution to embryonic and extraembryonic lineages. These cells, named 2-cell like (2-CL) cells, arise stochastically at low frequency (< 0.5% of the population) and are characterized by upregulation of the MuERV-L retroelement. To investigate whether high histone mobility is a feature of totipotency we asked if 2-CL cells display high histone mobility like 2-cell stage embryos. Together with another post-doc in the laboratory, a reporter ES cell-line was generated in which 2-CL cells can be identified by the presence of the tandem Tomato (tdTomato) fluorescence protein, which is under the control of the regulatory regions of MuERV-L. We transfected this cell line with plasmids harboring either H2A-GFP or H3.1-GFP and performed FRAP experiments in 2-CL cells as well as neighboring ES cells. Cells with totipotent features showed markedly higher histone dynamics compared to their pluripotent counterparts within the same population. This finding strongly suggests that acquisition of, or reprogramming to totipotency entails high levels of histone exchange.

9. Conclusions

In this work, we optimized the FRAP technique to investigate chromatin dynamics in the mouse embryo. We were able to probe histone mobility in the embryo at different stages of development. Our results indicate that chromatin of 2-cell stage mouse embryos is characterized by unusually high core histone exchange, which dramatically decreases as development progresses. Interestingly, the histone variant H3.3 remains relatively immobile on chromatin throughout early embryogenesis. Ultrastructure of embryonic

nuclei revealed progressive chromatin condensation during development and an almost complete absence of presumably higher-order chromatin structures at the 2-cell stage. After lineage allocation, pluripotent ICM cells exhibit higher dynamics of H3.1-GFP compared to the trophectoderm cells. Importantly, already at the 8-cell stage, cells destined to become ICM display higher histone mobility compared to neighbouring cells, implying that cellular states can be distinguished by the level of chromatin plasticity. Finally, ES cells with totipotent-like properties show higher chromatin dynamics compared to pluripotent ES cells in culture suggesting that high histone dynamics support totipotency in vivo and in vitro.

II. Publication 3

III. Unpublished results

1. Mobility of HP1β during mouse preimplantation development

HP1β is the major HP1 isoform present during early mouse development (van der Heijden, Dieker et al. 2005), (Arney, Bao et al. 2002). It contributes to the formation and maintenance of heterochromatin during cleavage stages of embryogenesis. HP1α, normally present in somatic cells as the main HP1 isoform associated with constitutive heterochromatin, is not expressed during early development.

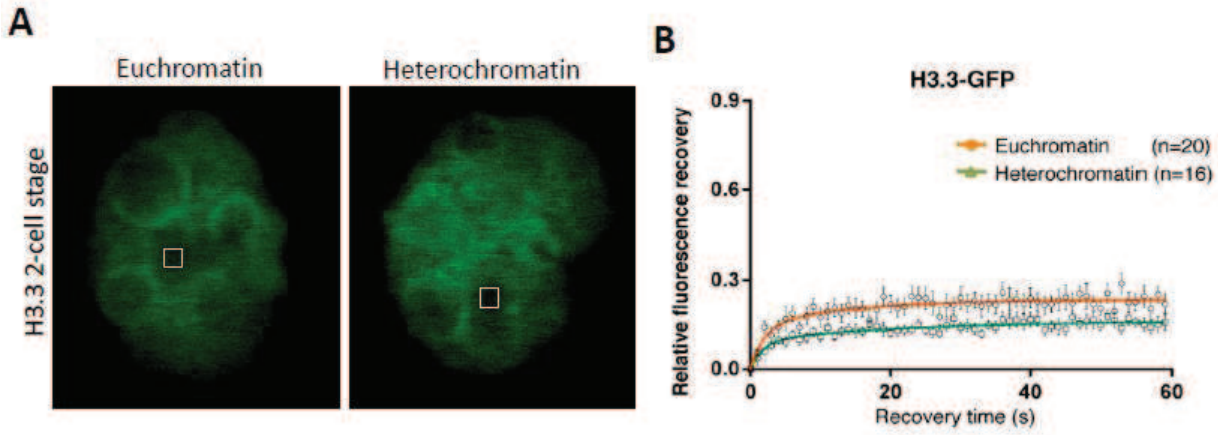

Figure 24. Region-dependent H3.3-GFP mobility at the 2-cell stage. (A) FRAP experiments for H3.3-GFP at the 2-cell stage, investigating small regions of interest (ROI) within the nuclei, to discriminate between mobilities in heterochromatin rings versus nucleoplasm (euchromatin). Mobility of H3.3-GFP was investigated in the ROI of 1 mm^2. (B) Euchromatic pool of H3.3-GFP is more mobile, and displays slightly higher recovery kinetics, compared to heterochromatic H3.3-GFP. Mobile fraction of H3.3 in euchromatin was 14±6% in the fast recovering fraction and 9.4±5% in the slow recovering fraction, compared to 9±2% (fast) and 7±1% (slow) in heterochromatin.

Because the embryonic heterochromatin is very unusual in its properties, discussed in the introduction, we wondered if HP1β dynamics in embryonic nuclei are also different compared to somatic cells or ES cells. Furthermore, we observed high

histone mobility during 2-cell stage of development and wondered if this property is shared by non-histone chromatin proteins in the early mouse embryo.

Firstly, we synthesized mRNA encoding for HP1β-GFP in vitro, and injected zygotes with this mRNA, at a concentration of 280 ng/μl. We could observe clear GFP signal in embryonic nuclei already in the zygote, suggesting that the mRNA is efficiently translated and HP1β-GFP is correctly localized in both pronuclei. At the 2-cell stage of development, we could distinguish two populations of HP1β-GFP: a) one uniformly distributed in the nucleoplasm, and b) another one in regions of high HP1β-GFP density. We reasoned that the former population corresponds to euchromatic HP1β and the latter to HP1β on heterochromatin.

To investigate if different subnuclear compartments exhibit distinct HP1β dynamics we decided to measure HP1β mobility in both euchromatin and heterochromatin by FRAP at the 2-cell and 8-cell stage of development. The experimental setup used for HP1β FRAP was the same as the one used for core histones, outlined in Publication 3.

We observed that HP1β is highly mobile in the two developmental stages analysed. The recovery rates in the embryo were comparable to previous reports of HP1β dynamics in different cell lines (Schmiedeberg, Weisshart et al. 2004). Interestingly, HP1β associated with either euchromatin or heterochromatin was highly dynamic, with virtually identical mobile fractions between the two populations (fig). However, the kinetics of HP1β recovery in different nuclear subcompartments were distinct. The HP1β pool associated with heterochromatin recovered more slowly compared to the nucleoplasmic pool. Close inspection of earliest timepoints of HP1β recovery after photobleaching revealed that the kinetic rates were about 1.4 times faster in euchromatin compared to heterochromatin (Figure 25). These results suggest that HP1β resides on the chromatin for a longer time in heterochromatin context.

We next investigated HP1β dynamics in the two cell types of the blastocyst. At the blastocyst stage, HP1α is expressed and becomes the predominant heterochromatin protein associated with H3K9 trimethylation and pericentric heterochromatin (Lachner,

O'Carroll et al. 2001). In line with this, microinjected HP1β–GFP was mostly uniformly distributed in blastocyst nuclei and was rarely observed in heterochromatic foci (fig). Distribution of HP1β at the blastocyst stage was also checked by immunostaining experiments against the endogenous HP1β protein (not shown).

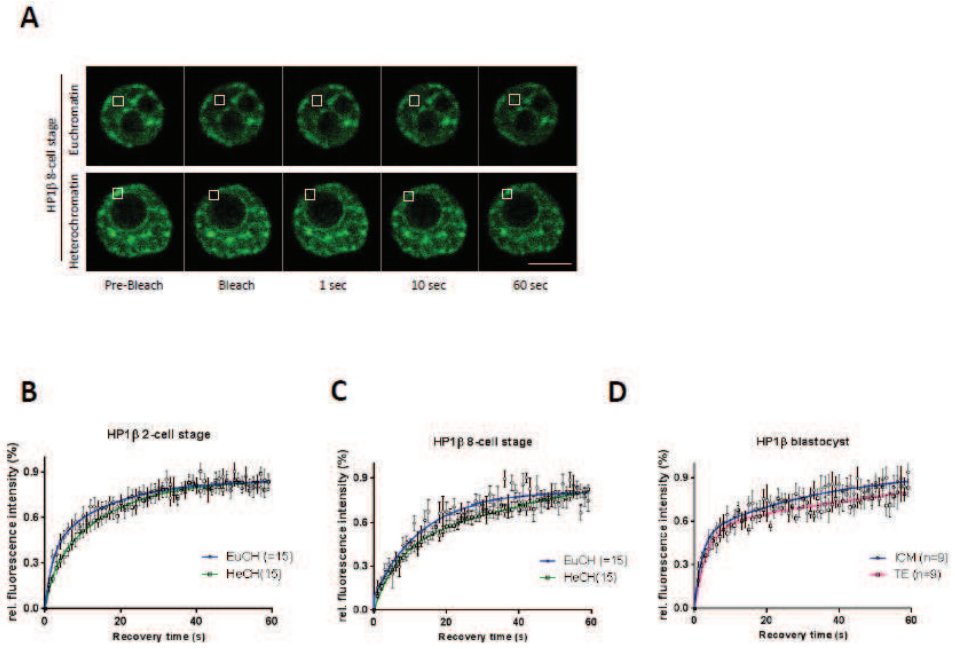

Figure 25. Mobility of HP1β during preimplantation mouse development. (A) Representative FRAP experiments for HP1β at the 8-cell stage. HP1β dynamics were investigated in euchromatin (top panel) and heterochromatin (bottom panel). Bleached regions are shown by a rectangle. Scale bar = 15 mm. FRAP curves for HP1β at the 2-cell stage (B) and the 8-cell stage (C) are shown. Green curves correspond to HP1β mobility in heterochromatin and the blue curves to HP1β recovery in euchromatin. (D) HP1β recovery curves in different cell types of the blastocyst. ICM is represented by blue and TE by pink curves.

As with H3.1-GFP, we measured HP1β recovery in single ICM and TE nucleus per embryo. Overall, HP1β retained high exchange rates after lineage allocation (fig), very similar to earlier developmental stages. Interestingly, kinetics of HP1β association with chromatin were slower in the TE nuclei compared to ICM, in both slow and fast recovering protein fractions. It should be pointed out, however, that the final average

curves consist of only 9 experiments for each cell type, and additional experiments will be performed to ascertain the reliability of these results.

2. Effect of H1.0 overexpression on histone dynamics

The first level of chromatin organization, which is also the least condensed form of chromatin, is the so-called 'beads on a string' 10-nm chromatin fiber. These fibres comprise nucleosomal arrays which are generally not associated with linker H1 (or H5) histones. As mentioned above, transmission electron micrographs of 2-cell stage embryonic nuclei revealed a global absence of electron dense regions, suggesting that the chromatin of early embryo exists in a state of low condensation, possibly consisting mostly of extended 10-nm fibres not arranged in higher-order chromatin structures. These results were indirectly corroborated by the high core histone mobility observed by FRAP at the same developmental stage. We reasoned that high histone mobility and general lack of electron dense regions at the 2-cell stage could be (at least partly) due to absence of linker H1 histone (Clarke, McLay et al. 1998). If this is the case, we hypothesized that expression of linker histones after fertilization could affect chromatin condensation and potentially histone mobility. To address this directly, we sought to express exogenously H1 after fertilization. We chose H1.0 because it was shown that linker histones H1.4 and H1.0 (the latter one being expressed in terminally differentiated and G0 cells) most strongly promote chromatin compaction. (Clausell, Happel et al. 2009). I thus first cloned human H1.0 with an N-terminal HA tag and synthesized the corresponding mRNA in vitro. We performed co-microinjection of H3.1-GFP along with HA.H1.0 mRNAs in zygotes and asked if H3.1-GFP mobility is affected at the 2-cell stage by performing FRAP as above. Two different concentrations of HA.H1.0 mRNA (100 ng/μl and 200 ng/μl) were used in different sets of experiments.

Firstly, we verified that H1.0 mRNA was efficiently translated and that H1.0 associates with embryonic chromatin. To do so, we performed immunostaining experiments on 2-

cell stage embryos against the HA tag, and observed a clear HA signal in the nuclei, marked by DAPI (not shown).

Next, we addressed whether H3.1-GFP mobility was altered in H1-injected embryos. FRAP experiments and data processing were as outlined in Publication 3. We compared H3.1-GFP recovery curves of H1.0-injected embryos to the recovery of H3.1-GFP (without H1.0 expression, Publication 3, figure 2B). Surprisingly, H3.1-GFP mobility was virtually unaffected by H1.0 expression, and the recovery curves were almost perfectly overlapping (Figure 26).

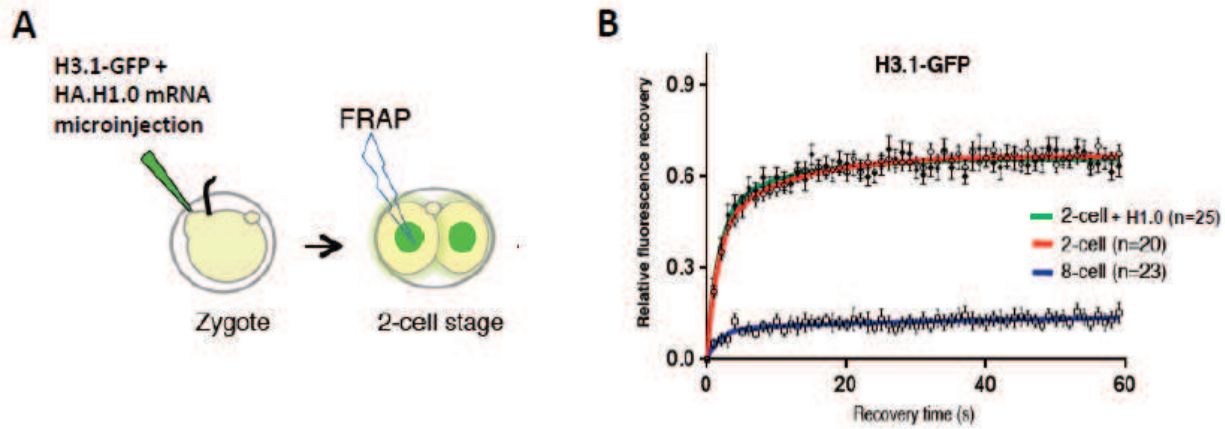

Figure 26. Effects of H1.0 overexpression in the zygote on H3.1-GFP mobility. (A) Experimental setup. Zygotes were co-injected with H3.1-GFP mRNA and HA.H1.0 mRNA. FRAP experiments for H3.1-GFP mobility were performed at the 2-cell stage. (A) Recovery curves for H3.1-GFP at the 2-cell stage (red), 8-cell stage (blue) and 2-cell stage after H1.0 injections (green). Mean values (±SEM) of indicated number of experiments are shown.

These results could point to several conclusions. Firstly, it is possible that H1.0 overexpression does not affect chromatin compaction and/or that there is an uncoupling of histone dynamics and chromatin compaction in the mouse embryo. However, since we did not investigate the ultrastructure of the H1.0-injected embryos, it is so far impossible to conclude on H1.0 effects on chromatin compaction at the 2-cell stage. Secondly, it is possible that the mRNA concentration that I used for H1.0 expression is not high enough to observe an effect on chromatin compaction, and experimental titration of H1 concentrations needs to be done. Finally, the choice of H1 variant could be an important factor when investigating changes in chromatin dynamics in the

embryo. Extensive profiling of H1 variants during preimplantation mouse embryogenesis is needed to understand their function in epigenetic regulation of development. Unfortunately, this has so far been impossible to address given the lack of antibodies specific for the somatic H1 variants.

3. The mobility of core histones in ES cells in different culture conditions

It is known that culture conditions and media composition can influence the biological properties of ES cells (Ying, Wray et al. 2008; Silva, Nichols et al. 2009). ES cells can be maintained in different states of pluripotency – 'ground-state' or 'primed'. We thus wondered if histone mobility differs depending on the stability of pluripotency of ES cell cultures. To address this, we performed FRAP experiments in ES cells grown in standard conditions (serum + LIF) and 2i (serum + 2 inhibitors) conditions.

ES cells were transiently transfected with plasmids harboring H3.1-GFP or H3.2-GFP using LIPOFECAMINE 2000 and FRAP experiments were performed 24 hours after transfections. The fluorescence levels were comparable between standard (S+L) and 2i-grown ES cells and all experimental settings and normalization methods were identical for both groups, making the obtained results directly comparable.

FRAP experiments in ES cells in different culture conditions yielded different recovery curves for H3.1-GFP and H3.2-GFP. The mobility of core histones was lower in 2i-grown ES cells, compared to S+L conditions (figure). The observed difference is most likely not due to inclusion of differentiating cells into analysis which might skew the recovery rates, as the majority of both populations (<90%) comprised of undifferentiated ES cells positive for OCT4 as assessed by immunostaining (fig). The observed H3.1-GFP and H3.2-GFP mobility were relatively low in our experiments, with mobile fractions for both histones of about 5-8% in 2i condition and 12-15% in serum.

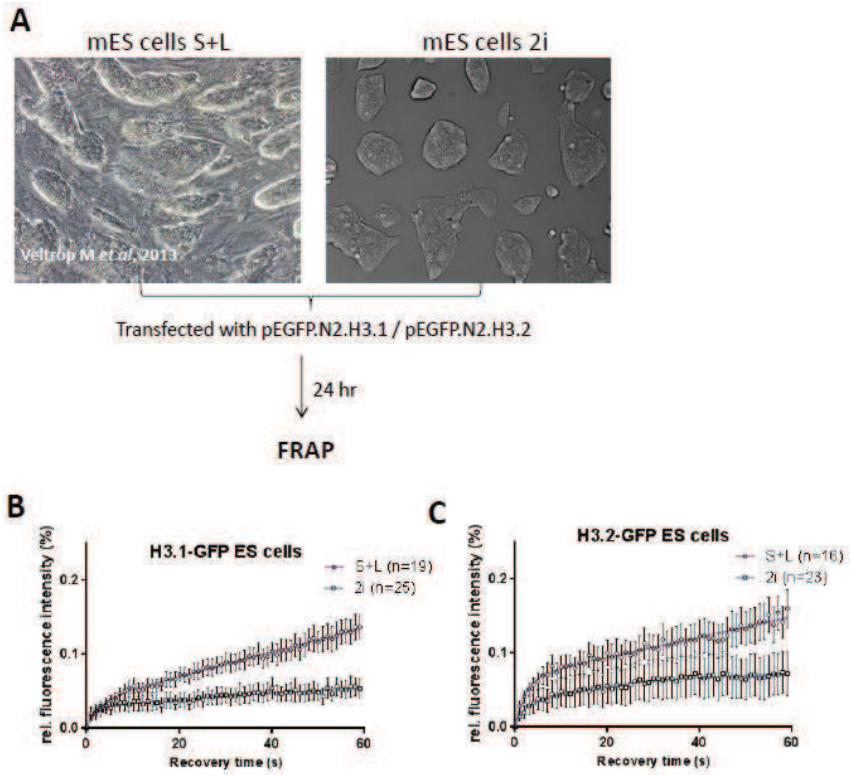

Figure 27. Investigating H3.1-GFP and H3.2-GFP mobility in mouse ES cells grown in different culture conditions. (A) Mouse ES (mES) cells, maintained either in standard (serum + LIF) or 2i (serum + 2 inhibitors) conditions were transfected with plasmids encoding for either H3.1-GFP or H3.2-GFP. FRAP experiments were performed 24 hours after transfection. (B) Recovery curves for H3.1-GFP in two ES cell populations. (C) Recovery curves for H3.2-GFP in two ES cell populations. (B and C) Shown are average values (±SEM) of an indicated number of experiments. Mean recovery values were fit into a two-phase exponential association equation.

These results suggest that pluripotency states could be distinguished by differential histone mobility and that chromatin plasticity correlates with the stability of cell states. However, further characterization of dynamics of other histones and chromatin factors is necessary to conclude on the extent of chromatin influence on stability of pluripotency.

IV. Discussion

Measurements of histone-GFP mobility in the early mouse embryo revealed high levels of core histone exchange and dynamics, unparalleled in other mammalian systems. Most mammalian cell lines in which histone dynamics were investigated revealed very low histone mobility, indicating stable association of core histones on chromatin (Kimura and Cook 2001), (Kanda, Sullivan et al. 1998). Notable exceptions were ES cells, which are characterized by elevated H3.1-GFP and H2A-GFP dynamics (Meshorer, Yellajoshula et al. 2006).The chromatin during early developmental stages thus seems to accommodate global rearrangements and changes in its composition. Interestingly, high histone mobility early in development was also reported in *Drosophila* embryos. When measuring H2B-GFP recovery rates in the *Drosophila* syncytial blastoderm by FRAP and FLIP, large scale histone exchange between the nuclei and the yolk was observed before cellularisation. The rates of H2B-GFP mobility were reduced to negligible levels after cellularisation (Bhattacharya, Talwar et al. 2009). Interestingly, immediately after cellularisation, H2B-GFP mobility remained high, but with time, and correlating with the formation of specialized domains of compacted chromatin, histone exchange was reduced to basal levels. The increase of chromatin rigidity at the time of H2B-GFP stable incorporation was further confirmed by fluoresce anisotropy imaging. These results suggest that high histone mobility before cell fate allocation and differentiation could be an evolutionarily conserved mechanism important for maintaining chromatin plasticity at the onset of development.

1. Chromatin dynamics and embryonic genome activation in the mouse embryo

In light of chromatin structure posing as a barrier for DNA-acting processes, including transcription (Prioleau, Huet et al. 1994); (Almouzni and Wolffe 1995) one could imagine that high histone mobility at the 2-cell stage contributes to embryonic

genome activation. On the other hand, it could be argued that the opposite is true – robust transcription of embryonic genome is the potential cause of high histone mobility. However, it is very well possible that the two interpretations are not fully mutually exclusive. Activation of the embryonic genome is likely facilitated by loose chromatin organization, but could also in turn contribute to the overall histone exchange rates that we observed at the 2-cell stage. Electron microscopy results obtained in this work suggest that the chromatin of 2-cell stage embryos is largely devoid of higher-order chromatin structures. As it was reported that enhancer engagement is important for the expression of the mouse embryonic genome (Majumder and DePamphilis 1995; Schultz 2002), it is plausible that in this context enhancer-promoter looping could occur on a global level. In line with this hypothesis, a study of gene expression in the mouse embryo reports that *hsp70*, usually requiring enhancer promoter association, is efficiently expressed with or without TSA at the 2-cell stage (Thompson, Legouy et al. 1995). The authors hypothesize that nucleosomes at that time are positioned so that they do not interfere with promoter binding sites, allowing for unhindered promoter engagement.

2. H3.3 – variant of choice during early development

The canonical core histones H2A, H3.1 and H3.2 displayed high mobility in 2-cell stage mouse embryos. On the other hand, the exchange of the histone variant H3.3 was very low, and remained constant during development. A similar observation about H3.3 dynamics comes from ES cells in which H3.3-GFP mobility does not change during differentiation. The authors attributed such low H3.3 dynamics in ES cells to high levels of transcription, as H3.3 is thought to be a marker of transcriptionally active chromatin (Ahmad and Henikoff 2002). In the mouse embryo, H3.3 was already described as an important histone variant in *de novo* establishment of pericentric heterochromatin on the male pronucleus (Santenard 2010). In this study, we observed that H3.3 is stably associated with embryonic chromatin regardless of its subnuclear localization. A potential explanation for the overall low mobility of H3.3 is that it could be the predominant H3 variant during earliest developmental stages, in the absence of stable

H3.1 or H3.2 binding. Indeed, in HeLa cells, H3.3 was shown to be incorporated through a nucleosome-gap filling mechanism when H3.1 incorporation is impaired, ensuring genome stability (Ray-Gallet, Woolfe et al. 2011). Our results raise the questions of what we consider as histone (replacement) variants. While RI-histones are generally less abundant than RD-histones, and play specialized roles at specific genomic loci, this is maybe not the case in the early mouse embryo. The relative abundance of different H3 variants during gametogenesis was investigated by mass spectrometry (unpublished). The results of that work indicated that during oogenesis, there is a shift in the relative proportions of H3 variants. While H3.1 (and H3.2, to a lesser extent) levels decrease, H3.3 becomes more abundant and represents about 30% of all H3 variants in fully grown oocytes, compared to 10% in somatic cells. One could argue that at the 2-cell stage H3.1 and H3.2 are in fact 'replacement' variants while the organization of embryonic chromatin relies mostly on H3.3. Later in development, at the 8-cell stage, there is a global incorporation of H3.1 and H3.2, concomitantly with changes in overall chromatin organization.

Interesting work in *Drosophila* by the lab of Kami Ahmad found non-redundant functions of histone H3 variants in heterochromatin silencing and specifically linked canonical H3 as the important histone variant in heterochromatin maintenance and transcriptional silencing. Using RNAi in fly eye cells to knock-down histone mRNAs during S-phase, they observed that heterochromatin maintenance was impaired when the canonical H3 (H3.2 in Drosophila) is depleted. This was presumably, and logically, due to more nucleosome free regions after DNA synthesis as H3 was unavailable to package newly synthesized DNA. 'Nucleosome-depleted' DNA was now more accessible to transcriptional machinery leading to increased expression of various genes, including the ones in heterochromatin. Interestingly, though, rescue experiments with H3.3 supplementation failed to restore heterochromatin silencing, and the active genes remained transcribed even in the presence of nucleosomes. Taking this into consideration, together with the fact EGA takes place at the 2-cell stage of mouse development, we can now envisage 3 potential roles of H3.3 incorporation into embryonic nuclei early in development. (1) H3.3 is important for de novo establishment of pericentric heterochromatin on the male genome. (2) The low mobility of H3.3 after

fertilization indicates a placeholder role in the absence of robust H3.1/H3.2 incorporation and (3) through global incorporation into the embryonic genome, H3.3 could facilitate high levels of transcription of at the beginning of development. While attractive, the connection between latter two hypotheses remains to be experimentally confirmed.

Whether other histone variants also exhibit low mobility after fertilization remains to be investigated. A potential starting candidate for investigation is H2A.X, as it was reported to be uniformly present during preimplantation development (Ziegler-Birling, Helmrich et al. 2009; Nashun, Yukawa et al. 2010), while other H2A variants, such as H2A.Z, show stage-specific abundance.

3. Linking cell fate decisions and chromatin mobility

Can cell fate be predicted even before the formation of the blastocyst, by measuring histone mobility? Ideally, one would be able to perform FRAP experiments in all blastomeres of an 8-cell stage embryo and develop them until the blastocyst. Then, lineage tracing could be done to correlate back the recovery curves of respective blastomeres to their cell progeny. This way we could discern if blastomeres with different levels of histone mobility at the 8-cell stage (high versus low) preferentially allocate to ICM or TE fate. However, this approach is technically unfeasible without perturbing development due to embryo sensitivity to phototoxicity.

Instead, we opted for an experimental approach in which we can induce cell fate from the 2-cell stage. CARM1 is an arginine methyltransferase catalyzing histone H3 arginine 2, 17 and 26 methylation (Chen, Ma et al. 1999). It was shown that overexpression of CARM1 already at the 2-cell stage directs cell fate to the ICM (Torres-Padilla, Parfitt et al. 2007).

Interestingly, when CARM1 is overexpressed in one of the 2-cell stage blastomeres, the daughter cells of the injected blastomere show higher H3.1-GFP mobility at the 8-cell stage compared to the neighbouring, CARM1-negative cells. Thus, cells destined to the

ICM, which could be considered ‘more pluripotent’ compared to their neighbours, retain elevated levels of histone exchange and chromatin plasticity. It was previously shown that daughter cells of CARM1-injected blastomeres exhibit elevated transcriptional rates and up-regulate *Nanog* expression. Taken together, these results suggest that CARM1 expression might also cause looser chromatin organization which is permissive to more efficient RNA polymerase II action. A potential mode of CARM1 action was previously proposed (Wu, Cui et al. 2012), whereby increased arginine methyation on H3R17 and H3R26 does not serve as a docking platform for effector proteins, but instead weakens the chromatin association with the NuRD and TIF1b transcriptional co-repressors. In conjunction with increased histone acetylation (An, Kim et al. 2004) (Daujat, Bauer et al. 2002), CARM1 could induce higher chromatin mobility, correlating with higher transcription levels.

Upon closer inspection of individual H3.1-GFP recovery curves at the 8-cell stage, we observed that about 25% of blastomeres exhibited higher H3.1-GFP mobility (‘8-cell high’), compared to others (‘8-cell low’) in wild-type embryos. This suggests that there are two populations of cells exhibiting two distinct chromatin dynamics in 8-cell stage embryos. Note however that since we averaged all the curves, the final result reflects a single, average of all experiments. Interestingly, H3.1 mobility in ‘8-cell high’ nuclei is comparable to the CARM1-positive cells at the 8-cell stage. The recovery curves of CARM1-positive blastomeres also comprise ‘high’ and ‘low’ populations, but with the inverse ratios. About 80% of CARM1-positive cells showed higher H3.1 mobility (mobile fraction of about 20%) compared to the 20% that had lower H3.1-GFP mobility (mobile fractions of ~10%, similar to CARM1-negative cells). These finding suggest that 8-cell blastomeres are not necessarily equivalent with regards to core histone mobility. By inducing cell fate through CARM1 overexpression and analyzing histone mobility in CARM1+ blastomeres, we are effectively enriching for the population of cells at the 8-cell stage with higher chromatin mobility. Previously, it was shown that the residence time of the pluripotency-associated transcription factor OCT4 on chromatin at the 8-cell stage correlates with different cell fates in the blastocyst, as revealed by a complementary optical technique, fluorescence decay after photoactivation (FDAP)(Plachta, Bollenbach et al. 2011). The authors showed that blastomeres in which

OCT4 spends more time on chromatin are more likely to become pluripotent ICM cells. It is possible that higher histone mobility in future ICM cells facilitates OCT4 action on chromatin by creating a more ‘open’ chromatin structure, making it easier for OCT4 to find and engage its target sites.

4. Cell plasticity versus potency of embryonic stem cells

ES cells are pluripotent cells with a unique ability to self-renew but also to differentiate into any cell type of the embryo proper. In principle, the self-renewal potential of an ES cell population is high, and ESCs can theoretically proliferate indefinitely. However, under standard culture conditions (serum + LIF), certain ES cells begin to differentiate spontaneously, suggesting that they display the propensity to exit the self-renewal pathway towards a lineage differentiation path. This state is defined as ‘primed’ pluripotency and is characterized by moderate levels of NANOG protein (Nichols (Nichols and Smith 2009). Interestingly, by modifying culture conditions, ES cells can be maintained at a level of so-called ‘ground-state’ pluripotency. In this case, the self-renewal pathway is predominant and cells virtually never differentiate spontaneously, forming uniform populations of pluripotent cells. This is a clear demarking between cell potency and plasticity, as the first is defined by the ability to generate different cell types while the latter refers to the relative ease by which they can do so. One could think that ‘ground-state’ ES cell populations are more pluripotent compared to their ‘primed’ counterparts. On the other hand, as the stability of pluripotency of ‘ground-state’ ES cells is so high, they are arguably less plastic; they constantly retain a defined and uniform cell fate (they give rise only to other ES cells unless signalling cascades are reactivated). Indeed, a study has shown that ES cells with lower levels of NANOG exhibit higher nuclear plasticity and deformability, as well as faster H2B-GFP recovery, compared to NANOG-high cells (Chalut, Hopfler et al. 2012). In our experiments, the mobility of the core histones H3.1-GFP and H3.2-GFP is also higher in ES cells maintained in standard conditions (serum+LIF) compared to those in 2i. It is thus possible to imagine that chromatin dynamics define the stability of cellular states and correlate with the cell’s ability to undergo changes in fate (cell’s

‘reprogrammability’). ES cells of primed pluripotency are more likely to exit self-renewal and change fate into a cell type other than ES cells, therefore their cell state is less-well defined and chromatin dynamics are higher in support of this potential. It would be very interesting to test if the increase in core histone mobility in 2i-grown ES cells would be sufficient to influence their differentiation frequency (i.e. whether they would behave like ‘primed’ ES cells).

In the mouse embryo, distinction between potency and plasticity is not so clear-cut. Embryonic nuclei are characterized by both high chromatin plasticity and high potency. This underscores the importance of choosing the appropriate experimental system when addressing questions related to cell potency versus plasticity.

5. What causes high histone dynamics in totipotent cells?

An important question arising from the observations in 2-cell stage embryos (and 2-CL cells) is: what causes high chromatin dynamics in totipotent cells? Given the timescale and extent of chromatin changes that take place during earliest embryonic stages, it is unlikely that this phenomenon is caused by a single factor, but rather by interplay of different chromatin-modifying events. The 2-cell stage of mouse development is very particular because it entails the global remodeling of heterochromatin (Probst and Almouzni 2011) and the activation of embryonic genome. Therefore, discerning which factors (or lack thereof) and processes contribute to histone hypermobility is not straight-forward. The histone mobility measured through FRAP is probably a cumulative result of local histone exchange rates but also of overall movements of chromatin fibres during heterochromatin reorganization. One could envisage that a combined action of chromatin remodeling complexes, histone chaperons and histone PTMs affects histone recovery rates. Systematic profiling of the expression of individual chromatin modifiers could be performed. Subsequently, analysis of histone dynamics upon their modulation through gain-of-function or loss-of-function approaches could be used as readout of the contribution of a particular modifier to overall chromatin mobility in the embryo. However, this approach is highly

demanding, especially in the early embryos. Limited material, challenging experimental procedures and the presence of maternal as well as embryonic transcripts and proteins are just some of the issues that require attention before conducting such experiments.

Transmission electron micrographs of 2-cell stage embryos revealed a general absence of heterochromatin, indicating that most of the chromatin fibers exist in a highly decondensed state. A report by Clarke (Clarke 1992) indicates that somatic H1 appears in the embryo only at the 4-cell stage of development. However, overexpression of the linker histone H1.0 in the zygote did not detectably affect H3.1-GFP recovery in the embryo. H1 itself is reported to be a highly dynamic chromatin component (Misteli, Gunjan et al. 2000). Nuclear transfer experiments in mouse oocytes showed that somatic H1 (H1c) associated with donor nuclei is rapidly replaced by an oocyte specific H1 (H1foo), similarly to what happens in Xenopus eggs ((Dimitrov and Wolffe 1996). The same study indicates that H1foo is more dynamic on chromatin and presumably contributes to destabilizing the chromatin of donor nuclei (Teranishi, Tanaka et al. 2004). Perhaps with the overall loose binding of core histones to chromatin, as well as the presence of H1foo until the 4-cell stage (Teranishi, Tanaka et al. 2004)), the chromatin of the early embryo is refractory to the formation of stable secondary chromatin structures.

Determining and modulating ATP levels in the embryonic nuclei could be a pursuable avenue, as chromatin remodeling complexes require energy for their function. However, since the metabolic activity of early embryos is high Schultz (Schultz 1993) optimal experimental conditions for a clear readout should first be established. As normal developmental progression is essential for continuation of the species, it is likely that a certain level of functional redundancy exists between different chromatin modifying pathways in the embryo. With the identification of 2-CL cells, biochemical and large-scale approaches will become more feasible. However, the molecular characteristics of these cells are still rather poorly understood and require further molecular characterization. They nonetheless provide an attractive in vitro alternative to the embryo and are a good starting point (at the very least) to set up larger-scale screens

for addressing the question of how histone mobility is regulated and what properties of the embryonic chromatin such high mobility reflect.

6. Is high chromatin dynamics necessary and/or sufficient for totipotency?

As I pointed out above, histone mobility is unusually high in the 2-cell stage blastomeres as well as 2-CL cells. Both of these cell types have totipotent features, including similar transcription profiles and chromatin organization. Our results suggest that high histone mobility is a characteristic of totipotency both in vivo and in vitro. Thus, the question following these observations is whether 'shutting down' chromatin dynamics at the 2-cell stage would cause premature loss of totipotency. While the comparison between 2-CL and normal ES cells within the same population suggests that high histone mobility is necessary for the acquisition of (or reprogramming to) totipotency, it only provides a strong correlation without formally addressing whether high histone turnover is sufficient to drive totipotency.

However, when considering experimental approaches to address this question, important points must be raised: How does one discriminate between loss of totipotency and developmental arrest experimentally? Is it possible to functionally separate the two? Totipotency is the ability to generate all cell types of the organism – therefore, strictly speaking, one should be able to prove that the progeny of cells which have lost totipotency cannot contribute to embryonic and extraembryonic tissues. But if developmental arrest occurs before lineage allocation normally takes place (for instance, at the 4-cell stage), it is difficult to decisively conclude if totipotency was lost. Furthermore, in vitro, if cultured cells cannot tolerate totipotent characteristics well and arrest or die, one cannot directly address their abilities to contribute to embryonic and extraembryonic tissues.

Conversely, the question could also be asked the other way around: is it possible to retain totipotency by keeping chromatin dynamics high? This provides an intriguing

hypothesis that remains to be experimentally tested. Due to tight regulation of development during cleavage stages, it is likely that finding an experimental approach able to induce high histone mobility in the developing embryo beyond the point when it normally becomes reduced will not be trivial. Following up with the considerations of the previous paragraph, identification of key factors regulating unusually high chromatin dynamics should be the starting point to experimentally address the importance –if any- of high histone mobility in sustaining high levels of cell plasticity.

Answers to these questions are still pending and will be of pivotal importance in our understanding of the molecular mechanisms behind the reprogramming process and the transitions in cellular states.

Conclusions

Mouse preimplantation embryo represents a unique system for studying cell plasticity and potency. From the chromatin perspective, development from the zygote to the blastocyst entails dramatic changes in chromatin structure and composition within a short time-frame. Indeed, many characteristics of nuclei of somatic cells are absent from earliest embryonic stages, and need to be established during development. Likewise, early mouse embryos are a powerful model for investigating changes in cell potency in vivo, ranging from totipotency to pluripotency and further differentiation.

The studies conducted during the first part of my doctorate contributed to the annotation of embryonic chromatin composition with regards to histone variants and post-translational modifications, and more specifically those correlated with active chromatin regions. Particularly, the histone variant H2A.Z was shown to be present on embryonic chromatin in a stage-specific manner. Ectopic expression of H2A.Z after fertilization reduced developmental progression, suggesting that absence of H2A.Z at the onset of development might be important for epigenetic reprogramming in the embryo and/or for the organization of the newly formed embryonic chromatin. Additionally, I have identified a new putative H2A.Z PTM, and have started a project to investigate its contribution to H2A.Z effects on chromatin. The spatio-temporal profiling in mouse and bovine preimplantation embryos of different PTMs known to be associated with transcription, conducted in collaboration with Dr N.Beaujean, revealed a species-specific pattern of chromatin modifications at the time of transition from maternal to embryonic regulation of development.

Secondly, instigated by rapid changes in chromatin composition and organization, I investigated histone dynamics in the developing mouse embryo. Our work represents the first report on histone mobility during early mammalian embryogenesis, encompassing totipotent stages as well as stages concomitant to and after cell fate allocation. Epigenetic reprogramming after fertilization is accompanied by strikingly high core histone mobility. As development proceeds, global chromatin maturation occurs, revealed by changes in chromatin ultrastructure and decrease in histone mobility. Interestingly, pluripotent cells, and those destined to pluripotency, retain higher

chromatin dynamics after lineage allocation, suggesting a link between chromatin plasticity and cell potency.

Together, the two studies conducted during my PhD contributed to the understanding of the molecular characteristics of chromatin in the mouse embryo, and their dynamic changes during preimplantation development. They indicate that chromatin and cell plasticity are functionally connected, underscoring the importance genome organization for developmental progression. Molecular mechanisms and key factors governing transitions in chromatin dynamics and (re)organization in the developing embryo will undoubtedly contribute to our understanding of changes in cell plasticity, with broad implications in reproductive and stem cell biology and personalized medicine.

Conclusion

L’embryon préimplantatoire de souris représente un système unique pour l'étude de la plasticité et la potence cellulaire. Du point de vue de la chromatine, le développement du zygote au blastocyste entraîne des changements radicaux dans la structure de la chromatine et sa composition dans un laps de temps court. En effet, de nombreuses caractéristiques du noyau des cellules somatiques sont absentes durant les premiers stades embryonnaires, et devrais être mises en place au cours du développement. De même, les embryons de souris précoces sont un modèle puissant pour étudier les changements dans la potence des cellules in vivo, allant de la totipotence vers la pluripotence et une différenciation plus poussée.

Les études menées au cours de la première partie de mon doctorat ont contribué à l'annotation de la composition de la chromatine embryonnaire en ce qui concerne les variantes des histones et des modifications post-traductionnelles, et plus particulièrement celles corrélées avec les régions actifs de la chromatine. En particulier, la variante d'histone H2A.Z s'est révélée être présente sur la chromatine embryonnaire d'une manière spécifique au cours du développment. L'expression ectopique de H2A.Z après la fécondation réduit la progression du développement, ce qui suggère que l'absence de H2A.Z au début du développement pourrait être important pour la reprogrammation épigénétique dans l'embryon et / ou pour l'organization de la chromatine embryonnaire nouvellement formée. En outre, j'ai identifié une nouvelle PTM putative de H2A.Z, et j'ai commencé un projet visant à étudier sa contribution aux effets de cette dernière sur la chromatine. Le profilage spatio-temporel, dans les embryons préimplantatoire de souris et bovins, de différentes PTM connues pour être associées avec la transcription, réalisée en collaboration avec le Dr. N. Beaujean, a révélé un modèle spécifique à l'espèce des modifications de la chromatine lors de la transition de la transcription maternelle à la réglementation embryonnaire du développement.

Deuxièmement, à l'instigation des changements rapides dans la composition et l'organization de la chromatine, j'ai étudié la dynamique des histones dans l'embryon de souris en développement. Notre travail représente le premier rapport sur la mobilité de l'histone au début de l'embryogenèse chez les mammifères, englobant les étapes totipotentes ainsi que les stades concomitants et après l'attribution du destin

cellulaire. La reprogrammation épigénétique après la fécondation est accompagnée par une étonnante forte mobilité des histones dans le noyau. Au cours du développement, la maturation globale de la chromatine se produit, révélée par des changements par l'ultrastructure de la chromatine et la diminution de la mobilité des histones. Fait intéressant, les cellules pluripotentes, et celles qui sont destinées à la pluripotence, conservent une dynamique plus élevée de la chromatine après l'attribution de la lignée, ce qui suggère un lien entre la plasticité de la chromatine et la potence de la cellule.

Ensemble, les deux études menées au cours de ma thèse ont contribué à la compréhension des caractéristiques moléculaires de la chromatine dans l'embryon de souris, et leurs changements dynamiques au cours du développement préimplantatoire. Ils indiquent que la chromatine et la plasticité des cellules sont reliées fonctionnellement, soulignant l'importance de l'organization du génome sur la progression du développement. Les mécanismes moléculaires et les facteurs clés régissant les transitions dans la dynamique de la chromatine et (ré)organization dans l'embryon en développement contribueront sans aucun doute à notre compréhension de l'évolution de la plasticité cellulaire, avec de larges implications en matière de reproduction et de biologie des cellules souches et de médecine personnalisée.

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