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HERITABLE SILENCING MECHANISMS IN MOUSE DEVELOPMENT

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I declare that the work in this thesis is my own, except were otherwise stated

José Silva

(signature)

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To Yael and my parents……

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Abstract (English)

In early embryo development pluripotent cells differentiate into many distinct differentiated cell types. These are characterised by having particular sets of active and inactive genes (the epigenome), that need to be maintained through cell division for the perseverance of cell identity. The epigenome of a given cell type is not established in a single step in the early embryo. Rather, it appears to undergo a maturation process, becoming more restricted and stable as development proceeds. Understanding how heritable gene activation and gene silencing are established and maintained is a key question in biology. Using embryonic stem (ES) cells and the X inactivation process as model systems I have investigated mechanisms of heritable gene silencing in early embryonic development. Using two different ES cell lines I also investigated whether nuclear transcriptional compartmentalisation is a general feature of heritable gene silencing. Previous work has demonstrated that in contrast to active genes, transcriptionally silent genes often localise to discrete heterochromatic compartments in the nucleus of lymphocytes and other haematopoietic cells. Importantly, this targeting has been implicated in the maintenance of gene silencing, giving rise to a model for gene regulation that takes into account the nuclear position of a given gene. I studied the nuclear localisation of the developmentally regulated genes *Oct4*, *Rex1*, and *Dnmt3b* (active in undifferentiated ES cells), and *Cyclin D1* (activated upon differentiation), in relation to pericentric heterochromatin during ES cell differentiation. I found that whilst silenced alleles showed an increased tendency to be associated with pericentric heterochromatin, the effect was relatively weak compared with results reported for lymphocytes. This data may indicate that nuclear localisation to heterochromatic domains is utilised to a lesser degree in early development, perhaps reflecting genome plasticity prior to terminal differentiation.

I then went on to investigate the role of Eed-Enx1 Polycomb Group complex in X inactivation. The Polycomb proteins are key regulators of heritable gene silencing in a species as diverse as plants and mammals. Previous studies have shown that the Eed-Enx1 Polycomb Group complex has a role in imprinted X inactivation specifically in trophectoderm. I found that Eed-Enx1 is also involved in random X inactivation in ES cells and in the embryo proper. The complex is temporally regulated, localising to the inactive X only in early development/differentiation stages. Localisation occurs coincident with *Xist* RNA accumulation, and precedes early N-terminal histone modifications. Analysis of an XY ES cell line carrying an *Xist* transgene demonstrated that ectopic *Xist* RNA expression is sufficient to recruit Eed-Enx1 complexes to their targets. Analyses of *eed* mutant mice demonstrated that Eed-Enx1 complexes are required to establish histone H3 methylation at lysine 9 (K9) and at lysine 27 (K27) on the inactive X. H3-K9/K27 methylation was

found to be required, in the embryo proper, for the stable maintenance of gene silencing and of chromatin modifications, namely H3-K9 hypoacetylation and H3-K4 hypomethylation.

Taken together my results suggest that distinct and specialised mechanisms of heritable gene silencing function in ES cells and in early development. These results have important implications in terms of understanding how the genome is regulated as cells differentiate from pluripotent lineages.

Résumé (Français)

Au cours du développement embryonnaire précoce, les cellules, d'abord pluripotentes, sont amenées à se différencier en un grand nombre de types cellulaires distincts. Ces cellules différenciées sont caractérisées par le patron d'expression de leurs gènes (épigénome). Les gènes sont ainsi spécifiquement activés ou réprimés, et ce patron doit être maintenu de façon stable au cours des divisions cellulaires pour maintienir l'identité cellulaire. L'épigénome d'un type cellulaire donné n'est pas établi en une seule étape au sein de l'embryon précoce. Au contraire, il est progressivement mis en place, devenant, au cours du développement, de plus en plus restreint, spécifique et stable. La compréhension des mécanismes d'établissement et de maintien de l'activité ou répression des gènes est une question centrale en biologie. En utilisant comme modèle de travail les cellules souches embryonnaires (ES) et le processus d'inactivation du chromosome X, j'ai entrepris d'étudier les mécanismes de répression stable et héritable des gènes au cours du développement embryonnaire précoce. L'utilisation de deux lignées distinctes de cellules ES, m'a aussi permis d'analyser l'implication de la compartimentalisation nucléaire des gènes inactifs dans le maintien de la répression transcriptionnelle. Des travaux antérieurs ont en effet démontré, qu'en contraste avec les gènes actifs, les gènes réprimés sont localisés au sein de compartiments hétérochromatiques discrets dans le noyau des lymphocytes et d'autres cellules hématopoïétiques. La position de ces gènes semble directement impliquée dans le maintien de la répression trancriptionnelle, ce qui a donné naissance à un modèle de régulation des gènes qui prend en compte leur position sub-nucléaire. J'ai ainsi étudié la localisation sub-nucléaire des gènes *Oct4, rex-1* et *Dnmt3b* (actifs spécifiquement dans les cellules ES indifférenciées), et du gène *Cyclin D1* (activé au cours du processus de différenciation), en rapport avec l'hétérochomatine péricentromérique au cours de la différenciation des cellules ES. Mes résultats montrent que si les allèles réprimés ont tendance à être associés plus fréquemment à l'heterochomatine pericentromerique, la corrélation reste faible en comparaison avec les résultats reportés pour les lymphocytes. Ces données suggèrent que la localisation sub-nucléaire des gènes inactifs au sein de domaines heterochromatiques n'est pas requise au cours du développement embryonnaire précoce, reflétant peut être la plasticité du génome qui précède la différentiation terminale.

J'ai ensuite analysé le rôle du complexe polycomb Eed-Enx1 dans le processus d'inactivation du chromosome X au cours de la différenciation des cellules ES. Les protéines du groupe polycomb (PcG) sont des éléments clefs du maintien de la répression transcriptionnelle, extrêmement conservés au cours de l'évolution. Des travaux antérieurs ont montré que le complexe Eed-Enx1 est spécifiquement impliqué dans l'inactivation du chromosome X dans le

trophectoderme. J'ai mis en évidence que ce complexe PcG est aussi impliqué dans l'inactivation aléatoire du chromosome X dans les cellules ES et dans l'embryon lui-même. Le complexe Eed-Enx1 subit une régulation temporelle. Il est localisé au niveau du chromosome X uniquement au cours des stades précoces du développement/différenciation. Cette co-localisation coïncide avec l'accumulation de l'ARN *Xist*, et précède les modifications précoces de la queue N-terminale des histones. L'analyse d'une lignée de cellules ES males (XY) portant un transgène *Xist* a permis de démontrer que l'expression ectopique de l'ARN *Xist* est suffisante pour le recrutement des complexes Eed-Enx1 au niveau de leurs cibles. L'étude de souris mutantes pour le gène *eed* a par ailleurs permis de mettre en évidence le rôle du complexe Eed-Enx1 dans l'établissement de la méthylation de la queue N-terminale de l'histone H3, au niveau des lysines 9 (K9) et 27 (K27) sur le chromosome X inactif. La méthylation K9/K27 de l'histone H3 s'est révélée nécessaire dans l'embryon en cours de développement, pour le maintien stable de la répression transcriptionnelle, et des modifications de la chromatine qui y sont associées (hypoacetylation de la lysine 9 de l'histone H3 et hypomethylation de la lysine 4 de l'histone H3).

L'ensemble de ces résultats suggère que des mécanismes distincts et spécifiques de répression stable et héritable prennent place au sein des cellules ES et au cours du développement embryonnaire précoce. Ils apportent une importante contribution à la compréhension des mécanismes mis en jeu pour la régulation de l'expression du génome au cours de la différentiation cellulaire précoce.

Resumo (Português)

Durante as fases iniciais do desenvolvimento embrionário, as células pluripotentes diferenciam-se em tipos celulares distintos. Estes tipos celulares são caracterizados pelos conjuntos de genes activos e inactivos (epigenoma) que contêm e que têm que ser mantidos nesses estadios durante a divisão celular para manter a identidade celular. O epigenoma de um dado tipo celular não é estabelecido num único evento durante as fases iniciais do desenvolvimento embrionário. Na verdade, parece sofrer um processo de maturação tornando-se mais restrito e estável à medida que o desenvolvimento ocorre. Compreender como esta activação e silenciamento heriditários são estabelecidos e mantidos é uma questão chave em biologia. Utilizando células estaminais embrionárias (ES) e o processo de inactivação do cromossoma X como sistemas modelo, procedeu-se à investigação de mecanismos heriditários de silenciamento genético nas fases iniciais do desenvolvimento embrionário. Recorrendo a 2 linhas celulares estaminais foi também investigado se a compartimentalização transcricional no núcleo é um evento geral do silenciamento genético hereditário. Estudos anteriores demonstraram que ao contrário de genes activos, os genes transcricionalmente silenciados localizam-se frequentemente em compartimentos heterocromáticos no núcleo de linfócitos e outras células hematopoiéticas. Esta localização particular foi implicada na manutenção do silenciamento genético, dando origem a um modelo de regulação genética que associa a posição no núcleo de um dado gene com o seu estado transcricional. Na presente tese, foi estudada a localização nuclear de genes regulados durante o desenvolvimento, *Oct4*, *Rex1* e *Dnmt3b* (que se encontram activos em células ES indiferenciadas) e *Ciclina D1* (que se encontra activa após a diferenciação das células ES), em relação à heterocromatina centromérica durante a diferenciação das células ES. Observou-se que os alelos silenciados mostram uma maior incidência para se encontrarem associados com a heterocromatina centromérica, embora esta observação seja menos obvia quando comparada com os resultados em linfócitos. Estes resultados podem indicar que a localização com domínios heterocromáticos é utilizada com menor frequência nas fases iniciais do desenvolvimento, o que poderá reflectir a plasticidade genómica antes de ocorrer completa diferenciação.

Na continuação dos estudos sobre mecanismos de slenciamento genético heriditário foi também investigado a importância do complexo Eed-Enx1, proteinas do grupo Polycomb, no processo de inactivação do cromossoma X. As proteinas do grupo Polycomb são reguladoras chave do silenciamento genético hereditário, desde plantas até mamíferos. Estudos anteriores mostraram que o complexo Eed-Enx1 está envolvido na inactivação não aleatória do cromossoma X, especificamente na trofectoderme. No presente trabalho observou-se que o complexo Eed-Enx1

também está envolvido na inactivação aleatória do cromossoma X em células ES diferenciadas e no embrião. O complexo é regulado durante o desenvolvimento, localizando-se com o cromossoma X inactivo apenas aquando dos estadios iniciais da diferenciação das células ES e do desenvolvimento embrionário. A co-localização ocorre simultaneamente com a acumulação do RNA transcrito pelo gene *Xist* e precede modificações N-terminais das histonas. Análise de uma linha celular estaminal XY que expressa um transgene do *Xist* demonstrou que expressão ectópica do RNA é suficiente para recrutar o complexo Eed-Enx1 para os seus alvos. Análises de ratinhos mutantes para o gene *eed* revelou que o complexo Eed-Enx1 é necessário para que ocorra a metilação da lisina 9 e 27 da histona H3 no cromossoma X inactivo. O mesmo padrão de metilação é também necessário no embrião, para que ocorra a manutenção do silenciamento genético e das modificações da cromatina, nomeadamente a hipoacetilação da lisina 9 e a hipometilação da lisina 4 da histona H3.

No conjunto, os resultados apresentados na tese sugerem que mecanismos distintos e especializados de silenciamento genético hereditário ocorrem em células ES e nas fases iniciais do desenvolvimento embrionário. Estes resultados tentam clarificar como o genoma é regulado à medida que as células pluripotentes se diferenciam.

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List of abbreviations:

BrdU- 5-bromodeoxyuridine
DAPI- 4,6-diamidino-2-phenylindole
dpc- Days post coitum
dsRNA- Double stranded RNA
EB- Embryoid bodies
ES- Embryonic stem
E(var)- Enhancer of variegation
FCS- Foetal calf serum
FISH- Fluorescent in situ hybridisation
GFP- Green fluorescent protein
HGMP- Human Genome Mapping Project
HMTase- Histone methyltransferase
ICM- Inner Cell Mass
K9- Lysine 9
Lif- Leukaemia inhibitory factor
me_3- tri-methylated
NFDM- Non-fat dry milk
PAC- Phage artificial chromosome
PcG- Polycomb group
PEV- Position effect variegation
PRE- Polycomb responsive element
PRC1- Polycomb repressive complex 1
RNAi- RNA interference
rt- Room temperature
siRNA- Small interfering RNA
Su(var)- Suppressor of variegation
TrxG- Trithorax group
TS- Trophoblast stem
TSA- Trichostatin A
X^{GFP}- GFP transgene localised in the X chromosome
Xic- X chromosome inactivation centre
Xm- Maternal inherited X chromosome
Xp- Paternal inherited X chromosome
5azadc- 5-azaedeoxycytidine

Chapter 1: Introduction

1.1- Heterochromatin and heritable gene silencing

During development of complex organisms specific subsets of genes are either activated or repressed in a heritable manner. The ability to establish and maintain expression/repression of parts of the genome is central to how different cell types define their identity. In this section I discuss heritable gene silencing mechanisms.

1.1.1- Heterochromatin and position effect variegation

The definition of heterochromatin was originally based on morphological criteria. The term was introduced by Heitz (1928) to describe chromosomal regions that under the light microscope appear deeply stained throughout the cell cycle. The remaining chromosomal regions were found to condense only during mitosis, changing to a diffuse state during interphase, and were named euchromatin. Heitz also noticed that heterochromatic regions are mainly located at pericentric and telomeric locations. Subsequent studies on metaphase chromosomes demonstrated that heterochromatic regions may change over time and with cell type, and that boundaries between heterochromatin and euchromatin may not be precisely defined (John, 1988).

Heterochromatic regions were found to be highly enriched in repetitive DNA (Lohe and Roberts, 1988). Repetitive DNA generally consists of short sequences repeated many times in tandem in large arrays. These include satellite sequences which are the main component of pericentric heterochromatin (Elgin, 1996). Repetitive DNA is present in almost all eukaryotic genomes, although to markedly varying extents. Unicellular eukaryotes have low levels of repetitive DNA, the majority of the genome is transcriptionally competent, while more than 60% of the mammalian genome is permanently silenced and only 3% of its DNA encodes structural genes (reviewed in Jenuwein *et al*, 1998). Although not devoid of genes, heterochromatic regions are typically gene poor.

The heterochromatin of the fruit fly (*Drosophila*) was found to aggregate together, promoting the association of non-homologous chromosomes and influencing the organisation of chromosomes in the nucleus (reviewed in Eissenberg *et al*, 1995). This characteristic of *Drosophila* heterochromatin was also reported in the interphase nucleus of some mammalian cell types (Haaf and Schmid, 1989; Brown *et al*, 1997).

Investigation of the relationship between heterochromatin and gene expression started with the observation that heterochromatin had a repressive effect on euchromatic genes. This phenomenon was named "position effect variegation" (PEV). In these studies mutations of the *Drosophila* white gene (w^+), that resulted in variegated eye colour phenotypes, were associated with chromosome rearrangements that placed the gene adjacent to heterochromatin (Muller, 1930; Schultz, 1936). The unique characteristics of *Drosophila* polytene salivary gland chromosomes provided the opportunity to study the cytological aspects of PEV. These studies have shown that chromosomal breakpoints created by novel junctions between euchromatin and heterochromatin can induce the mosaic silencing phenotypes of euchromatic genes (Spradling and Karpen, 1990). Moreover, the position effect induced on an euchromatic gene could be relieved by recombining the gene away from heterochromatin or by inducing a second chromosomal rearrangement which placed the gene in an euchromatic environment. Another interesting observation was that a single breakpoint could affect more than one gene, with the strength of repression diminishing with the increase of distance from the breakpoint. Importantly, several investigators noticed that the silencing effect of the PEV phenomenon appeared to be stably maintained throughout successive cell divisions, representing an excellent model to study heritable gene silencing (reviewed in Spradling and Karpen, 1990; Weiler and Wakimoto, 1995).

1.1.2- Modifiers of position effect variegation

To identify factors that affect PEV, large scale mutational genetic screens were performed (Sinclair *et al*, 1983; reviewed in Reuter and Spierer, 1992). Altogether more than 120 loci acting as dominant supressors of variegation (Su(var)s), or enhancers of variegation (E(var)s), of the w^{m4} phenotype (Figure 1.1.2.1), were identified and mapped. The suppressor mutations convert a variegating phenotype to one that is virtually indistinguishable from wild type. All dominant Su(var) and E(var) loci studied to date display haplo-abnormal effects, suppressing or enhancing PEV phenotypes when present as single dosage. A small proportion of these genes also exhibit triplo-abnormal opposite phenotypes, resulting from increase in gene copy number to three or more, or by induced over-expression (Locke *et al*, 1988; Wutsmann *et al*, 1989; Reuter *et al*, 1990; Eissenberg *et al*, 1992). Examples of these include the *Su(var)205* and *Su(var)3-9* loci.

1.1.3- Su(var)3-9

Su(var)3-9 homologues have been found in human, mouse and fission yeast, named SUV39H1, Suv39h1 and Clr4 respectively (Aagaard *et al*, 1999). Alignment of all four proteins highlighted three regions of sequence identity: A cysteine-rich region and the highly evolutionary conserved chromodomain and SET domain, which are also found in other proteins. The latter was named with the initials of the three founder members of SET domain containing proteins, **S**u(var)3-9, **E**nhancer of zeste and **T**rithorax. The two latter proteins belong to the Polycomb group (PcG) and Trithorax group (TrxG) respectively (see section 1.1.6 for further details), and are involved in the regulation of gene expression. Interestingly, immunofluorescence of SUV39H1/Suv39h1 localisation revealed enrichment at heterochromatic foci during interphase, and centromeric localisation during metaphase (Aagaard *et al*, 1999). Schotta and Reuter (2000) also subsequently demonstrated association of Su(var)3-9 with heterochromatic regions in *Drosophila*.

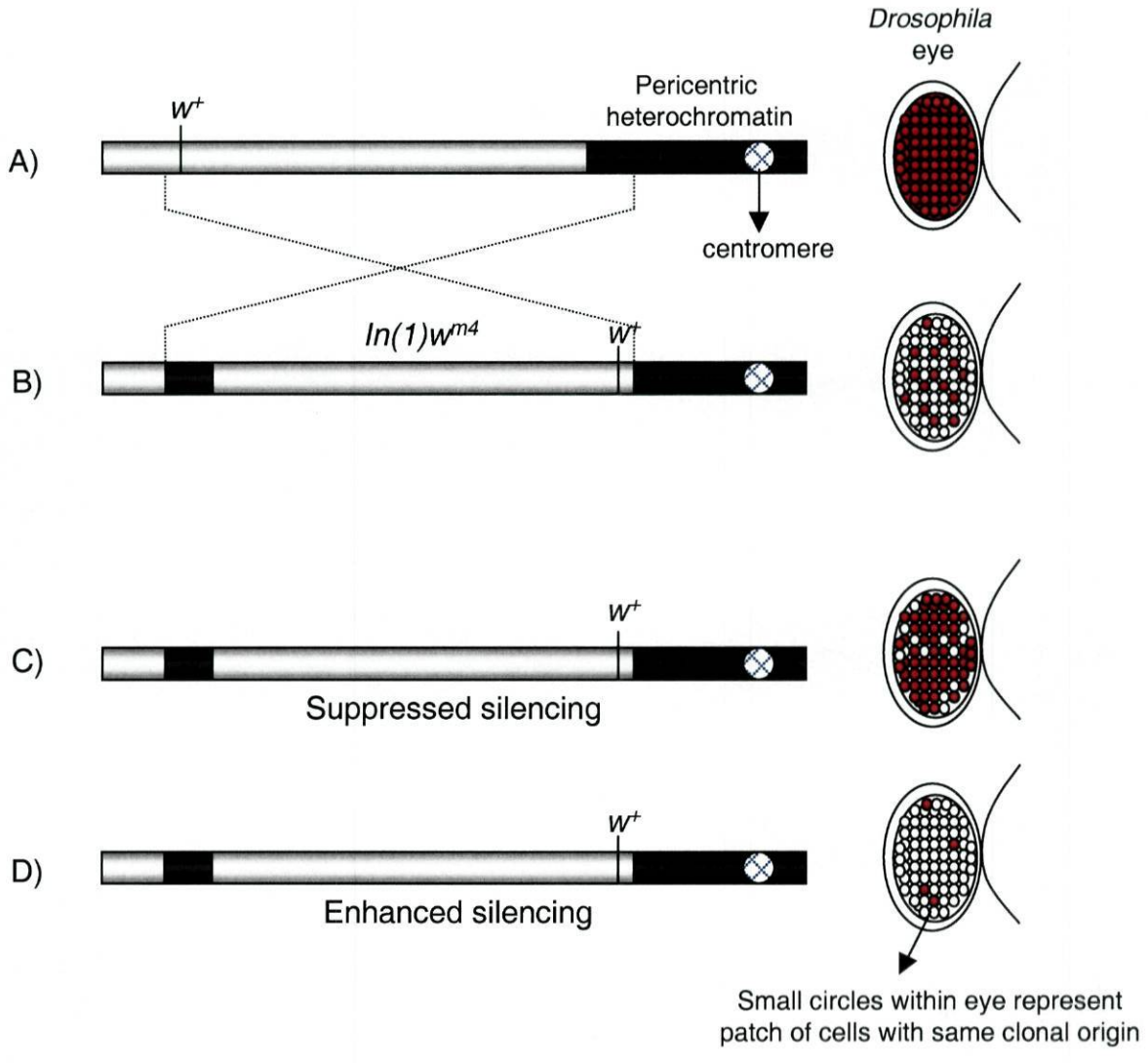


Figure 1.1.2.1- **Position effect variegation of *Drosophila* white gene.**
Euchromatic genes like the white gene (w^+), which is required for red eye pigmentation, undergo clonal inactivation when juxtaposed to pericentric heterochromatin as in the case of the X chromosome inversion $In(1)w^{m4}$. The resultant mosaic expression pattern of the affected gene reflects an altered transcriptional state and is a useful tool for the investigation of heritable gene silencing. Mutations affecting position effect variegation (PEV) can either suppress the silencing effect (suppressor of position effect variegation (Su(var))) resulting in more red eye pigmentation or enhance the silencing causing white eyes (enhancer of PEV, E(var)). A) Wild type. B) Mutant with chromosomal inversion $In(1)w^{m4}$ C) Mutant for Supressor of PEV. D) Mutant for enhancer of PEV. Illustration was adapted from Laible *et al* (1997).

Based on similarity to potential plant histone N-methyltransferases Rea *et al* (2000) investigated whether the SUV39H1/Suv39h1 proteins have enzymatic activity. Histones were tested as a possible substrate for *in vitro* methylation. Interestingly, results demonstrated that SUV39H1/Suv39h1 transfer methyl groups to histone H3 (histone methyltransferase (HMTase) activity). The SET domain of these proteins was found to encode the catalytic activity. More detailed analysis determined that lysine 9 of the histone H3 N-terminal tail was the preferred residue for SUV39H1/Suv39h1 mediated HMTase activity. A similar function was shown for the yeast homologue Clr4 and for *Drosophila* Su(var)3-9 (Czermin *et al*, 2001; Nakayama *et al*, 2001). Interestingly, SUV39H1 HMTase activity was found to be restricted by specific pre-existing histone modifications, namely histone H3 lysine 9 (H3-K9) acetylation, and histone H3 serine 10 (H3-S10) phosphorylation (Rea *et al*, 2000).

The murine Su(var)3-9 homologues are encoded by two loci, *Suv39h1* and *Suv39h2* (O'Carroll *et al*, 2000). These show entirely overlapping expression patterns in developing embryos, which suggested redundant roles. To address their functional role Peters *et al* (2001) generated *Suv39h1* and *Suv39h2* double knockout mice by gene targeting. Results showed that the Suv39h1/2 regulate H3-K9 methylation *in vivo* in pericentromeric heterochromatin. In addition, the deficient mice exhibited increased tumurogenesis risk and chromosomal instability.

1.1.4 – *Su(var)205* / Heterochromatin Protein 1

The *Su(var)205* gene was found to encode a heterochromatin protein termed heterochromatin protein 1 (HP1) (James and Elgin, 1986; Eissenberg *et al*, 1990). HP1 was found to be predominantly localised to pericentric heterochromatin when visualised in *Drosophila* salivary gland nuclei (James *et al*, 1989). HP1 contains the highly evolutionary conserved chromodomain motif. Importantly, immunofluorescence and co-immunoprecipitation studies demonstrated that SUV39H1/Suv39h1 co-localise and interact with HP1 (Aagaard *et al*, 1999). Further analysis, performed concurrently in different laboratories, revealed that correct localisation of the fission

yeast HP1 homologue, Swi6, and mammalian HP1 to heterochromatic domains, depends on Clr4 and Suv39h HMTase activity respectively (Nakayama *et al*, 2001; Lachner *et al*, 2001; Bannister *et al*, 2001). Also, methylated H3-K9 peptides were found to possess a binding site for Swi6 and HP1 proteins (Figure 1.1.4.1). This interaction required a functional chromodomain in Swi6/HP1 (Bannister *et al*, 2001; Lachner *et al*, 2001). Together, these data outlined an evolutionarily conserved mechanism for maintaining pericentric heterochromatin between yeast and mammalian HP1.

In studies to investigate if histone H3 methyltransferase activity is associated with the ability of the Retinoblastoma (RB) protein to silence genes, SUV39H1 was identified as a candidate by co-immunoprecipitations (Nielsen *et al*, 2001). Previous experiments had shown the promoter of *CYCLIN E* gene to be repressed by RB (Brehm *et al*, 1988; Magnaghi-jaulin *et al*, 1988; Luo *et al*, 1988). When the human Hela cell line was transfected with both RB and SUV39H1 expression constructs, along with a reporter construct containing luciferase under the control of CYCLIN E promoter, repression of the luciferase gene was observed. However, when the SUV39H1 SET domain was removed RB was unable to silence the reporter gene. Further detailed experiments have shown that HP1 also interacts with RB both *in vivo* and *in vitro*. Moreover, HP1 was able to recognise methylated H3-K9 peptides while associated with Rb. Finally, chromatin immunoprecipitation (ChIP) analysis on $RB^{+/+}$ *versus* $RB^{-/-}$ cells demonstrated that RB is necessary to direct HP1 binding to the CYCLIN E promoter transgene. In summary, these results implicated the SUV39H1-HP1 complex in the repressive functions of the Rb protein. Importantly these findings extend the role of SUV39H1 and HP1 in heterochromatin to individual gene repression.

1.1.5- The role of RNA in pericentric heterochromatin

In a surprising series of experiments, addressing how pericentric heterochromatin is maintained, RNAse A treated fibroblast cells were found to lose concentration of HP1 at pericentric heterochromatin (Maison *et al*, 2002). The normal pattern could be rescued by the addition of small

amounts of purified nuclear RNA. This result implied the existence of an RNA component in constitutive heterochromatin which could either function as a structural component, giving it a specific configuration, or provide additional binding sites for heterochromatin associated proteins. However, the identity of this RNA remains unknown.

In conclusion, homologues of the *Drosophila* modifiers of PEV Su(var)205 and Su(var)3-9 are found in many eukaryotes, ranging from fission yeast to mammals. The mechanisms by which these proteins modulate heterochromatin formation and gene silencing are found to be at least broadly conserved. H3-K9 methylation is now thought to provide an epigenetic mark for the establishment and maintenance of stably repressed regions and heterochromatic domains (Figure 1.1.4.1) (Zhang and Reinberg, 2001; Jenuwein and Allis, 2001). There is also evidence for the SUV39H-HP1 complex being involved in the repression of one euchromatic gene, *CYCLIN E*, although it remains to be tested how widespread this mechanism is. For the future, the characterisation of other suppressors of PEV will be of great interest. Such work will shed more light on our understanding of the mechanism of heritable gene silencing.

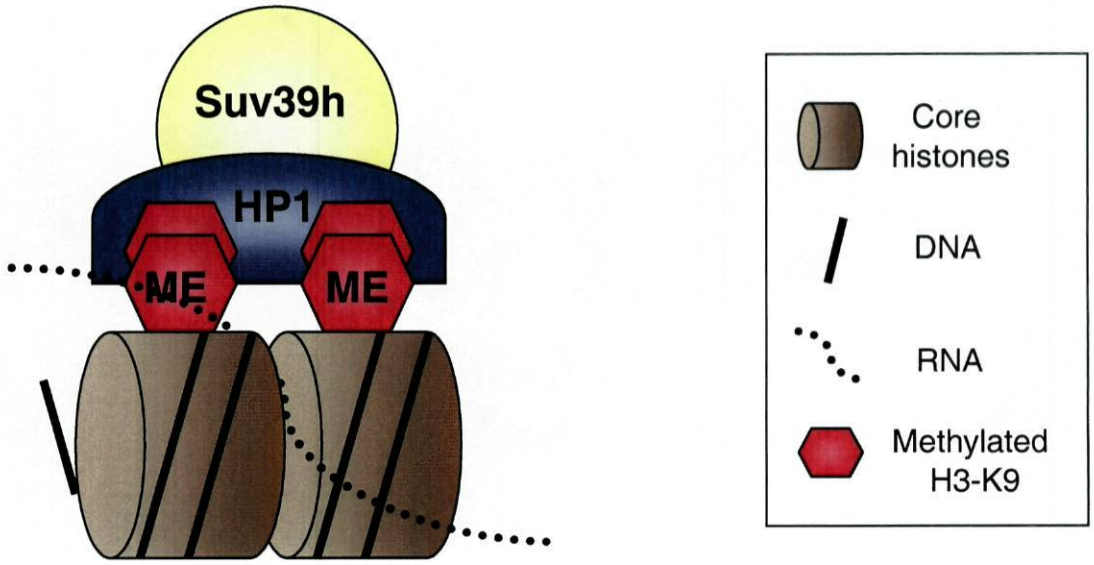


Figure 1.1.4.1- **Components of pericentric heterochromatin**
Suv39h methylates histone H3 at lysine 9. This mark provides a binding site for HP1. Recently, an RNA component was also found to be essential for proper heterochromatin recognition by HP1. Importantly, these factors seem to work together to induce heterochromatin formation and gene silencing. This schematic representation was adapted from Lachner and Jenuwein (2002).

1.1.6- The Polycomb Group (PcG) and cell memory

PcG genes were identified in genetic screens for mutations that affect stable maintenance of homeotic gene silencing in *Drosophila*. Homeotic genes are fundamental for proper formation of the anterior-posterior axis of the developing embryo (Lewis, 1978). This is achieved by the differential expression of these genes on each individual segment. All segments are characterised by the activity of a specific set of homeotic genes with the others being inactive in that segment. Genetic screens of *Drosophila* mutants exhibiting homeotic transformations revealed that two classes of genes are involved in the transmission of correct homeotic expression patterns through development: those necessary to maintain homeotic gene activity in appropriate segments (the trithorax group (TrxG)) and those required to stably repress homeotic genes (PcG (see below)). Due to my interest in heritable gene silencing I will only discuss the latter class of genes.

In 1947 the *Polycomb* (*Pc*) gene was identified by its specific phenotype of an extra sex comb tooth on the second and third leg of heterozygous *Drosophila* mutants (Lewis, 1947). Subsequently it was elucidated that the phenotypic consequences of the mutation were caused by defective regulation of homeotic genes of the bithorax complex (*Bx-c*) (Lewis, 1978). *Bx-c* genes were found to express in anterior embryonic segments where they are normally silenced. More careful analysis of *Pc* mutants identified single segments of *Drosophila* embryos displaying features normally specific to several segments (Dennell and Frederick, 1983). This result led to the suggestion that the effects observed resulted from instability in the transmission of correct homeotic expression patterns, rather than a local misexpression.

A number of loci were identified that when mutated show a similar phenotype as the *Pc* mutant (Table 1.1.6.1) (Satijn and Otte, 1999). This group of genes were classified as belonging to the Polycomb Group (PcG). In general, PcG mutants exhibit normal expression patterns of homeotic genes in early development, but at later developmental stages the homeotic genes start being expressed in body segments where they normally are repressed (Kennison and Tamkun, 1988; Paro,

1990). This indicates that PcG proteins are involved in the maintenance of the silencing state of homeotic genes. In recent years PcG homologues were also found in many species including human, mouse, *Xenopus*, *Caenorhabditis elegans* (worm) and *Arabidopsis* (plant) .

1.1.7- PcG proteins interact and form large multi-subunit complexes

Studies of double PcG mutants, which resulted in the enhancement of PcG phenotypes, and immunofluorescence data on *Drosophila* polythene chromosomes, showing overlapping signals for different PcG antibodies, led to the suggestion that PcG proteins form complexes containing different PcG proteins (Kennison and Tamkun, 1988; Lewis, 1978; Paro and Hogness, 1991; Campbell *et al*, 1995; Zink and Paro 1989; Rastelli *et al*, 1993). Subsequent biochemical studies led to the identification of two PcG complexes; the Extra sex combs Enhancer of zeste (ESC-E(Z)) and the polycomb repressive complex 1 (PRC1) (see below).

1.1.7.1- The ESC-E(Z)/Eed-Enx1/EED-ENX1 complex

Drosophila PcG proteins are required and expressed continuously during embryonic development. However, extra sex combs (ESC) is an exception because this protein is expressed only transiently and at very early embryonic stages (between 0 and 12 hours of embryogenesis) (Struhl and Brower, 1982; Simon *et al*, 1995; Ng *et al*, 2000). Moreover, the *Esc* murine homologue, embryonic ectoderm development (*eed*), showed that embryos carrying a null mutation die early in development, at around 8.5 days post coitum (dpc), displaying disrupted anterior-posterior (A-P) patterning of the primitive streak during gastrulation (Faust *et al*, 1995; Shumacher *et al*, 1996).

Drosophila genes	Mouse homologues	Human homologues
Extra sex combs (Esc)	*eed*	*EED*
Enhancer of zeste (Ez)	*Enx1, Ezh1*	*ENX1,EZH1*
Polycomb (Pc)	*M33, MPc2*	*HPC1, HPC2, HPC3*
Posterior sex combs (Psc)	*Mel18, Bmi1*	*MEL18, BMI1*
Polyhomeotic (Ph)	*MPh1, MPh2*	*HPH1, HPH2*
Sex comb on midleg (Scml)	*Scml1, Scml2*	*SCML1, SCML2*
dring	*Ring1a, Ring1b*	*RING1A, RING1B*
Multi sex combs (Msc)	-	-
Pleiohomeotic (Pho)	*Yy1*	*YY1*
Super sex combs (Ssc)	-	-
Polycomblike (Pcl)	*Phf1*	*PHF1*
Aditional sex combs (Asx)	*Asxl1, Asxl2*	*ASXL1, ASXL2*
Suppressor of zeste 2 (Su(z)2)	-	-
Enhancer of polycomb (E(Pc))	*Epc1, Epc2*	*EPC1, EPC2*

- Esc-Ez complex - PRC1 complex

Table 1.1.6.1- **Polycomb group genes.** This table shows the *Drosophila* polycomb group genes and the respective, mammalian homologues. Note that some of these gene products were found to interact with each other in both *Drosophila* and mammalian ESC-EZ (red box) and in PRC1 (green box) protein complexes.

This early requirement in mouse development was only paralleled by two other PcG genes, the *Drosophila* enhancer of zeste *E(Z)* murine homologue *Enx1,* and *Drosophila* pleihomeotic (*Pho*) murine homologue yen yang 1 (*Yy1*) (Donohoe *et al*, 1999; O'carrol *et al*, 2001). *Enx1* and *Yy1* mutants in mouse were found to stop developing soon after implantation (O'Carrol *et al*, 2001; Donohoe *et al*, 1999).

Studies carried out concurrently in three independent laboratories demonstrated that *Drosophila* ESC, mouse Eed and human EED interacted with E(Z), Enx1 and ENX1 respectively (Jones *et al*, 1998; Sewalt *et al*, 1998; van Lohuizen *et al*, 1998). Similarly, biochemical analyses of *C. elegans* maternal-effect sterile proteins MES-2 and MES-6, and of *Arabidopsis* Medea (MEA) and fertilisation-independent-endosperm (FIE), homologues of E(Z) and ESC respectively, demonstrated that these interact directly (Xu *et al*, 2001; Yadegari *et al*, 2000). These results show that this molecular partnership has been maintained throughout evolution. In addition, co-immunoprecipitation studies showed that EED binds to YY1 (Satijn *et al*, 2001). The latter result is of particular significance as PHO/YY1 is the only PcG protein that has been shown to bind DNA specifically (Brown *et al*, 1998). Despite this reported interaction several studies to date failed to identify PHO/YY1 as a component of the purified ESC-E(Z)/EED-ENX1 complex (Tie *et al*, 2001; Cao *et al*, 2002; Czermin *et al*, 2002; Muller *et al*, 2002; Kuzmichev *et al*, 2002).

Sequence alignments between the *Drosophila* ESC and the murine homologue Eed protein highlighted the presence of a conserved motif, the WD40 domain, showing 67% identity (Shumacher *et al*, 1996) (Figure 1.1.7.1.1A). This motif is thought to be involved in protein-protein interactions (Neer and Smith, 1996).

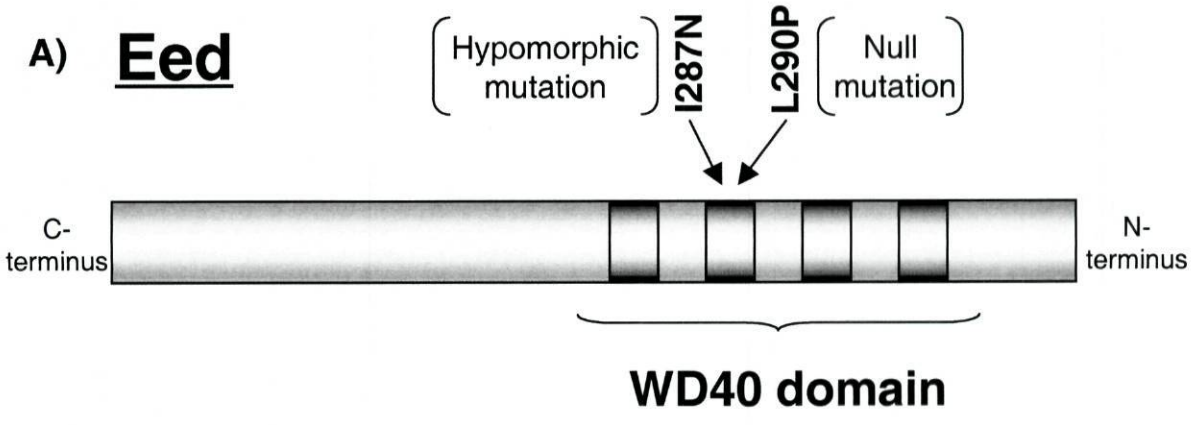


Figure 1.1.7.1.1- **Schematic representation of mammalian Eed and Enx1 proteins.** (A) Illustration of Eed highlighting the conserved WD40 domain. The localisation of hypomorphic and null mutations at the WD40 domain are also indicated (see text for additional information). The former results from an amminoacid change (Isoleucine (I) → Aspargine (N)) at position 287 and the latter results also from a single amminoacid change (leucine (L) → proline (P)) at position 290. (B) Schematic representation of Enx1highlighting two conserved domains: an 115 amino acid, cystein rich region and the SET domain. The latter has been shown, in *Suv39* mammalian homologues, to add methyl groups to lysines in histone H3 tails, an activity known as histone methyltransferase (HMTase) (Rea *et al*, 2000). However, this same function has not yet been demonstrated for Enx1 SET domain. See text for additional information.

Mammalian homologues of the *Drosophila* E(Z) are encoded by two loci, murine *Ezh1* and human *EZH1*, and also murine *Enx1* and human *ENX1* (Laible *et al*, 1997). Comparisons between the *Drosophila* E(Z) and the four mammalian homologues revealed the existence of regions of high conservation (Figure 1.1.7.1.1B) (Laible *et al*, 1997). Among these regions is the evolutionary conserved SET domain with 86% identity.

The mammalian EED-ENX1 complex was found to interact with histone deacetylases providing an indication of the mechanism of repression (van der Vlag and Otte, 1999). Importantly, repression of a transgene artificially targeted by EED was found to require histone deacetylase activity (van der Vlag and Otte, 1999). Purification of ESC-E(Z) complex in *Drosophila* showed that it also contains a histone deacetylase, Rpd3, and the histone binding protein p55 (Tie *et al*, 2001). However, histone deacetylase activity is thought not to be the only mechanism of repression by PcG proteins, because repression of transgenes artificially targeted by PcG proteins in *Xenopus laevis* oocytes were not affected by inhibitors of histone deacetylase activity (Strouboulis *et al*, 1999).

1.1.7.2 - The polycomb repressive complex 1 (PRC1)

Studies of *Drosophila* PcG double mutants and of extra copies rescuing haplo-insufficiency phenotypes strongly suggested that additional sex combs (ASX), polycomb-like (PCL), sex combs on midleg (SCM), PC and polyhomeotic (PH) proteins act synergistically (reviewed in Paro, 1990; Kennison, 1995). *Drosophila* null mutations for *Asx*, *Pcl*, *Scm*, *Pc* and *Ph* genes cause misregulation of homeotic genes, resulting in multiple anterior-posterior axis defects, and are embryonic lethal (reviewed in Kennison, 1995). In subsequent studies PCL, SCM, PC and PH, but not ASX, were found to be part of a purified *Drosophila* protein complex termed PRC1 (Shao *et al*, 1999). In addition, a fifth PcG protein, DRING1, was also identified as a component of this complex (Saurin *et al*, 2001). Recently, Levine *et al* (2002) purified a human PRC1 (hPRC1) complex from HeLa cells, which contained homologues of PcG proteins found in *Drosophila* PRC1. A similar complex is thought to exist in mouse as biochemical analysis revealed that PC murine homologue M33

interacts with PSC and DRING murine homologues Bmi11 and Mel18, and Ring1A and Ring1B respectively (Alkema *et al*, 1997; Schoorlemmer *et al*, 1997; Hemenway *et al*, 1998). In addition Bmi1 was shown to interact with PH murine homologues MPh1 and MPh2 (Hemenway *et al*, 1998; Hashimoto *et al*, 1998).

Like their *Drosophila* counterparts, mice deficient for *M33, MPh1*, *Mel-18* and *Bmi1*, all exhibit transformations of the axial skeleton, providing evidence of conservation of function (van der Lugt *et al*, 1994; Akasaka et al, 1996; Core *et al*, 1997; Takihara *et al*, 1997). These mouse mutants result in lethality late in embryonic development or shortly after birth. As *eed* and *Enx1* mutant mice die at earlier embryonic stages (see section 1.1.7.1), PRC1 and Eed-Enx1 complexes appear to act independently. Supporting this, PRC1 homologues, in contrast to ESC-E(Z), have not been identified in the *C. elegans* (Worm) genome (Korf *et al*, 1998).

The purified *Drosophila* PRC1 complex was shown to block *in vitro* the ability of Trithorax group (TrxG) complexes to remodel nucleosome arrays (Shao *et al*, 1999). TrxG complexes are involved in maintaining an active chromatin state and were shown *in vitro* to modify DNA topology (supercoiling) in an ATP-dependent manner (Guyon *et al*, 1999). In addition, Shao *et al* (1999) tested whether histone tails are required for the ability of PRC1 to repress this remodelling. Similar to intact histones, tailless histones in nucleosome arrays did not interfere with PRC1 inhibition of TrxG remodelling. Together, these data suggested, at least *in vitro*, that histone tails are not required for formation of a repressive chromatin state by PRC1 PcG proteins, again contrasting with the proposed mechanisms of ESC-E(Z) complex function.

1.1.8- Targeting of PcG proteins

The specific expression profile of PcG regulated genes implies the existence of a highly selective mechanism targeting PcG mediated repression. In *Drosophila* recruitment of PcG proteins to homeotic genes require DNA sequences known as polycomb responsive elements (PREs) (Simon *et*

al, 1993). These are DNA elements of 100-300 base pairs, constituted by multiple protein binding sites, which together regulate homeotic gene expression (Chan *et al*, 1994; Horard *et al*, 2000). Most PcG proteins do not have sequence specific DNA binding activity, so the simplest model for PRE function is that recruitment factors bind to PREs sequences and interact with PcG proteins (reviewed in Francis and Kingston, 2001). The knowledge for the other organisms is more limited, because no PREs have been identified. Consequently, the identification of the latter and of factors involved in the PcG recruitment to target genes are important challenges.

1.2- Nuclear localisation and gene silencing

The patterns of gene expression established and maintained during development appear to rely on a number of different mechanisms. An emerging view is that nuclear localisation could establish states which oppose or facilitate transcription. In this section I describe recent studies which suggest that the spatial organisation of chromosome loci to centromeric heterochromatin may play a role in gene silencing and cellular memory.

1.2.1- The *Drosophila brown*^{Dominant} mutation

One of the most intriguing examples of PEV is the *brown*^{Dominant} (bw^D) mutation caused by an insertion of a large block of pericentric heterochromatin into the proximity of the brown gene, which lies in the distal portion of *Drosophila* chromosome 2 (Slatis, 1955). To investigate whether the variegated expression of the eye colour gene bw^D is influenced by a putative association of the mutated allele with pericentric heterochromatin (repressive domains), Dernburg *et al* (1996) performed fluorescence in situ hybridisation (FISH) in *Drosophila* embryonic and larval nuclei. These two stages represent early and late *Drosophila* development. Results with both mutant (bw^D/bw^D) and wild type (bw^+/bw^+) embryonic nuclei showed that the alleles are always spatially separated from pericentric heterochromatin. However, during larval development the mutant alleles, in contrast with wild type alleles, were found to associate with heterochromatin in a stochastic manner. This observation implied that the bw^D gene is silenced through its association with pericentric heterochromatin. Another feature of bw^D described by Slatis (1955) is that heterozygous (bw^D/bw^+) embryos silence the wild type allele. This phenotype makes heterozygous (bw^D/bw^+) indistinguishable from homozygous (bw^D/bw^D). In order to investigate how the mutated allele (bw^D) caused inactivation of the wild type copy (bw^+) Dernburg *et al* (1996) analysed heterozygous (bw^D/bw^+) embryos. Surprisingly, FISH in bw^D/bw^+ larval nuclei found the bw^D allele paired with the wild type allele, and both to be associated stochastically with pericentric heterochromatin. The results suggest that this association of alleles with pericentromeric heterochromatin caused them both to be silenced. This phenomenon was possibly due to specific interchromosomal associations

that were occurring during *Drosophila* development. Importantly, these results highlighted a possible involvement of nuclear organisation in heritable gene silencing.

1.2.2 - Centromeric association of silenced genes in mammalian cells

Further evidence for a role of nuclear organization in heritable gene silencing came from studies on the localisation of the murine Ikaros protein, a transcriptional regulator present in activated lymphocytes, and its target genes (Brown *et al*, 1997). Ikaros was found to localise to nuclear 4,6-diamidino-2-phenylindole (DAPI) dense regions. DAPI is an intercalating DNA stain that preferentially visualises heterochromatin due to its high DNA compactness. This localisation was confirmed by labelling cells with antibodies against both Ikaros and HP1, a protein marker of heterochromatin (see section 1.1.4 for further detail), and also with a γ-satellite DNA probe which specifically recognises centromeric heterochromatin regions in mouse chromosomes. In order to study the relationship between Ikaros localisation and their target genes, immunoDNA FISH analysis was performed with locus specific probes for the genes *Cd2*, *Cd4*, *Cd8α*, *Cd19*, *Cd45* and *λ5* in mouse pre-B and mature cell lines (Brown *et al*, 1997). Inactive genes were found to be associated with Ikaros at centromeric heterochromatin domains (Figure 1.2.2.1). In contrast, active genes were localised elsewhere in the nucleoplasm. These results provided a striking correlation between gene position within the nucleus and transcriptional activity, raising the possibility that centromeric heterochromatin recruitment is a widespread feature of gene silencing in mammals.

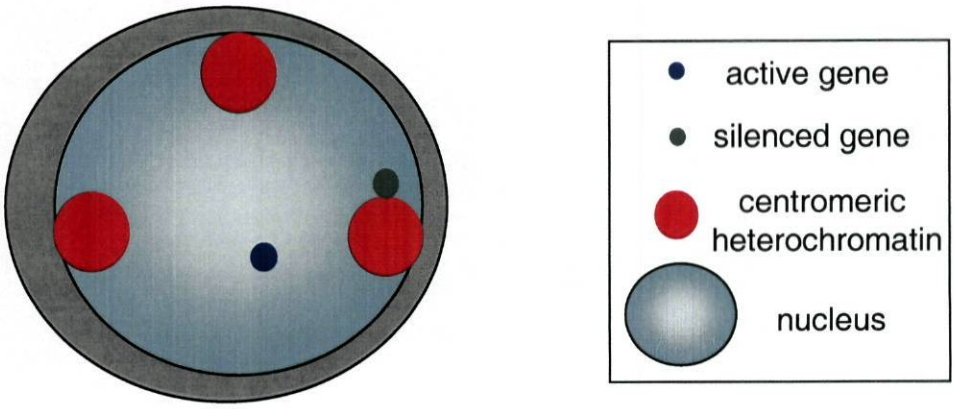


Figure 1.2.2.1- **Association of transcriptionally silent genes with centromeric heterochromatin.**
This illustration represents a model based on data discussed in section 1.2.2. Note that silenced genes localise to centromeric heterochromatin, considered to be a repressive enviroment, while transcriptional active genes localise elsewhere in the nucleoplasm.

In a subsequent study Brown *et al* (1999) showed that the Ikaros cellular distribution is different in non-cycling (G_0) cells when compared with cycling cells. In these studies noncycling B cells purified from the spleen of wild type mice showed very little or no Ikaros staining in their nuclei. After stimulation with mitogenic agents, which induce cell division, Ikaros proteins were seen initially as a faint nuclear haze before progressively displaying the heterochromatin associated distribution pattern described above. In order to analyse whether this Ikaros redistribution reflected a relocation of Ikaros target genes, three-dimensional FISH was performed for pericentromeric heterochromatin, using a γ-satellite probe, and for the *Cd2*, *Cd8α*, *Rag*, *Sox1*, *Hoxb4-b9* and *λ5* genes using locus specific probes. Results showed a selective repositioning of genes as cells activated and entered the cell cycle. Transcriptionally inactive genes alone were recruited to centromeric domains following cellular activation.

To investigate whether centromeric recruitment is a cause or a consequence of gene silencing, Brown *et al* (1999) examined *Tdt* and *Rag* gene expression in the nucleus of developing thymocytes (T cells). Immature $Cd4^+/Cd8^+$ thymocytes express *Tdt* and *Rag* but following T cell stimulation their expression is downregulated (Turka *et al*, 1991; Borgulya *et al*, 1992; Brandle *et al*, 1994; Groves *et al*, 1995; Merkenschlager *et al*, 1997). In this study major histocompatibility (MHC) deficient mice were used to ensure that the $Cd4^+/Cd8^+$ thymocytes were MHC naive. The majority of naive $Cd4^+/Cd8^+$ thymocytes showed *Rag* and *Tdt* alleles positioned away from centromeric domains, but following stimulation the *Rag* and *Tdt* genes were associated with centromeric domains. However, this repositioning appeared to be delayed relative to the transcriptional silencing of the *Rag* and *Tdt* genes. Moreover, in cell lines in which *Rag* and *Tdt* silencing is not stably maintained, centromeric localisation did not occur. These studies suggested that the recruitment of transcriptionally inactive alleles to centromeric domains is a consequence of gene silencing and perhaps is involved in maintenance of gene silencing.

To assess whether nuclear localisation is involved in gene regulation during cell lineage commitment, Grogan *et al* (2001) looked at the differentiation of naive $Cd4^+$ T cells into type 1 (Th1) or type 2 (Th2) phenotypes. They used FISH to analyse the nuclear localisation of *Il-4* and *Inf-γ* genes in relation to centromeric heterochromatin in this system. Following specific Th1 or Th2 polarisation, *Inf-γ* is activated and *Il-4* remains silenced in Th1 cells, while *Ifn-γ* remains silenced and *Il-4* is activated in Th2 cells. Results showed that in Th1 cells 52% of *Il-4* alleles were associated with pericentric heterochromatin. Conversely, in Th2 cells 67% of *Ifn-γ* alleles showed association with heterochromatic domains. Also, more than 95% of the expressed alleles, *Il-4* in Th2 and *Ifn-γ* in Th1 cells, were localised away from heterochromatic domains. Thus, gene association with heterochromatic regions correlates with control of expression and cell fate in the two different lineages. However, heterochromatin association was incomplete and thus was not an absolute requisite for gene silencing and correct differentiation.

Differences in nuclear localisation also appear to be involved in the monoallelic expression of the heavy chain Immunoglobulin (*IgH*) gene (Skok *et al*, 2001). A combination of immunofluorescence with DNA and RNA FISH demonstrated that in activated splenic B cells a single *IgH* allele, the silenced one, is localised to heterochromatic domains.

Together, these data suggest that gene compartmentalisation to heterochromatic domains may play a role in establishing and/or helping to maintain a gene repression state. However, functional data supporting gene compartmentalisation as an epigenetic mechanism is lacking and therefore it's significance is still speculative. In conclusion, these results emphasise that studies of the genome in the context of nuclear architecture are indispensable for the better understanding of cell lineage fate in terms of gene activity.

1.2.3 - Regulatory elements of gene nuclear localisation

Various studies have identified DNA elements flanking genes, termed enhancers, which act to maintain genes in an active state (Walters *et al*, 1995, 1996; Sutherland *et al*, 1997). In order to assess the ability of an enhancer to influence silencing and nuclear localisation, Francastel *et al* (1999) performed FISH analysis in erythroleukemia cells containing transgenes with wild type or derivative mutant enhancers linked to a reporter gene. Active transgenes linked to functional enhancers were seen to localise away from heterochromatin. However, transgenes with enhancer mutations, which caused increased transgene silencing, were localised at centromeric domains. Together, these results suggested that functional enhancers antagonise gene silencing by preventing localisation near centromeric heterochromatin, or by recruiting a gene to a nuclear compartment in which transcription is favoured and stably heritable.

To investigate the mechanisms of transcriptional activation in heterochromatin Lundgren *et al* (2000) performed FISH analysis on pre-B cells and fibroblasts from transgenic mice containing intact or mutated *λ5* multicopy transgenes with centromeric integration. *λ5* is expressed in pro- and pre-B cells in mice and encodes a component of the light chain that forms part of the pre-B cell receptor (Melchers *et al*, 1993). The transgene also contained a set of DNAse I hypersensitive sites, located downstream of the *λ5* gene, which were previously shown to be important for the regulation of the endogenous allele (Sabbattini *et al*, 1999). DNAse I hypersensitive sites are regions of chromatin more sensitive to digestion by DNAse I than bulk chromatin. The intact *λ5* transgene was found to be located on the outside of heterochromatic domains in pre-B cells, which normally express endogenous *λ5*, and in fibroblasts, where the endogenous *λ5* gene is silenced (Lundgren *et al,* 2000). However, deletion of the hypersensitive sites caused association of the *λ5* mutated transgene with heterochromatin in fibroblasts but not in pre-B cells. These data provided evidence for the involvement of DNAse I hypersensitive sites in nuclear positioning of a transgene away from heterochromatin. To test whether the Ebf transcription factor, which is present in pre-B cells and regulates the *λ5* gene (Kee and Murre, 1988; Sigvardsson *et al*, 1997; O'Riordan and Grosschedl,

1999), is involved in maintaining the transgene away from heterochromatic domains, Lundgren *et al* (2000) analysed pre-B cells of heterozygotic *Ebf* knockout mice ($Ebf^{+/-}$) mice which also contained the *λ5* transgene. These $Ebf^{+/-}$ pre-B cells were shown to have a reduced dosage of Ebf relative to wild type pre-B cells and the majority of them had λ5 transgenes associated with centromeric heterochromatin. These data indicated that transcription factors are involved in the nuclear positioning of a transgene away from heterochromatin.

1.3- X chromosome inactivation

1.3.1- The discovery of the X chromosome inactivation phenomenon

In 1949 Barr and Bertram found a cytological difference between nuclei of female and male cat neuronal cells. This was a dark heterochromatic stained body which they termed the sex chromatin, today known as the Barr body. Further cytological analysis identified the Barr body as being the X chromosome (Ohno *et al*, 1959). Studies of X:autosome translocations and the mosaic phenotypes of X-linked genes led to the hypothesis that in early female mammalian development one X chromosome, per cell, undergoes random inactivation (Lyon, 1961). The inactive state was proposed to be heritably maintained in all subsequent cell divisions. This hypothesis was subsequently proven by experimental evidence.

1.3.2 - The inactive X chromosome

X chromosome inactivation is a very stable mechanism of gene silencing and represents an excellent model for the study of heritable gene repression. Analysis of female somatic cells found that the inactive X differs from its active homologue in many ways (Figure 1.3.2.1). Such differences will be described and discussed in this section.

Cytogenetic studies of mouse female embryos, using 5-bromodeoxyuridine (BrdU) or fluorescent DNA stains, found that the two X chromosomes replicate asynchronously, with the inactive X replicating later in S-phase than the active (Takagi, 1974). Subsequent analysis of individual X-linked genes, using either interphase fluorescent *in situ* hybridisation (FISH) and BrdU labelling as an indicator of the cell cycle stage within S-phase, or by southern blot analysis using restriction enzymes recognising newly synthesised DNA labelled with BrdU, confirmed that genes replicate early when expressing (active X) and late when silenced (inactive X) (Schmidt and Migeon, 1990; Boggs and Chinault, 1994).

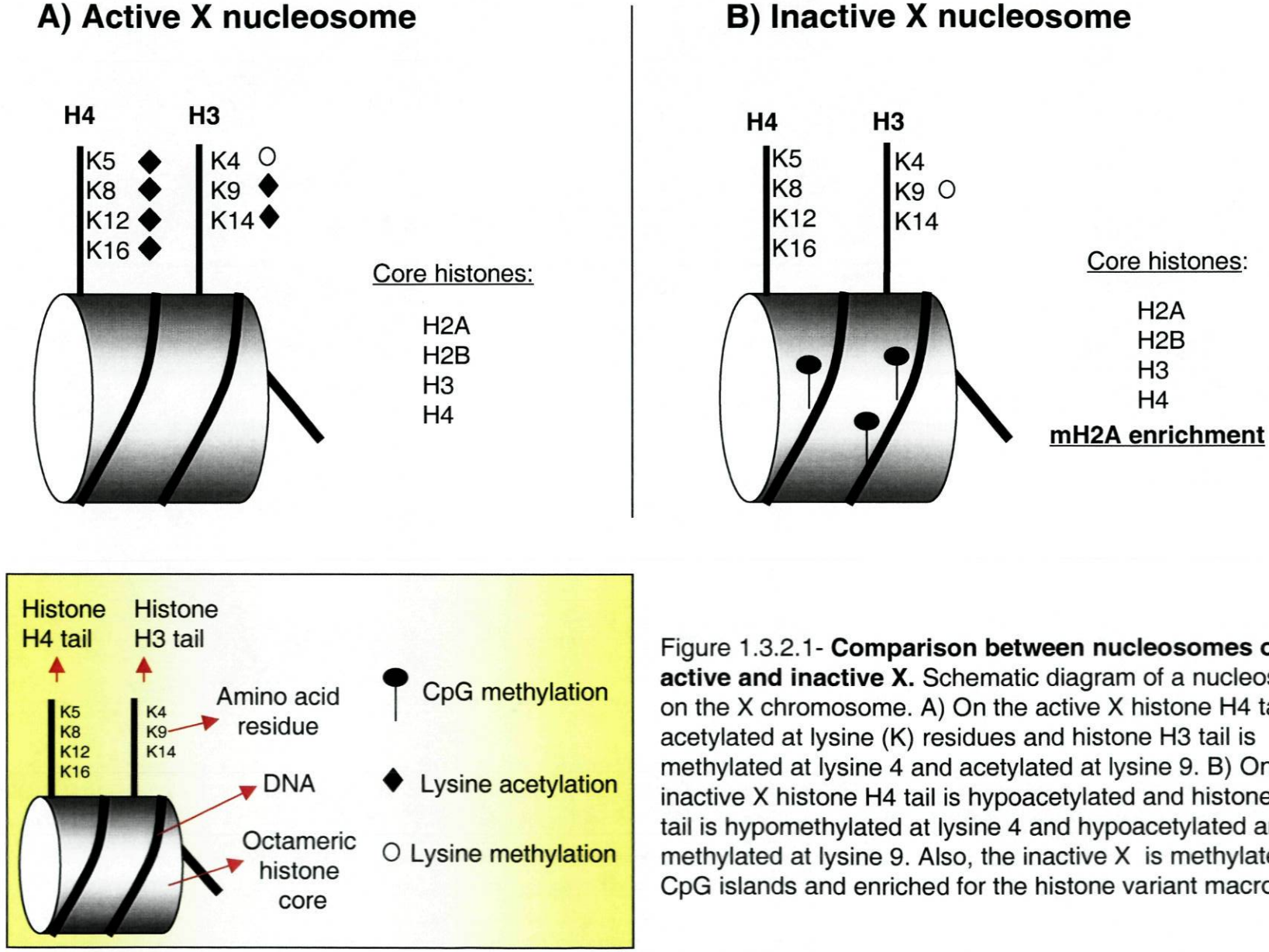


Figure 1.3.2.1- **Comparison between nucleosomes of the active and inactive X.** Schematic diagram of a nucleosome on the X chromosome. A) On the active X histone H4 tail is acetylated at lysine (K) residues and histone H3 tail is methylated at lysine 4 and acetylated at lysine 9. B) On the inactive X histone H4 tail is hypoacetylated and histone H3 tail is hypomethylated at lysine 4 and hypoacetylated and methylated at lysine 9. Also, the inactive X is methylated at CpG islands and enriched for the histone variant macroH2A.

Late replication is also a feature of pericentric heterochromatin and some silent autosomal genes, and has been suggested to be part of the cellular memory mechanism (Riggs and Pfeifer, 1992).

Southern blot analysis of genomic DNA digested with DNA methylation sensitive enzymes found that the inactive X, in contrast to the active X, is methylated at CpG islands (Lock *et al*, 1986; Keith *et al*, 1986; Norris *et al*, 1991). CpG islands are usually concentrated at promoter regions, and the presence of methylation in these domains can cause gene silencing (Bird *et al*, 1985; Boyes and Bird, 1991). Importantly, these results represented the first observed molecular difference between the active and inactive X chromatin.

To investigate the genomic distribution of histone H4 acetylation, Jeppesen and Turner (1993) performed immunofluorescence on human metaphases using antibodies which specifically recognise acetylated lysine residues in histone H4 tails. Results revealed that all chromosomes, with one exception, were strongly labelled. This poorly stained chromosome was the inactive X. The authors also noticed that chromosomal staining was specific to R bands (gene rich euchromatic regions). A previous paper had reported that human metaphase chromosomes were underacetylated in centromeric heterochromatin, which are gene poor regions (Jeppesen *et al*, 1992). Together, these data documented a very interesting relationship between the presence of histone H4 acetylation in euchromatin, and its absence at both heterochromatin and inactive X (facultative heterochromatin). However, immunofluorescence microscopy is limited in resolution and thus cannot address the acetylation status of individual genes. This was subsequently achieved through chromatin immunoprecipitation (ChIP) assays, performed by Sharp and Gilbert (1999). In these experiments human/hamster cell hybrids were used, containing either the human active X or the inactive X, to allow the examination of the acetylation status of particular DNA sequences on the X chromosome. Their results demonstrated that transcribing genes have high levels of acetylation at the promoter regions, whereas the promoters of silenced genes were clearly hypoacetylated. The DNA methylation status of the genes studied was also assessed and it was found that lack of acetylation

coincided with methylation at the CG dinucleotides. Today it is quite well accepted that, in general, histone deacetylation correlates with transcriptional silencing and high levels of acetylation with transcriptional activity (Cheung *et al*, 2000).

The inactive X was more recently shown to be enriched for a histone variant, macroH2A (mH2A) (Constanzi and Pehrson, 1998). This protein contains a histone component, similar to histone H2A, and an additional C-terminal domain which is totally unrelated to any known histone. Immunofluorescence for mH2A revealed a large, dense body in female somatic cells which corresponded to the inactive X and was named the macrochromatin body (MCB). mH2A staining is also present throughout most of the nucleus in both male and female cells, suggesting other functions as well as being involved in X inactivation.

Recently, in an immunofluorescence study to investigate the nuclear distribution of methylated histone H3 lysine 9 (H3-K9), it was found that antibodies recognising methylated (methyl) H3-K9 stained the Barr body of female human primary cells from foetal amniocentesis (Peters *et al*, 2002). Double labelling with an X chromosome paint confirmed the presence of methyl H3-K9 staining in one of the two X chromosomes. Interestingly, the staining was mitotically stable and when analysed in more detail was found to be distributed in a banded manner along the inactive X chromosome. Another independent group obtained similar results, showing that methyl-H3-K9 antibodies preferentially stained the inactive X (Boggs *et al*, 2002). In contrast, the staining of an antibody specific for methylated histone H3 lysine 4 (H3-K4) was distributed throughout the nucleus but absent from the inactive X (Boggs *et al*, 2002). ChIP assays for promoters of genes located on the X chromosome confirmed that methyl-H3-K9 antibodies precipitated chromatin corresponding to transcriptionally inactive genes, while methyl-H3-K4 antibodies precipitate chromatin corresponding to active genes (Boggs *et al*, 2002). Importantly, these results suggest H3-K9 methylation as a possible imprint for the maintenance of the inactive state.

1.3.3- Imprinted X inactivation

Two types of X inactivation have been described in female mammals: random X inactivation, where there is an equal chance of either the paternal or maternal inherited X undergoing transcriptional silencing, and imprinted X inactivation, where the paternal inherited X is always the one to undergo chromosomal silencing. Both mechanisms are developmentally regulated, with inactivation occurring during cell differentiation (Monk aand Harper, 1979) (Figure 1.3.3.1). Imprinted X inactivation was first observed in marsupial species (Sharman, 1971), and further studies, using cytologically distinguishable X chromosomes, found that imprinted X inactivation also occurs in the extraembryonic membranes of the mouse (Takagi and Sasaki, 1975). In evolutionary terms marsupials are considered primitive mammalians, and since these were shown to have exclusively imprinted X inactivation, and eutherians mainly random X inactivation (with the exception of extraembryonic membranes), it is thought that random X inactivation might have evolved from imprinted X inactivation (Moore *et al*, 1995). The putative evolution from imprinted to random X inactivation would have brought the advantages of heterozygosity, but would also have increased the complexity of the X inactivation mechanism.

1.3.4- The X inactivation centre and the *Xist* gene

Studies using X:autosome translocations in female mice revealed that a silencing signal from the inactive X spread into autosomal euchromatic regions causing repression of adjacent autosomal genes (Russell, 1963). This silencing was not complete and genes closest to the breakpoint were more likely to be inactivated compared to those further away, resembling the position effect variegation phenotype of *Drosophila white* (W^+) gene described in section 1.1.2. Importantly, these studies suggested the presence of a cis acting locus on the X chromosome that was required for gene repression. This locus was termed the X inactivation centre (Xic). Subsequent studies demonstrated that the Xic is involved in the process whereby cells sense the number of X chromosomes that are present (counting), and determine which, if any, to inactivate (choice) (Rastan, 1983; Rastan and Robertson, 1985).

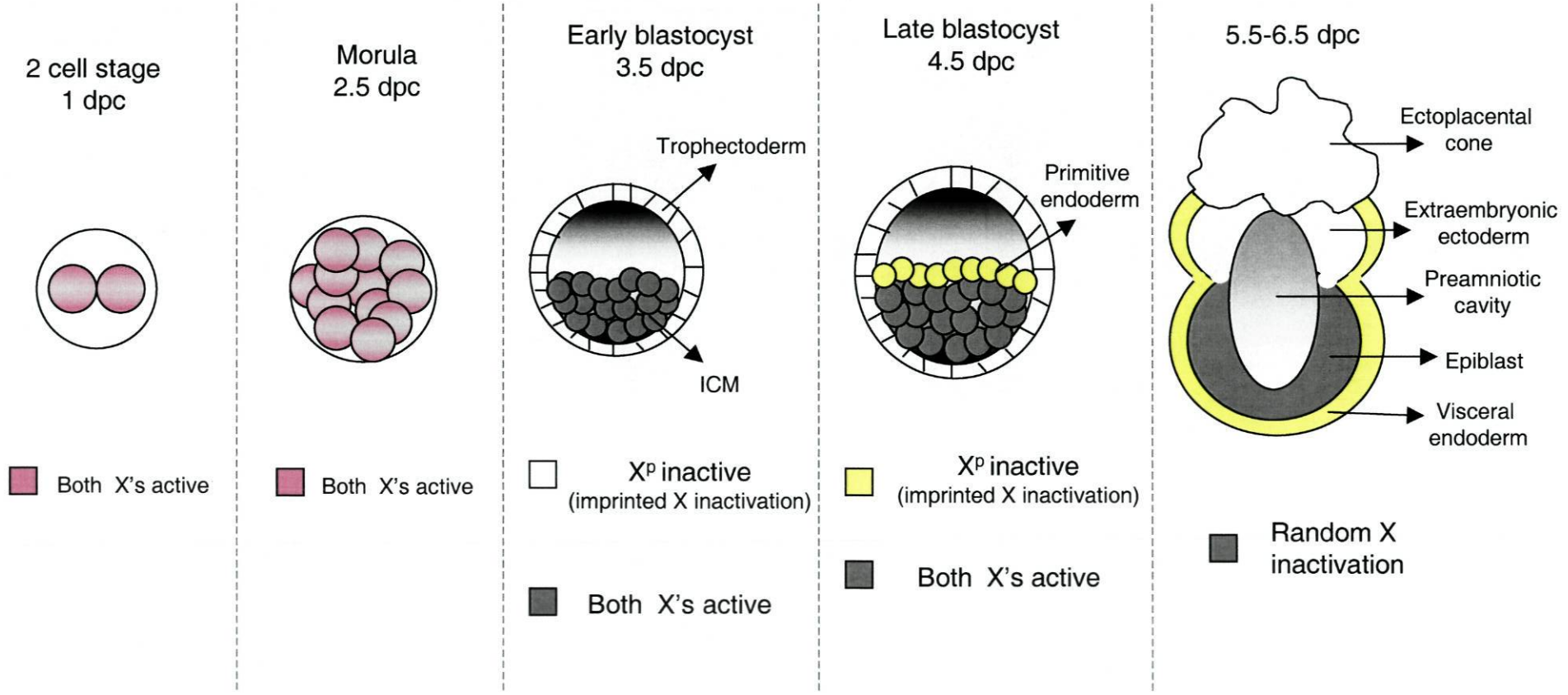


Figure 1.3.3.1- **X inactivation in female mice during embryonic development.**
Imprinted X inactivation occurs in the trophectoderm and primitive endoderm, which will give rise to extraembryonic tissues. Random X inactivation occurs in the epiblast cells which will form the embryo proper. X^p - paternal inherited X chromosome, dpc.- days post coitum, ICM- inner cell mass.

In the early 1990s a gene which was only expressed from the inactive X in XX somatic cells, and which was located within the Xic region was cloned from both human and mouse (Borsani *et al*, 1991; Brockdorff *et al*, 1991; Brown *et al*, 1991a; Brown *et al*, 1991b). It was named *XIST/Xist* (X inactive specific transcript). This gene produces a large RNA molecule, 17 kb in human and 15 kb in mouse, with no open reading frame for protein translation. Also, subcellular fractionation and RNAFISH experiments demonstrated that *Xist* RNA is located within the nucleus in close association with the Barr body (Brockdorff *et al*, 1992; Brown *et al*, 1992).

DNA/RNA fluorescent in situ hybridisation (FISH) experiments on XX human somatic cells, using three dimensional imaging technology, revealed a striking overlap between an X chromosome paint and the *XIST* RNA signal (Clemson *et al*, 1996). This kind of pattern was absolutely distinct from other known RNAs where signals are usually at the chromosome surface. In a subsequent study, *Xist* RNA was shown to be associated with the inactive X throughout most stages of the cell cycle, the only exception being telophase (Duthie *et al*, 1999). Further analysis showed that *Xist* RNA is localised along the X chromosome in a banded manner (Figure 1.3.4.1), selectively associated with gene-rich G-light bands and excluded from heterochromatic regions (Duthie *et al*, 1999).

Detailed comparative analysis of *Xist* genomic sequence between human, mouse and vole revealed relatively poor conservation, with homologies varying between 47 and 57%, which can perhaps be attributed to having no coding potential (Nesterova *et al*, 2001). However, the overall gene structure remained similar. In addition, six types of tandem repeats within the *Xist* gene showed a high degree of homology between the three species studied. The authors suggested the involvement of these tandem repeats in the function of the *Xist* gene, either at a DNA level or as a functional domain of the *Xist* RNA molecule.

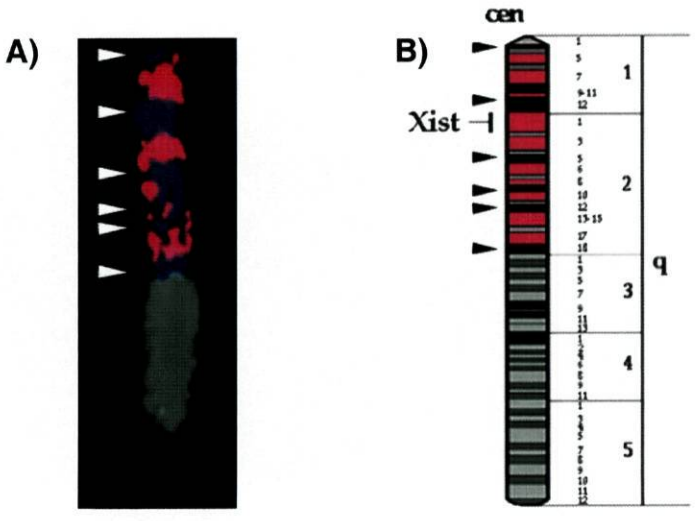


Figure 1.3.4.1- ***Xist* RNA chromosomal localisation.**
A) Banded localisation of *Xist* RNA to *M. transcaspicus* (field vole) metaphase X chromosome. FISH for *Xist RNA* (red) and heterochromatin block (green). Chromosome is counterstained with DAPI (blue). B) *M. transcaspicus* X chromosome cytogenetic map. Arrowheads mark G-dark bands. Note the absence of *Xist* RNA at G-dark bands and at heterochromatin. Illustrations were adapted from Duthie *et al* (1999).

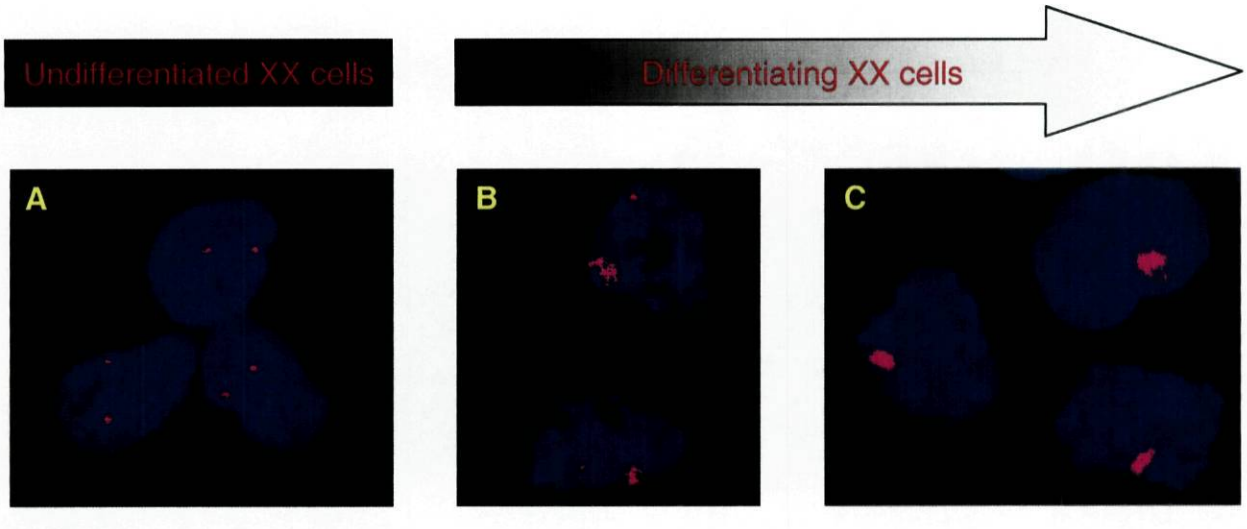


Figure 1.3.7.1- ***Xist* RNA patterns during XX female development.**
Fluorescent *in situ* hybridization (FISH) for *Xist* RNA (red) in undifferentiated and differentiated XX cells. Cells are counterstained with DAPI (blue) A) Active X chromosomes expressing unstable *Xist* RNA (punctate red signal). B) Early differentiated cells expressing one unstable *Xist* RNA signal, from the active X chromosome, and one accumulating signal, from the future inactive X. C) Terminally differentiated cells exhibiting one accumulated *Xist* RNA signal which is covering the inactive X.

1.3.5- *Xist* is necessary for X inactivation

Despite the strong evidence suggesting the involvement of the *Xist/XIST* gene in the X inactivation process, a clear definitive answer only came when Penny *et al* (1996) deleted a 7 kb fragment containing the *Xist* promoter and exon 1 to create a heterozygous *Xist* null mutation in XX ES cells. This ES cell line contained polymorphisms which allowed the detection of gene expression activity in individual X chromosomes. Upon ES cell differentiation, when X inactivation would usually be random, the X chromosome bearing the *Xist* mutation remained active whereas the wild-type X expressed *Xist* and was inactivated. In order to assess the situation *in vivo*, chimeric mice were produced by aggregating the targeted female ES cells with XY host embryos. Analysis of these mice revealed that the X chromosome bearing the wild-type *Xist* gene was always inactive. The incapacity of the mutated X chromosome to undergo inactivation proved that the *Xist* gene is essential for the X inactivation process.

Marahrens *et al* (1997) created a second *Xist* deletion, spanning exons 1 to 5. Targeted male mice were phenotypically normal, but the deletion proved to be lethal in females which inherited the mutated paternal X. This was due to failure of imprinted X inactivation in extraembryonic tissues. Females which inherited the mutated maternal X were viable but showed X inactivation exclusively from the wild-type X.

Neither of the deletions affected the counting mechanism for X inactivation, but in subsequent experiments this was mapped to downstream regulatory elements (Clerc and Avner, 1998).

1.3.6- *Xist* is sufficient for X inactivation

Further progress came with the analysis of XY ES cells carrying a multicopy 450 kb *Xist* transgene (Lee *et al*, 1996). The transgene was shown to recapitulate all of the functions of the Xic when integrated at an autosomal site (Lee and Jaenisch, 1997). Analysis of gene expression produced molecular and cytogenetic evidence for long range inactivation of autosomal genes. Moreover, the

inactive autosome displayed late replication and histone H4 hypoacetylation. Simultaneously, another group obtained similar results using XY ES cells and a smaller construct, which contained 9 kb upstream to 6 kb downstream of *Xist* gene sequence alongside one reporter gene (Herzing *et al*, 1997). The multicopy transgene was integrated into an autosomal locus and showed the ability to silence the reporter gene upon ES cell differentiation. RNA/DNA FISH analysis also revealed accumulated *Xist* RNA signal close to the autosome carrying the *Xist* transgene. Taken together, these results demonstrated that the *Xist* gene is sufficient to initiate inactivation and drive heterochromatin formation. Moreover, it was also shown that there is no need for X chromosome specific elements for propagation of inactivation.

1.3.7 – Expression of *Xist* in development

Early studies demonstrated that *Xist* expression starts at the morula stage, one day before the inactive X becomes cytologically evident (Kay *et al*, 1993). Importantly, these data demonstrated that *Xist* expression was from the paternal allele, which was in agreement with previous findings where the paternal X chromosome was shown to undergo X inactivation in the first differentiated tissues, trophectoderm and primitive endoderm (Takagi *et al*, 1978). Expression of the maternal *Xist* allele was first detected before formation of the primitive streak, the time at which random X inactivation is thought to start (Rastan, 1982).

In order to visualise *Xist* RNA patterns in XX ES cells, an *in vitro* model for random X chromosome inactivation, and during embryo development, RNA FISH was performed (Figure 1.3.7.1) (Panning *et al*, 1996, 1997; Sheardown *et al*, 1997; Johnston *et al*, 1998). XX ES cells were found to have two punctate *Xist* RNA signals and XY ES cells only one. At around 1.5 days of XX ES cell differentiation it was noticed that a proportion (approximately 25%) of cells were exhibiting both punctate and accumulated *Xist* RNA signals. This *Xist* RNA pattern is dominant until day 5 of differentiation, after which the majority of cells start displaying the typical somatic cell pattern of

one accumulated signal only. In XY ES cells the punctate *Xist* signal disappears during differentiation.

Analysis of XX pre-implantation embryos showed that the majority of cells at 8-16 cell embryo stage (morula) had one accumulated *Xist* RNA signal (Sheardown *et al*, 1997). This pattern is likely to represent preferential X inactivation of the paternal inherited X chromosome in trophectoderm cells. Subsequently, in blastocysts, two *Xist* RNA patterns emerge: cells exhibiting accumulated *Xist* RNA and cells with punctate *Xist* RNA only. The latter is thought to originate from an erasure of the *Xist* imprint in the inner cell mass (ICM), which is composed of cells that will undergo random inactivation in the embryo proper at a later time. After implantation, XX embryos from 5.5 to 8.5 days post coitum (dpc), show a decrease of the number of cells with punctate *Xist* RNA while the frequency of cells exhibiting the single accumulated *Xist* RNA signal increases.

1.3.8- Post-transcriptional regulation of *Xist* RNA

Further analysis, revealed that *Xist* transcription rates are similar between XY ES and XX somatic cells (Sheardown *et al*, 1997; Panning *et al*, 1997). This unexpected observation led to the suggestion that the increased levels of *Xist* RNA observed at the onset of X inactivation may be due to post-transcriptional regulation. To investigate this, RNA slot blot analysis was performed at different time points after treating cells with the transcriptional inhibitor actinomycin D. Results revealed that the *Xist* transcript half-life was much higher in XX somatic cells than in XY ES cells. These findings demonstrated that upregulation of *Xist* RNA at the onset of X inactivation is due to increased half-life of the *Xist* transcript and implied that punctate signal seen in ES cells by RNA FISH represented unstable *Xist* transcripts.

Subsequent studies led to the discovery of a gene antisense to *Xist*, which was named *Tsix* (Lee *et al*, 1999). *Tsix* produces a 40 kb transcript spanning the *Xist* gene, which is similarly not thought to encode a protein. RNAFISH analysis revealed that *Tsix* is detected as a punctate signal overlapping

Xist punctate signal in undifferentiated XX and XY ES cells (Lee *et al*, 1999). Interestingly, upon XX ES cell differentiation *Tsix* punctate signal is no longer observed with accumulating *Xist*, coating the future inactive X. In contrast, *Tsix* expression from the active X is still detected for several days and last longer than the punctate *Xist* signal. These results implicated *Tsix* in the modulation of *Xist* expression. Confirming this, recent data supports a model where *Tsix* acts by blocking *Xist* accumulation (reviewed in Plath *et al*, 2002).

1.3.9- *Xist* and maintenance of X chromosome inactivation

Brown and Willard (1994), using a mouse somatic hybrid cell line carrying one human inactive X chromosome, deleted the XIC (containing *XIST*) from the inactive X. Unexpectedly, the deleted human X chromosome remained silenced. This result demonstrated that the *XIST* gene is not necessary for the maintenance of X inactivation, even though *Xist* is continuously transcribed in female somatic cells.

Csankovszki *et al* (1999) revisited the issue of the role of *Xist* in maintenance of X chromosome inactivation by generating targeted XX embryonic fibroblasts deficient for both *Xist* alleles. As expected, the targeted inactive X remained silenced, but in contrast to control XX fibroblasts the targeted XX fibroblasts did not have macroH2A (mH2A) enrichment at the inactive X (henceforth named macrochromatin body (MCB)). This result suggested that *Xist* RNA is required for MCB formation. Further analysis showed that the inactive X remained late replicating and hypoacetylated at histone H4. This result implicated that, like *Xist* RNA, mH2A association with the inactive X is not necessary for the maintenance of X inactivation. To assess whether *Xist* recruits mH2A Rasmussen *et al* (2001) used an inducible *Xist* transgene system integrated at autosomic sites. Results demonstrated that *Xist* expression caused MCB formation in fibroblasts and differentiated ES cells, but not in undifferentiated ES cells. Together, these results demonstrated that *Xist* RNA expression alone is not sufficient to recruit mH2A.

Subsequent studies from Csankovszki *et al* (2001) were designed to assess more accurately the stability of X silencing in *Xist* deficient XX embryonic fibroblasts. These cells carried a green fluorescent protein (GFP) reporter transgene located on the inactive X. Following Cre mediated deletion of *Xist*, cells were three times more likely to reactivate the GFP transgene. Although the overall effect was small, with only 1/2000 cells showing GFP transgene reactivation, the three fold difference observed was a significant result. Therefore *Xist* RNA loss indeed causes some destabilisation of the silenced state. In addition the authors treated the cells with demethylation agent 5-azadeoxycytidine (5azadC) and trichostatin A (TSA), a potent inhibitor of histone deacetylases (Yoshida *et al*, 1995). Individually the drugs also caused reactivation of the X-linked GFP transgene in some cells and when used together, in the cells carrying the targeted inactive X, a synergistic interaction was observed leading to an increased reactivation of the GFP transgene. Taken together, these data demonstrate that silencing on the inactive X works at multiple levels and *Xist* RNA, DNA methylation and histone H4 hypoacetylation are just some of the components that help maintain the highly stable inactive state.

1.3.10- A developmental window of opportunity for X chromosome inactivation

Clemson *et al* (1998) reactivated the silenced *Xist* gene (on the active X chromosome) by treating XY somatic cells with the demethylating agent 5-azadeoxycytidine (5azadC) (Jones and Taylor, 1980). The *Xist* RNAFISH signal was found to be large and properly localised to the X chromosome. To address whether *Xist* was causing gene silencing the authors looked at the expression of two X-linked genes which normally undergo X inactivation. RNAFISH results clearly showed that these two genes were being transcribed. Together, these data led the authors to suggest that when *Xist* RNA expression, stabilisation and proper localisation is removed from the normal developmental context it no longer causes X inactivation.

Elegant experiments performed by Wutz and Jaenisch (2000) confirmed this idea and defined a window of opportunity during which *Xist* RNA can effect gene silencing in differentiating ES cells

(Figure 1.3.10.1). In these studies a full length *Xist* cDNA inducible transgene, integrated into an autosome, was shown to cause chromosome-wide silencing in XY ES cells. Interestingly, the transgene could only effect chromosomal silencing when induction occurred before cells reached day 2 of ES cell differentiation. After this time point, transgene induction caused normal *Xist* RNA accumulation but failed to effect silencing. Moreover, if the transgene was switched off after day 3 of ES cell differentiation, chromosomal silencing was irreversible, but if the transgene was switched off prior to day 3, silencing was reversible. The former result demonstrated that from day 3 of ES cell differentiation chromosomal silencing is *Xist* independent while the latter implied that primary silencing, which is reversible, is *Xist* dependent. In addition, the *Xist* transgene was also able to cause chromosomal late replication and histone H4 hypoacetylation, but only following ES cell differentiation. Interestingly, these results separated *Xist* mediated silencing from late replication and histone H4 hypoacetylation.

1.3.11- Defining functional domains of *Xist* RNA

In order to identify functional domains in *Xist* RNA Wutz *et al* (2002) generated several mutations within the previously described inducible *Xist* transgene (Figure 1.3.11.1). Deletion of a 0.9 kb fragment located upstream of the *Xist* RNA sequence created a mutation which was found to abolish silencing activity without affecting localisation. This region contains a highly conserved tandem repeat shown by computer analysis to fold into stem-loops (Brown *et al*,1992). These stem-loops were suggested to represent binding sites for interacting factors. Further deletions within the *Xist* transgene sequence identified three separate domains involved in RNA localisation. These results confirmed that *Xist* effects are mediated by the cis-spreading of the RNA, and moreover demonstrate that the silencing and cis-localisation functions are separable.

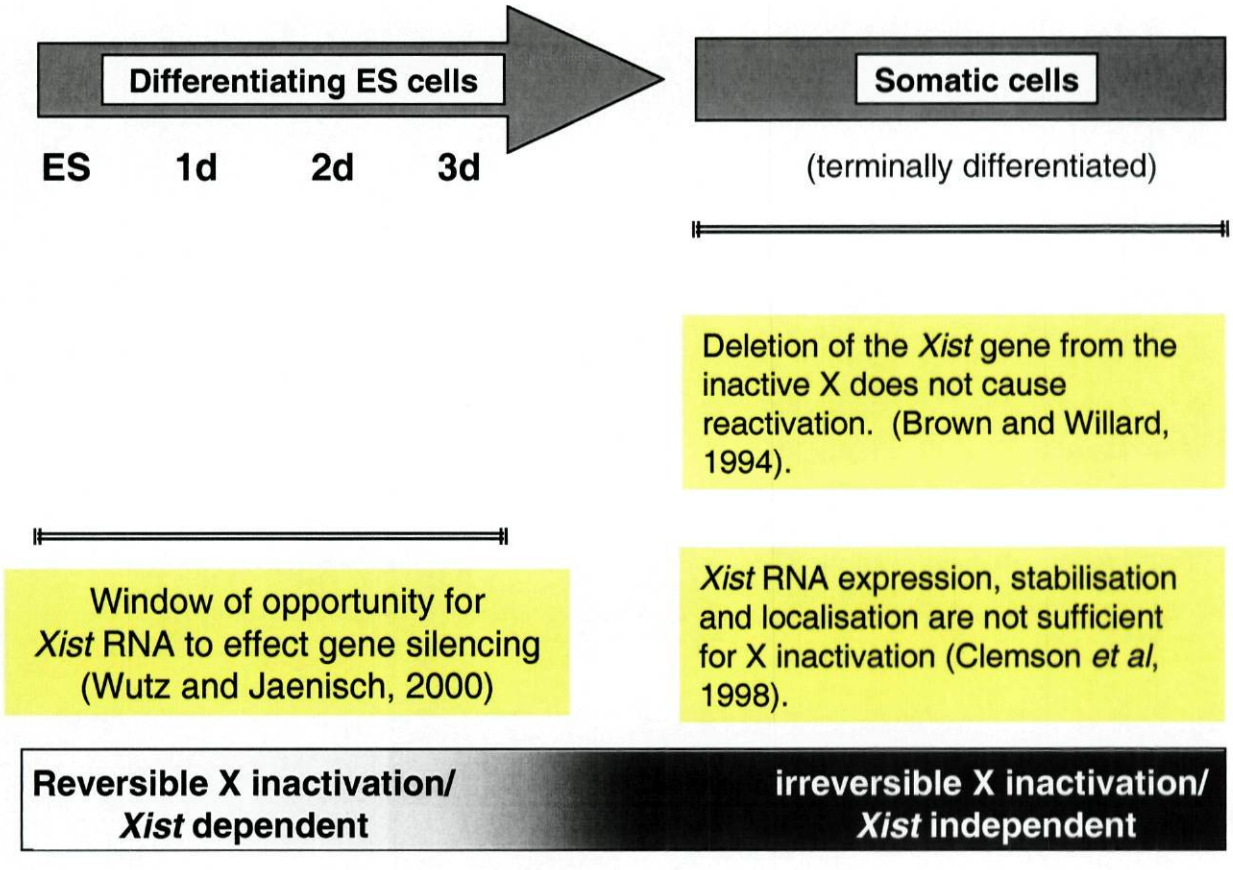


Figure 1.3.10.1- **Window of opportunity model.** This model outlines the window of opportunity hypothesis (section 1.3.10), and highlights the key evidence (yellow boxes). The model implies the existence of *Xist* RNA dependent factors, involved in the initial silencing, and *Xist* RNA independent factors which maintain the silent state.

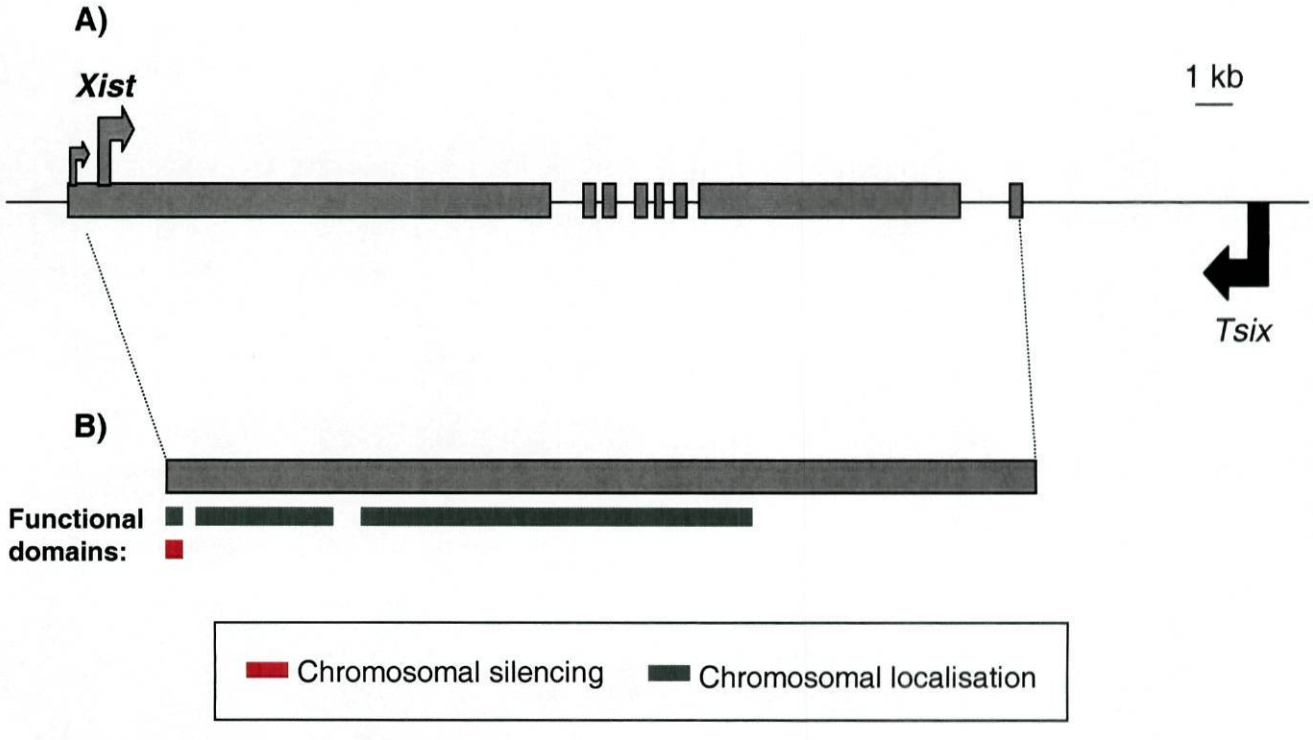


Figure 1.3.11.1- **The *Xist* gene structure and RNA functional domains**.
(A) Schematic representation of the *Xist* locus, grey boxes and grey arrow indicate respectively, *Xist* gene exons and promoters. Black arrow represents the main promoter of the antisense *Tsix* gene (see text for additional information). (B) Localisation of *Xist* RNA functional domains. (see Wutz *et al* (2002)).

1.3.12- Sequence of events in X inactivation

One approach used to identify and characterise factors involved in the X inactivation process has been through study of the sequential order of events in differentiating XX ES cells, which mimics random X inactivation *in vivo* (Figure 1.3.12.1).

As mentioned previously *Xist* expression is the earliest detectable event, occurring between 1-5 days of differentiation. To investigate the role of histone H4 deacetylation in X inactivation Keohane *et al* (1996), used immunofluorescence and the embryonic stem cell system, to analyse the appearance of hypoacetylation on the inactive X in comparison with other known markers such as late replication and DNA methylation. Hypoacetylation was first seen at day 4 of ES cell differentiation, later than the occurrence of transcriptional silencing and appearance of a late replicating X at around days 2 to 3. These data suggested that histone H4 deacetylation is not required for the initial silencing process, implying instead an involvement in maintenance of the silencing state. In these studies the authors also looked at the methylation status of the *Hprt* gene, which undergoes X inactivation and is methylated in female somatic cells. They first detected methylation of the locus at day 21 of ES cell differentiation making methylation on the inactive X a late stage event. This result is consistent with previous studies indicating that DNA methylation is a late event both in embryos and in XX embryonic carcinoma (EC) cells (Lock *et al*, 1986). The latter are thought to be equivalent to ES cells in many ways (Martin, 1980).

Mermoud *et al* (1999), showed that mH2A enrichment on the inactive X occurred after histone deacetylation at day 7 of XX ES cell differentiation. The timing of mH2A accumulation on the inactive X therefore also suggested an involvement of this protein in the maintenance of X inactivation.

In order to characterise the kinetics of histone H3 modifications in the X chromosome inactivation process, Heard *et al* (2002) performed immunoRNA FISH in differentiating female ES cells.

Histone H3 lysine 9 (H3-K9) deacetylation and methylation, and histone H3 lysine 4 (H3-K4) demethylation on the inactivating X were found to immediately follow *Xist* RNA accumulation. In a parallel study Mermoud et al (2002) demonstrated that H3-K9 methylation on the inactive X closely mirrors accumulation of stable *Xist* RNA in differentiating XX ES cells. The H3 modifications thus precede global histone H4 deacetylation. Importantly, the timing suggests that histone H3 modifying activities could be directly recruited by *Xist* RNA.

The data discussed in this section indicates that the inactive X chromosome undergoes a series of stepwise changes which establish and then stabilise the silent state (Figure 1.3.12.2). Following from this, the identification of chromatin modifiers carrying out these steps is an important focus for future research.

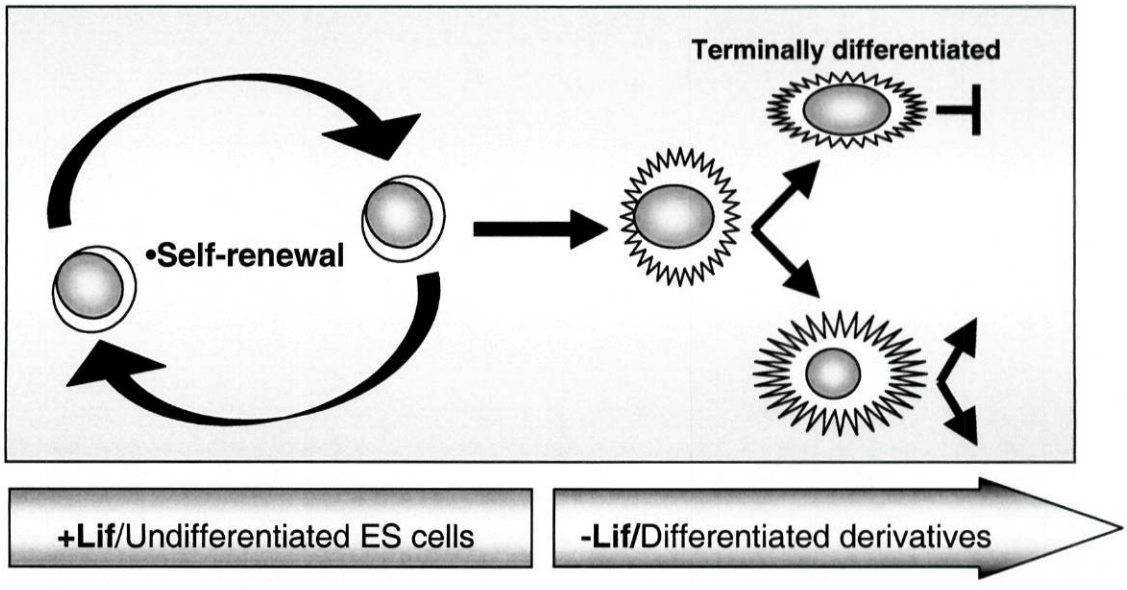


Figure 1.3.12.1- **Embryonic stem cell system.** Illustration of alternative fates of a stem cell. Mouse embryonic stem (ES) cells are derived directly from the pluripotential inner cell mass of the preimplantation embryo. They are capable of proliferating indefinitely *in vitro* in an undifferentiated and pluripotent state in presence of the leukaemia inhibitory factor (Lif) (Smith *et al*, 1988; Williams *et al*,1988). ES cells cultured in presence of Lif can contribute to the development of the whole embryo when they are injected back into the blastocyst (Bradley *et al*, 1984). Withdrawal of Lif is sufficient to induce differentiation of ES cells *in vitro*. Differentiation gives rise to embryoid bodies composed of a large spectrum of differentiated tissues, mimicking gastrulation and early embryo organogenesis (reviewed in Smith, 2001).

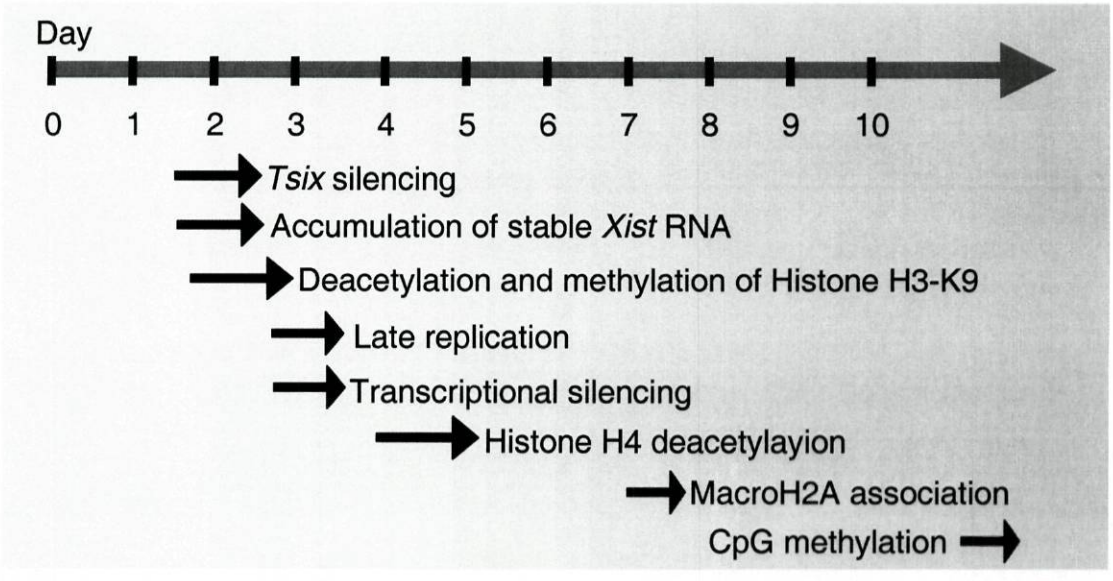


Figure 1.3.12.2- **Timing of X inactivation events in differentiating XX ES cells.** This chart represents a summary of the data described in the text concerning the sequence of events in X inactivation (section1.3.12). The top arrow indicates differentiation time points and the short bold arrows indicate initiation of events in the X inactivation process.

Chapter 2: Materials and methods

2.1- Cell culture

2.1.1- Cell lines and culture conditions

All cell lines used in this study were from murine origin. XX PGK12.1, XX LF2, XY Efc1, XY 129/1 and XY LH1 Embryonic Stem (ES) cells were grown in ES cell media (Dulbecco's Modification of Eagles Medium (DMEM) (Gibco)/20% foetal calf serum/2 mM glutamine/50 μg Streptomycin/50 units of penicillin/1 times MEM Non-essential amino-acids/10mM beta-mercaptoethanol) supplemented with 1000Units/ml of leukemia inhibitory factor (LIF) as described before (Kay *et al*, 1993; Norris *et al*, 1994; Sheardown *et al*, 1997; Herzing *et al*, 1997). In addition to ES medium, LH1 and 129/1 ES cells were grown on inactivated feeder SNL cells (XY STO fibroblasts transfected with neomycin resistent LIF expression plasmid). The pre-B cell line B3 was grown in Iscove's modified Dulbecco's medium (Gibco) containing 10% foetal calf serum and antibiotics as described previously (Fisher *et al*, 1995; Brown *et al*, 1997). All cells were grown at 37^0C/ 5% CO_2/ 100% humidity.

Differentiated ES cells were generated by withdrawal of LIF from the ES cell media (EC10) as described previously by Mermoud *et al* (1999). Cells were grown in tissue culture plates up to day 3 and re-plated onto bacterial petri-dishes between day 3 to 7, during which they were unable to adhere and generated spherical aggregates (embryoid bodies (EBs)) of differentiating cell types. At day 7, EBs were transferred to standard tissue culture plates, where cells adhered and formed differentiating outgrowths. Cells were harvested by trypsinization at selected time points during 0 to 11 days of differentiation and analyzed by immunofluorescence, *in situ* hybridization, Western blotting and Northern blotting.

Trichostatin A (TSA) (WAKO cat. No 204-11991) ES cell treatment was carried out as described in section 5.2.3.

2.1.2- Passaging of ES cells

When cultures had reached 70-90% confluence they were washed with PBS, and then treated with trypsin (0.25% trypsin (Gibco-BRL), 0.04% EDTA) that had been supplemented with 2% chicken serum (Gibco-BRL). The cells were incubated at 37^0C until fully digested (typically 5 minutes), and the digestion terminated by the addition of an excess of the appropriate growth medium. ES cell lines were not split more than 1:8.

2.1.3- Freezing and thawing of cells

Aliquots of $3x10^6$ cells were frozen in a volume of 1 ml. Briefly, cultures were trypsinised and ressuspended at $6x10^6$ cells/ml in growth medium. An equal volume of 2 times freezing medium (20% DMSO in foetal calf serum) was added dropwise to the cells, with continual mixing. The aliquoted cells were placed at -70^0C in a cryo 1^0C freezing container (Nalgene) overnight and subsequently stored under liquid nitrogen. Cells were thawed rapidly at 37^0C in a waterbath, and immediately placed into a 15 ml falcon tube were 10 ml of fresh medium was added dropwise. Then, cells were sequentially centrifuged for 5 min at 1000 rpm, ressuspended in 5 ml of fresh ES cell medium and plated onto a $25cm^2$ flask.

2.1.4- Preparation and use of feeder layers

SNL cells were treated with 0.01mg/ml mitomycin C (sigma) for 3 hrs, before being thoroughly washed with PBS and trypsinised and replated at 1:3 confluence. Since fibroblasts are considerably more adherent than ES cells, feeder cells can be easily removed from an ES cell culture by double replating. Briefly, the culture is trypsinised, replated on non-gelatinised tissue culture plasticware and incubated at 37^0C for 20 min, during which time the majoraty of feeder cells will have attached

to the growth surface. The medium containing the non attached ES cells is then removed and plated out again for further 20 min to give a highly enriched ES cell culture.

2.2- Mouse work

All licensed animal procedures were carried out by Ruth Appanah and Dr. Neil Brockdorff at the Medical Research Council (MRC) Clinical Sciences Centre (CSC).

2.2.1- Post-implantation stage embryos

Embryos were obtained from crosses between CD1 or PGK strain females and either PGK strain or X^{GFP} males. The latter were from Jackson Labs. This strain carries a GFP transgene on the X chromosome, and, when paternally inherited X, it allows for XX and XY embryos to be distinguished (Hadjantonakis *et al*, 1998). *eed*^{3354SB} mutant mice were obtained from the Oak Ridge National Laboratory.

Embryos used in immunofluorescence and immunoRNA FISH analysis were dissected free of decidua and Reicharts membrane. PCR for sexing/genotyping analysis of post implantation embryos was carried out on DNA from extraembryonic ectoderm or embryo fragments. To genotype adult animals and post implantation embryos I amplified by PCR a 570bp fragment from genomic DNA using primers eed3 (tgtgcacctcatcagtctctttt) and eed4 (ggtttgcccccagccttatg) (annealed at 56^0C). The *eed*^{3354SB} and wild-type alleles could then be distinguished after restriction digestion with AluI (Figure 5.2.4.1A). To sex post-implantation embryos I used the UBE1XA (tggtctggacccaaacgctgtccaca) and UBE1XB (ggcagcagccatcacataatccagatg) primers (annealed at 66^0C), which amplify a 198 bp and a 217 bp band from the Y and X chromosome respectively.

2.3- Phage-artificial-chromosome (PAC) library

The mouse PAC library (RPCI21), was provided by the UK HGMP Resource Centre and consists of 7 individual filters (Hybond N nylon membranes) containing 18.432 spotted clones/filter. The

clones correspond to 129/SvevTACfBr mouse genomic DNA inserted in pPAC4 vectors (for further information on vector consult http://bacpac.med.buffalo.edu). The estimated insert average size is 147 kb.

2.3.1- PAC library screening

Membranes were pre-hybridised in 300 ml of hybridisation solution (1.5 times SSPE/ 10% PolyEthyleneGlycol-8000/ 7% SDS/ 100μg/ml salmon sperm DNA) for 1hour at 65^0C. In parallel, approximately 10ng of cDNA was radiolabelled (α^{32}P-dCTP) by random hexamer priming (ROCHE) for 30 min at 37^0C. Probe was subsequently purified from unincorporated nucleotides, using S-300 HR microspin columns (Amersham biosciences), and denatured for 5 min at 100^0C. Then, probe was added to hybridisation solution and membranes were hybridised overnight at 65^0C.

The probes used for PAC library screening were: the insert of image clone 1247019 spanning the full *Cd8α* transcript, pUHD D1 insert containing 1.3 kb homologous sequence to the 5' *Cyclin D1* transcript, the 0.8 kb pOct4 insert corresponding to HindIII and HaeII sites of the full Oct4 cDNA sequence, the product of MT3B1F (ccacccaagcgcctcaagacaaat) and MT3B1R (tggcagcgctgagggaggcacata) primers that amplify a 413 bp fragment with homology to the 5' *Dnmt3b* transcript (specially designed to avoid homology with other Dnmt's), and the pRex1 insert of 846bp comprising homology to the full *Rex1* coding sequence.

On the second day membranes were washed in 1 times SSC/ 0.1% SDS, for 15 min at 65^0C, and in 0.1 times SSC/ 0.1% SDS for 15 min at 65^0C (the number of washes and length of time varied according to the stringency desired). Membranes were then wrapped in saran film, while still moist, and exposed by autoradiography to X-omat X ray film (KODAK) with the use of intensification screens (Hi Speed X/Hoeffer Scientific), and left at -70^0C for 2-10 hrs.

2.4- Southern

Samples of PAC clones (Figure 2.2.1.1) preparations, digested with BamHI, were run on a 1% agarose gel. Then, the gel was treated sequentially with 0.25M HCl for 15 min at room temperature (rt), denatured with denaturing solution (0.5 M NaOH/ 1.5 M NaCl) on shaker for 45 min, and transferred to neutralizing solution (1M Tris/ 3 M NaCl) for 30 min. Subsequently, DNA was transferred overnight in 10 times SSC by capillary action to a Hybond N^+ membrane.

Southern membrane was then incubated in 10 ml of Rapid-hyb buffer (Amersham), containing denatured ^{32}P-labelled probe, for 4 hrs at 65^0C (The probes used for Southern analysis are described in section 2.3.1). Subsequent washes and exposure were performed as described in section 2.3.1.

2.5- Northern

2.5.1- Isolation of RNA

Total RNA extracts were prepared from ES cells, lymphocytes and thymus using RNAzol B (Biogenesis Ltd) method. This Kit is based on phenol/trizol extraction, in which DNA, RNA and protein are extracted into different phases of the mixture. Briefly, pellets of ES cells, lymphocytes and thymus tissue were homogenized mechanically in RNAzol B reagent (2 ml/100mg of tissue or $1x10^7$ cells) using an Ultra Turrax T8 micro-homogeniser (IKA Labortechnik). Then, 0.2 ml of chloroform per 2 ml of homogenate were added followed by 15 seconds vigorous shaking and 5 min incubation on ice. The suspension was then centrifuged at 12000g (4^0C) for 15 min and the top aqueous phase was transferred to a fresh tube. To induce RNA precipitation, an equal volume of isopropanol was added to the aqueous phase and sample was incubated for 15 min on ice. Subsequently, samples were centrifuged for 15 min at 12000g (4^0C) and the supernatant discarded. The RNA pellet was then washed with 1 ml of 75% Ethanol, centrifuged for 8 min at 7500g (4^0C), ressuspended in RNAse free distilled water and stored at -70^0C.

2.5.2- Northern blot procedure

Samples of approximately 10μg total RNA were mixed with sample loading buffer (62.5% deionized formamide/ 1.14M formaldehyde/ 1.25 times MOPS-EDTA-sodium acetate buffer/ 200μg/ml bromphenol blue/ 200μg/ml xylene cyanole/ 50μg/ml ethydium bromide) (SIGMA) in a ratio of 1:3. Then, samples were heated to 65^0C for 10min and chilled on ice before running on formaldehyde gel (15ml of 10 times running buffer (200mM MOPS/ 10mM EDTA.Na_2/ 100mM NaAc, pH 7.2)/ 127ml H2O/ 1.8g of agarose/ 8.1ml formaldehyde (only added after agarose cooling at 50^0C)).

Subsequently gel was soaked twice in 10 times SSC for 20 min each at rt, with gentle shake, and RNA was transferred overnight in 10 times SSC by capillary action to a Hybond N nylon membrane. Then, RNA was fixed to the membrane by baking for 2 hrs at 80^0C.

The northern hybridization was carried by pre-wetting the membrane in 2 times SSC and pre-hybridizing in northern Pre-Hybridization (PH) solution (50% deionized formamide/ 0.5 times deinhardt's solution/ 1% SDS/ 5 times SSC/ 10% dextran sulfate/ 100μg/ml denatured salmon sperm solution) at 42^0C for 2-4hrs with gentle shake.

The northern probes used were: image clone: 1125218 spanning the full *Oct4* transcript, image clone: 894578 spanning the full *Rex1* transcript, the product of primers D3.1 (acagcggtagggatgaaatagtga) and D3.2 (cccccgttgcccaataaaagacc) spanning 684 bp of the *Cyclin D1* 3' transcript, image clone 1247019 spanning the full *Cd8α* transcript, the product of primers 3BF1 (ctacagccagcctcacgacag) and 3BR1 (caccatgccgggctactcta) spanning the full *Dnmt3b* coding transcript, and β-actin (Ambion). Approximately 2 ng of template DNA per ml of hybridisation solution was used for radiolabelling (α^{32}P-dCTP) by random hexamer priming (ROCHE). Probe was subsequently purified from unincorporated nucleotides with S-300 HR microspin columns (Amersham biosciences). Then, probe was denatured for 5 min at 100^0C and added directly to the

membrane in PH solution. Hybridization was carried out overnight at 42^0C with agitation. Subsequently, membranes were washed twice in 2 times SSC for 15 min at rt and if background was high additional washes in 2 times SSC/ 2 % SDS at 65^0C for 45 min and in 0.1 times SSC for 45 min at rt were performed. Exposure was performed as previously described in section 2.3.1.

2.6- Bioinformatics

The genomic sequences used to predict the BamHI restriction sites illustrated in Figure 3.2.1.1 were obtained from Ensembl databases. The transcripts IDs are: ENSMUST00000028980 for *Dnmt3b*, ENSMUST00000033387 for *Cyclin D1*, ENSMUST00000025271 for *Oct4*, and ENSMUST00000032007 for *Cd8α*.

2.7- Characterization of antibodies

Eed, Enx1, Bmi1, MPc2 and Mph1 antibodies have been described elsewhere (Satijn *et al*, 1997; Sewalt *et al*, 1998; van der Vlag and Otte, 1999). Antisera to di/trimethyl H3-K9 are described in Peters *et al* (2001). The trimethyl H3-K27 antibody is described in detail elsewhere (Peters and Jenuwein, submitted). Antisera to methylH3-K4 were a kind gift of Dr. David Allis and are described in Boggs *et al* (2002). Antisera to Ikaros are described in Hahm *et al*, 1994 and were a kind gift of Amanda Fisher. Antisera to HDAC2 and Lamin B were from Santa Cruz Biotechnology. Antisera to Pcna were from SIGMA. Antisera to acetyl H3-K9, acetyl H4, and HDAC1 were from Upstate Biotechnology. The following dilutions were used for immunofluorescence (IF) and Western blotting (WB): Eed mouse monoclonal IgG1, 1:200 (IF) and 1:1000 (WB); Enx1 rabbit polyclonal, 1:200 (IF) and 1:2000 (WB); Bmi1 rabbit polyclonal, 1:200 (IF) and 1:10,000 (WB); MPc2 rabbit polyclonal, 1:200 (IF) and 1:500 (WB); Mph1 rabbit polyclonal, 1:200 (IF) and 1:1000 (WB) Lamin B goat polyclonal, 1:2000 (WB); HDAC1 rabbit polyclonal, 1:100 (IF) and 1:2000 (WB); HDAC2 rabbit polyclonal, 1:100 (IF) and 1:1000 (WB); methyl H3-K9 rabbit polyclonal, 1:1000 (IF); trimethyl H3-K27 rabbit polyclonal, 1:500 (IF); methylH3-K4 rabbit polyclonal, 1:1000 (IF); acetyl H3-K9 rabbit polyclonal, 1:200 (IF); acetyl H4, 1:200 (IF); Pcna mouse monoclonal

IgG2a, 1:3000 (IF); Ikaros rabbit polyclonal, 1:100 (IF). Secondary antibodies for immunofluorescence were from Molecular Probes, and those for western blot were from Amersham Pharmacia.

2.8- Western

2.8.1- Sample preparation

Total cell extracts were prepared from ES cells and lymphocytes as described by Mermoud *et al* (1999). Briefly, cells were washed and harvested twice with cold TB buffer (20mM Hepes/ pH 7.3, 110 mM KOAc/ 5mM NaOAc/ 2 mM MgOAc/ 1mM EGTA/ 2 mM DTT/ protease inhibitors). They were then incubated for 40 min at 37^0C in TB buffer with 0.1% NP-40/ 10 mM $MnCl_2$/ 20 µg/ml DNAseI followed by the addition of SDS-PAGE loading buffer.

2.8.2- Western Blot procedure

Protein samples were boiled for 5 min at 100^0C and run into a ready precast 10% polyacrylamide gel (BioRad) in pre-chilled 1 times running buffer (10% of 10 times running buffer (250 mM Tris Base pH 8.3/ 1.92 M Glycine)/ 0.1% SDS) at 200V for 60 min in ice bucket. Before transfer, the PVDF membrane (BioRad, Cat. 162-0181) was pre-wet in 50% MeOH and equilibrated in 1 times transfer buffer (25 mM Tris Base pH 8.3, 192 mM Glycine) for 5 min. The transfer was performed in pre-chilled 1 times transfer buffer at 100V for 60 min with ice pack and apparatus in ice. Then, the membranes were sequentially incubated with blocking buffer (20 ml of 5% non-fat dry milk (NFDM)/ 1 times TTBS (0.05 M Tris (pH 7.5)/ 0.15 M NaCl/ 0.1% Tween-20) for 60 min on shaker, with primary Ab diluted in 5% NFDM/ 1 times TTBS in sealed bag on rocker overnight, and rinsed 3 times for 5 min with 1 times TTBS. Subsequentelly, detection was carried out using the ECL method (Amersham Pharmacia, Cat. RPN2108) in accord to manufacturer's recommendations. Briefly, the membrane was incubated for 60 min with shaking in 10 ml containing 1 times TTBS and secondary HRP-conjugated antibody. Then, membrane was washed once in 1 times TTBS for

15 min followed by 4 washes in 1 times TTBS for 5 min each. Detection solution (equal volume of detection solutions 1 and 2 (125 µl/cm^2 membrane)) was then added to the membrane, allowing surface tension to keep solution on membrane without spilling off sides, and incubated for exactly 1 min at rt. Thereafter, the membrane was drained and wrapped in Saran film (carefully removing air pockets). Finally, the membrane was exposed to Hyperfilm (Amersham Pharmacia, cat. RPN2103H) for a desired length of time. A color marker (Sigma, cat. C3437) with wide molecular weight range was run in parallel to assess labelled protein seizes.

2.9- Metaphase preparations

ES cells grown nearly to confluence were used to prepare metaphase spreads. Ethydium bromide (1.5 µg/ml) and Colchicine (0.1 µg/ml) were added to the culture 3 hrs and 40 min, respectively, before cell harvesting. Carefully, the medium was poured off, cells were harvested by trypsinisation at rt, and centrifuged down at 1500 rpm for 5 min. Subsequently, cells were ressuspended and incubated in 5 ml of hypotonic solution (75 mM KCl) for 10 min at rt. Then, 100 µl of freshly prepared fixation solution (methanol:acetic acid (3:1)) (drop by drop) was added before spinning cells down at 1500 rpm for 5 min. Subsequently, cells were fixed in 1 ml methanol:acetic acid (3:1) for 30 min at 4^{0}C, without shaking the tube, and centrifuged down once more at 1500 rpm for 5 min. Then, the cell pellet was ressuspended in 1 ml of freshly prepared fixation solution, and using a small quantity of cell suspension in a Pasteur pipette, holded vertically with the end about 10 cm above the slide, a single drop was released onto the slide generating metaphase spreads. Slides were then placed in dessicator (under vacuum) overnight before carrying on FISH analysis.

2.10- Probes used for *in situ* hybridization

The probes used in the detection of *Brx* nascent transcripts were a mixture of partially overlapping λ clones, IIIF9 and IVC3, spanning the *Brx* locus (Clerc and Avner, 1998). *Pgk-1* RNA was detected using λPGK probe, a 17 kb λ clone spanning mouse *Pgk-1* locus (Sheardown *et al*, 1997). *Xist* RNA was detected with the DNA plasmid probe pEFB containing a 3 kb BamHI fragment from *Xist* exon

1. The probe used for detection of nascent *Tsix* transcript, pTsix5', was a gift from Dr. Tatyana Nesterova and contains the *Tsix* promoter and 5' region. The locus specific probes used were: PAC clone 529-B1 for *Rex1*, PAC clone 390-F16 for *Oct4*, PAC clone 579-H3 for *Cyclin D1*, PAC clone 544-L3 for *Cd8α*, and PAC clone 498-A8 for *Dnmt3b*. All PAC clones were provided by the UK HGMP Resource Centre. Detection of γ-satellite repeats (pericentric heterochromatin) was performed using pγSat. This plasmid contains 2 times 4 γ-satellite repeats, and is described in Lundgren *et al* (2000).

2.11- Probe labelling

The standard labelling reaction used 1 μg of template DNA in 16 μl of sterile H_2O (if DNA is eluted in anything else but H_2O remove all the salt before proceeding with labelling reaction) and 4 μl of biotin or digoxigenin-Nick translation mix (Roche). Reaction was incubated for 5 min at 37^0C and then for 80 min at 15^0C. Subsequently, samples were collected from the labelling reaction and run on a 1.5% agarose gel to make sure that probe smear was bellow 400 bp. After this the reaction was stopped by the addition of 1 μl of 0.5 M EDTA (pH 8.0) and by heating the labelling reaction at 65^0C for 10 min. The probe was then cleared from unincorporated nucleotides using S-300 HR microspin columns (Amersham biosciences).

Before *in situ* application an appropriate amount of labelled probe (2ng/kb of target) was aliquoted into a tube and mixed with 5 μg salmon sperm (invitrogen), with 1-3 μg mouse Cot-1 DNA (invitrogen) (except when using pγSat) and with 2.5 times of 100% ethanol. Then, tube was centrifuged under vacuum to concentrate DNA. Subsequently the pellet was ressuspended in hybridisation buffer (50% formamide/ 10% dextran sulphate/ 5% BSA (biolabs)/ 5% vanadyl ribonucleoside (biolabs)) and denatured at 80^0C for 10 min. Finally, the denatured probe was cooled on ice for 1 min, allowed to pre-anneal for 20-25 min, and added to the slide. In the case of pγSat probe use, this was mixed with pre-annealed locus specific probe just prior to the addition onto the slide. This way Cot-1 will not compete with repetitive pγSat sequences.

2.12 - Metaphase FISH

The prepared slides described in section 2.9 were incubated in RNAse A (100 μg/ml in 2 times SSC) for 1 hr at 37^0C. Then, slides were rinsed in 2 times SSC, dehydrated through 70%, 90% and 100% ethanol for 2 min each, and dried in dissecator (under vacuum). Then, slides were warmed to 70^0C for 5 min and denatured in pre-warmed 70% formamide/ 2 times SSC for 3 min at 70^0C. Slides were then sequentially transferred to ice cold 70% ethanol, to 90% and to 100% ethanol for 2 min each. Then, slides were dried in dessicator (under vacuum) and transferred to a 37^0C heated plate where DNA probes were applied. Hybridization was performed overnight at 37^0C in humid chambers. On the second day slides were sequentially washed 4 times for 3 min each in 50% formamide/ 2 times SSC at 37^0C, washed 4 times for 3 min each in 2 times SSC at 37^0C, 4 times for 3 min each in 0.1 times SSC at 60^0C, and 4 times SSC/ 0.1% tween 20 at 37^0C. Then, slides were sequentially incubated in 4 times SSC/ 5% non fat skimmed milk (NFSM) for 15 min at rt (blocking buffer), incubated with aividin FITC (for locus specific probe), biotynilated anti-avidin, and avidin FITC or anti-dig rhodamine (for γ-satellite probe), and anti-Sheep texas red. All antibody incubations were performed in 4 times SSC containing NFSM, for 1 hr at rt. After each antibody layer step slides were washed 3 times for 5 min in 4 times SSC/ 0.1% tween 20. Finally, slides were rinsed once in PBS, mounted in vectashield mounting medium with DAPI and sealed with nail vanish.

2.13- ImmunoDNA FISH

In situ hybridization in whole cells was modified from protocols developed by Funabicki *et al* (1993) and Brown *et al* (1997). Undifferentiated and differentiated ES cells at selected time points were placed onto gelatinized (0.1% gelatin/ PBS) glass coverslips inside electrocharged petri-dishes. These were subsequently filled with media and allowed to adhere inside cell incubator overnight. B3 cells (pre-B lymphocytes) were attached to glass coverslips using poly-L-lysine (5 min at rt). Subsequently, coverslips were rinsed 3 times in PBS and then fixed for 30 min with fixation and permeabization buffer (20 mM KH_2PO_4/ 130 mM NaCl/ 10 mM EGTA, 20 mM $MgCl_2$/ 0.15%

triton X-100/ 0.2% glutaraldehyde (Sigma, grade 1, 70% aqueous)). Then, coverslips were washed 3 times in PBS and twice (15min/wash) with sodium borohydride solution (1mg/ml prepared freshly in water). The coverslips were sequentially incubated with blocking solution (PBS/ 5% goat serum/ 5% Foetal calf serum (FCS)) for 30 min at 37^0C, with anti-Ikaros (1:100) or Pcna antisera (1:3000) for 1hr at 37^0C, and with Texas red coupled anti-rabbit IgG or alexa 488 coupled anti-mouse IgG2a respectively for 1hr at 37^0C. All antibodies were diluted in blocking solution. After antibody incubations coverslips were washed 3 times for 5 min in PBS. Thereafter, cells were kept in the dark, washed and incubated with a 50 mM solution of ethylene glycol bis succinimidyl succinate (EGS) in PBS for 30 min at 37^0C to allow post fixation cross linking of proteins. Cells were then incubated for 1 hr with RNAse A (100 μg/ml in 2 times SSC), washed in PBS, and chromosomal DNA denatured by placing the coverslips (inverted) in 100 μl drops of 85mM NaOH for 2 min. Then, cells were rinsed in ice cold PBS prior to applying DNA probes. Hybridization was performed overnight at 37^0C in humid chambers. On the second day coverslips were rinsed in 4 times SSC, washed twice in 2 times SSC for 30 min at 37^0C and washed once in 1 times SSC for 30 min at rt. Thereafter, coverslips were sequentially incubated in 4 times SSC/ 5% non fat skimmed milk (NFSM) for 15 min at rt (blocking buffer), incubated with aividin FITC and anti-dig rhodamine (for locus specific and γ-satellite probe respectively) or avidin FITC only (if locus specific probe and Ikaros). Subsequently, cells were sequentially incubated with biotynilated anti-avidin and anti-Sheep texas red, and with avidin FITC. All antibody incubations were performed in 4 times SSC containing NFSM, for 1 hr at rt. After each antibody layer cells were washed 3 times for 5 min in 4 times SSC/ 0.05% tween 20. Finally, coverslips were rinsed once in PBS, mounted with vectashield mounting medium (Vector Laboratories) containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and sealed with nail vanish.

2.14- Immunofluorescence and ImmunoRNA FISH

Undifferentiated and differentiated ES cells harvested at selected time points were placed onto Superfrost Plus glass slides (BDH) and allowed to adhere by incubation in a humidified chamber for approximately 3 hrs.

For 6.5 dpc and later postimplantation stages, dissected embryos (or embryo fragments) were washed in PBS, transferred to 5 µl of trypsin solution, and dissociated into small cell clumps with a fine drawn-glass capillary. After the addition of 95 µl of EC10 medium, cells were placed onto Superfrost Plus glass slides and allowed to adhere as described above.

2.14.1- Immunofluorescence

Slides containing medium and attached cells were sequentially: rinsed with PBS, fixed in 2% paraformaldehyde for 15 min, quenched with 50 mM NH_4Cl solution for 10 min, and permeabilized with 0.4% Triton X-100 for 5 min (three PBS washes between each step). Cells were then incubated in blocking solution (0.2% fish gelatin (Sigma) in PBS) for 30 min and in primary antibody for 1 hr in a humidified chamber at 37^0C. After three consecutive 3 min washes in blocking solution, cells were incubated for further 45 min at 37^0C with secondary antibody (all antibody dilutions were carried out in 5% normal goat serum). The cells were washed again with blocking solution (three times), once with PBS, and then mounted in Vectashield antifade containing DAPI.

2.14.2- ImmunoRNA FISH

The immunoRNA FISH was modified from protocols developed by Heard *et al* (2001). Briefly, cells were permeabilized for 3 min in 0.5% Triton X-100 cytoskeletal (CSK) buffer (100 mM NaCl/ 300 mM sucrose/ 3mM $MgCl_2$/ 10 mM PIPES pH 6.8) and fixed in 4% paraformaldehyde (PFA) for 10 min (all solutions on ice). At this stage, slides can be stored in 70% ethanol or in PBS (use PBS storage for optimal Eed and Enx1 detection). Then, slides were sequentially rinsed in 2 times SSC, mounted with a pre-annealed probe, sealed with rubber solution and hybridized overnight at 37^0C.

ICBAS

On the second day slides were washed in 2 times SSC/ 50% formamide for 20 min at rt and washed in 2 times SSC for 20 min at rt. The *Xist* detection was performed simultaneous with *Tsix* or proteins (Eed and Enx1). See bellow for detection of methylated H3-K4/K9/K27 and acetylated H3-K9. Slides were sequentially incubated in blocking buffer (4 times SSC/0.1% Tween 20/ 40% BSA) for 30 min at 37^{0}C in a humidified chamber, incubated with appropriate primary antibodies (diluted in detection buffer (4 times SSC/0.1% Tween 20/ 10% BSA)) for 1 hr at 37^{0}C, and washed 3 times with 4 times SSC/0.1% Tween 20 for 5 min each at 37^{0}C. Subsequently, slides were incubated for 1 hr at 37^{0}C in the remaining antibody layers and washed 3 times for 5 min at 37^{0}C with 4XSSC/0.1% Tween 20 after each antibody incubation. Finally, slides were washed once in PBS and mounted in DAPI-Vectashield.

The detection of methylated H3-K4/K9/K27 and acetylated H3-K9 was carried out on the first day of immunoRNA FISH procedure. The following method was based in a protocol developed by Dr. Jacqueline Mermoud (unpublished). After permeabilization and fixation, slides were incubated sequentially in blocking buffer for 30 min at 37^{0}C, with primary antibody for 2 hrs at 37^{0}C, with secondary antibody (alexa 568 anti-rabbit IgG (H+L)) for 1 hr at 37^{0}C and washed 3 times after each antibody layer with 4 times SSC/0.1% Tween-20 at 37^{0}C. Then, slides were post-fixed in 4% PFA for 10 min at rt, rinsed with PBS, rinsed in 2 times SSC and hybridized overnight as described before. All subsequent steps were carried out as described above.

2.15- Microscopy

The GFP signal in X^{GFP} embryos was detected with a Leica M2FLIII dissection microscope. Confocal images were collected using a Leica TCS-Sp1 confocal microscope (63x and 100x oil immersion) and processed with the Leica software and Adobe Photoshop.

Chapter 3: Analysis of gene localisation in relationship to heterochromatin in Embryonic Stem cells

3.1- Introduction

3.1.1- Degree of heterochromatic association of silent genes in lymphocytes

A focus of study in biology concerns the understanding of how gene expression is regulated during development. As described in section 1.2 an emerging model for gene regulation takes into account the chromatin context and the nuclear positioning of a given gene.

A growing number of genes characterised in the lymphocyte system, show selective positioning to heterochromatic domains when transcriptionally inactive (Brown *et al*, 1997, 1999, 2001; Grogan *et al* 2001; Skok *et al*, 2001). Studies in lymphocyte cell lines (pre-B and mature B cells) for silent alleles of *Cd8α*, *Cd19*, *Cd2*, *Cd4*, *λ5* and *Cd45* revealed a striking (98 to 100%) co-localisation with Ikaros protein localised to heterochromatic domains (Brown *et al*, 1997). In contrast, active alleles showed only 0 to 5% association. In activated splenic B cells, silent loci (*λ5*, *Cd8α*, *Rag*, *Sox1* and *Hox4-b9*) had one or both alleles associated with heterochromatin in 74 to 88% of the cases (Brown *et al*, 1999). In contrast, the Cd2 gene, which is expressed in activated splenic B cells, showed only 13% association to these domains. However, the correlation between expression status and localisation is not allways so clear cut. Grogan *et al* (2001) studies showed for Th1 cells that the silent *Il4* allele is associated in 52% of the cases with heterochromatic domains (see section 1.2.2 for additional information). In contrast, the active *Ifn-γ* allele showed only 5% association. Furthermore, in Th2 cells *Il4* is active while *Ifn-γ* is silent, and the latter showed 67% association with heterochromatic domains and the former only 5%.

The α and *β-globin* loci are two of the most extensively studied gene loci (reviewed in Higgs *et al*, 1998; Grosveld, 1999; Bulger and Groudine, 1999). The human *α-globin* gene occupies a gene rich domain on chromosome 16p which has a high density of unmethylated CpG islands and is constitutively early replicating (Vyas *et al*, 1992; Smith and Higgs, 1999). In contrast, the human *β-globin* gene shows late replication in non-erythroid cells (reviewed in Engel and Tanimoto, 2000). Comparison of human α and *β-globin* nuclear position in haemopoeitic cells revealed that they occupy different nuclear domains (Brown *et al*, 2001). In erythroblasts both α and *β-globin* genes are active and, in more than 86% of the cases both alleles were found to localise away from heterochromatic domains. In ex vivo activated T cell blasts both α and *β-globin* genes are silent. *β-globin* showed 90% allele association to heterochromatic domains while *α-globin* was never associated. It was suggested that the chromosomal context of loci might influence whether or not heterochromatin localisation occurs.

3.1.2- Question: Does heterochromatin association of silent genes occur in ES cells?

It is interesting to consider whether targeting of silenced genes to heterochromatin observed in lymphocytes forms the basis of wider (other cell types) cellular memory during development. In order to investigate this I analysed the nuclear localisation of genes in embryonic stem (ES) cells and their differentiated derivatives (Figure 3.1.2.1). ES cells differentiated *in vitro* by withdrawal of the leukaemia inhibitory factor (Lif) (see material and methods), form a large spectrum of differentiated cell types, mimicking embryonic development (Robertson, 1987). To address the hypothesis I was interested in those genes that are expressed in ES cells and then silenced upon differentiation, and also genes that are silent in ES cells and then expressed upon differentiation. The murine DNA methyltransferase 3b (*Dnmt3b*) and the transcription factors *Rex1* and *Oct4* are genes that fall into the latter class. *Oct4* is a transcription factor that regulates the expression of multiple genes via interaction with other transcription factors present in pluripotent cells (Scholer *et al*, 1991; Saijoh *et al*, 1996).

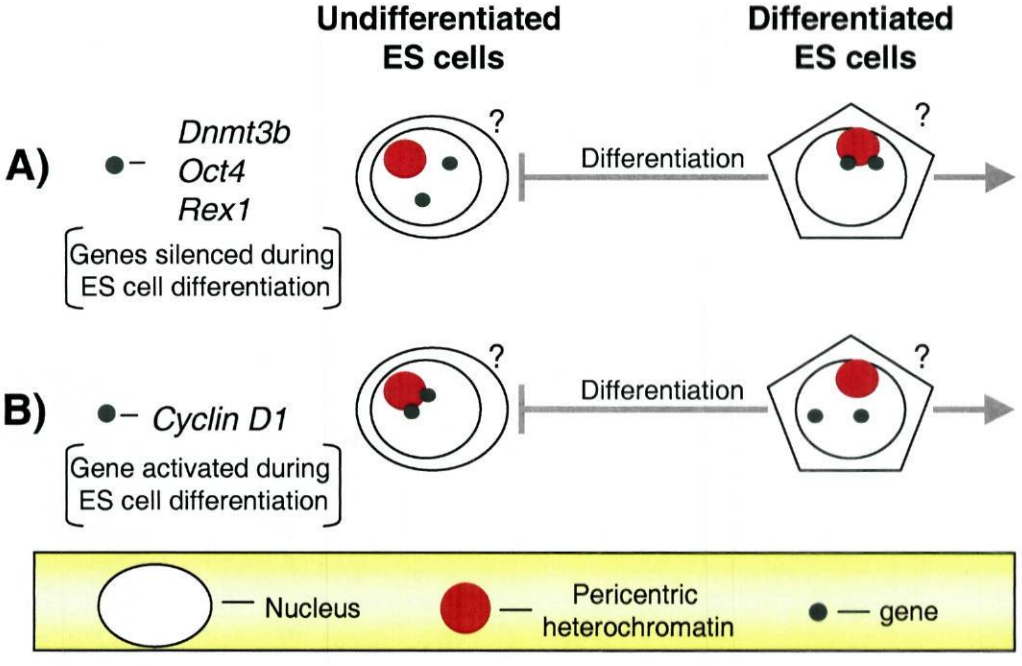


Figure 3.1.2.1- **Hypothesis model.** This scheme represents the hypothesis being tested in chapter 3. The objective is to find whether heterochromatin recruitment of silent genes forms the basis of wider (other cell types) cellular memory during development. To address it the localisation of genes, with particular expression profiles (described in section 3.1.2), in relation to pericentric heterochromatin was analysed using the Embryonic Stem (ES) cell system. See text for additional information.

Its expression levels define differentiation and self-renewal of embryonic stem cells (Nichols *et al*, 1998; Niwa *et al*, 2000). The *Rex1* gene is also developmentally regulated and encodes a transcription factor that contains a zinc finger motif (Ben-Shushan *et al*, 1998). The presence of a zinc finger motif suggests that *Rex1* protein binds DNA and regulates transcription. The *Dnmt3b* gene is involved in genome wide *de novo* methylation (Okano *et al*, 1999). Examples of genes that are switched on at the onset of differentiation includes the D-type cyclins genes (*Cyclin D1*, *D2* and *D3*) (Savatier *et al*, 1996). These are involved in promoting G1 phase progression and G1/S transition in cycling cells (reviewed in Weinberg, 1995).

3.2- Results

3.2.1- Deriving locus specific probes

To obtain locus specific probes for FISH analysis of *Dnmt3b*, *Oct4*, *Cyclin D1* and *Rex1*, cDNA clones or the products generated by RT-PCR were hybridised to a mouse Phage-artificial-chromosome (PAC) library gridded out onto nylon membranes (provided by the UK Human Genome Mapping Project (HGMP) resource centre). The library consists of approximately 128000 genomic clones with an average insert size of 140 Kb. Positive PAC clones were verified by southern blot analysis following digestion with BamH1 (Figure 3.2.1.1). As illustrated, PAC clones for Cyclin D1, Cd8α, Oct4, Dnmt3b and Rex1 were confirmed as true positives. The BamHI fragments detected were compared to the expected fragment size based on available genomic sequence from databases (Ensembl, 2003). As illustrated in Figure 3.2.1.1, labelled southern fragments corresponded to the expected sizes. Only Rex1 PAC clones were not checked this way, due to the absence of genomic sequence information on the databases.

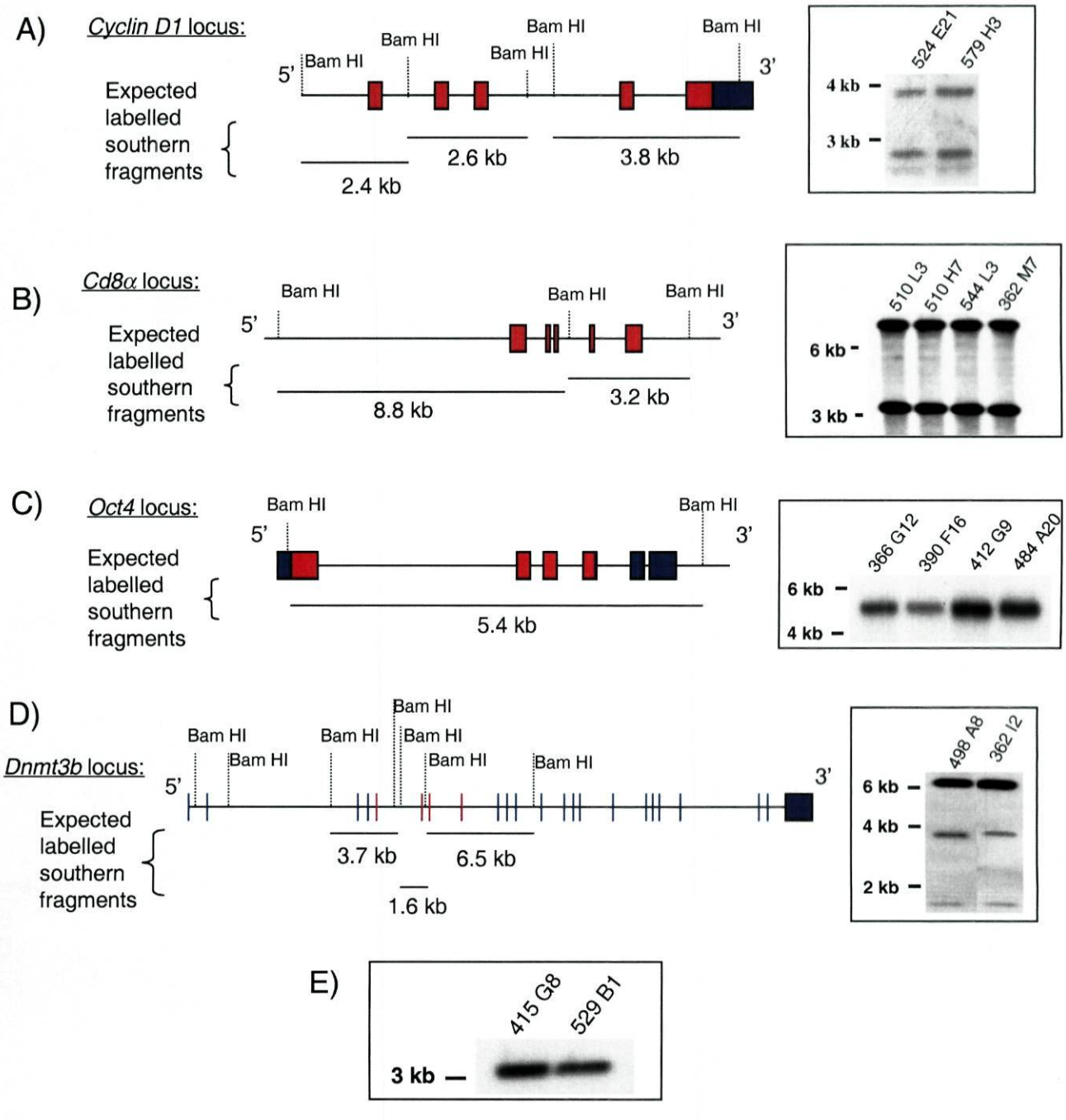


Figure 3.2.1.1 - Derivation of locus specific probes

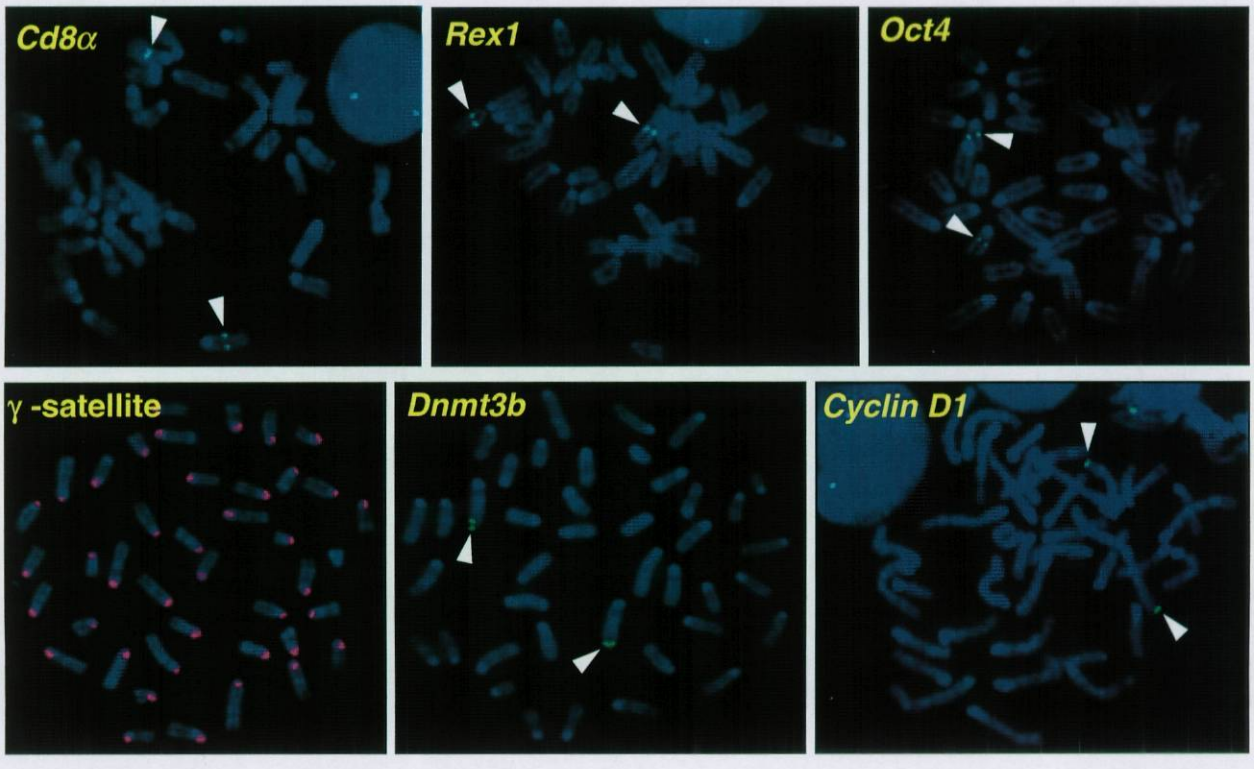
The right panels of Figure A to E show positive clones screened from the mouse PAC library (provided by the UK Human Genome Mapping Project (HGMP) resource centre), tested by southern blot analysis. All clones were digested with BamHI. A high mass ladder was run in parallel with digested PAC clones providing a reference size (as indicated) for the labelled fragments. The middle panels of Figures A to D show the gene locus structure as described in Ensembl (2003) (see material and methods). The red and blue boxes represent exons. Red boxes represent area covered by the cDNA or the product of RT-PCR probe. Dashed lines indicate the restriction sites for BamHI. Below is indicated the expected labelled fragment sizes. Note that in Figures A to D labelled southern fragments corresponded to the expected sizes based on available genomic sequence from databases (Ensembl, 2003). A) Southern blot analysis for Cyclin D1. PAC clones membrane was hybridised with pUHD D1 radiolabelled cDNA probe containing homology sequence to the first 1.3 kb of *Cyclin D1* transcript. B) Southern blot analysis for Cd8α. PAC clone membrane was hybridised with the radiolabelled image clone:1247019 cDNA probe containing homology sequence for the full *Cd8α* transcript. C) Southern blot analysis for Oct4. PAC clone membrane was hybridised with pOct4 radiolabelled probe containing a cDNA fragment size of approximately 0.8 kb corresponding to Hind III and Hae II sites of the full Oct4 cDNA sequence. D) Southern blot analysis for Dnmt3b. PAC clone membrane was hybridised with the radiolabelled product of MT3B1F, MT3B1R primer pairs. This amplifies a 0.4 kb product of Dnmt3b cDNA and was specially designed to avoid homology with other Dnmt's. E) Southern blot analysis for Rex1. PAC clones membrane was hybridised with pRex1 radiolabelled probe containing a 0.9 kb cDNA fragment size corresponding to the *Rex1* full coding sequence. Unfortunately, there is no available genomic sequence for Rex1. Therefore, analysis was limited to checking whether screened PAC clones were positive by southern.

To be useful for immunoDNA FISH it is important that probes hybridise efficiently to the locus of interest, and also that they do not cross hybridise to other sites. To assess this, selected PAC clones were labelled with biotin or digoxigenin by Nick Translation reaction (see material and methods), and hybridised to metaphase chromosome spreads by FISH analysis (Figure 3.2.1.2A). In all cases hybridisation was only detected at a single locus (on two homologous chromosomes). In addition, a probe that recognises specifically repetitive centromeric sequences was also tested (γ-satellite). As illustrated, γ-satellite probe labelled centromeric regions among typical acrocentric mouse chromosomes.

3.2.2- Optimisation of immunoDNA FISH conditions

The immunoDNA FISH technique used to assess gene positioning in relation to pericentric heterochromatin requires conditions that allow DNA hybridisation without disturbing overall three-dimensional structure of the nucleus. This method requires careful optimisation of several parameters. Experiments carried out in lymphocytes by Brown *et al* (1997) used Ikaros protein staining pattern as a control for integrity of the nucleus. Since ES cells do not express Ikaros, a different protein control with a well known distribution pattern was needed. The proliferating cell nuclear antigen (Pcna) protein was a suitable candidate. Pcna is involved in DNA replication and is a marker of the S-phase of cell cycle. In early S-phase Pcna is distributed at numerous small foci throughout the nucleoplasm, whereas in late S-phase Pcna is present in relatively few large foci (Madsen and Celis, 1985). Using immunoDNA FISH fixation and permeabilization conditions Pcna antisera was tested in undifferentiated ES cells (Figure 3.2.2.1A). Nuclear staining was observed in a proportion of cells (presumably representing cells in S-phase). These cells exhibited either multiple small foci or large clusters of Pcna protein. As expected, cells at mitotic cell cycle stages were Pcna negative.

A)



B)

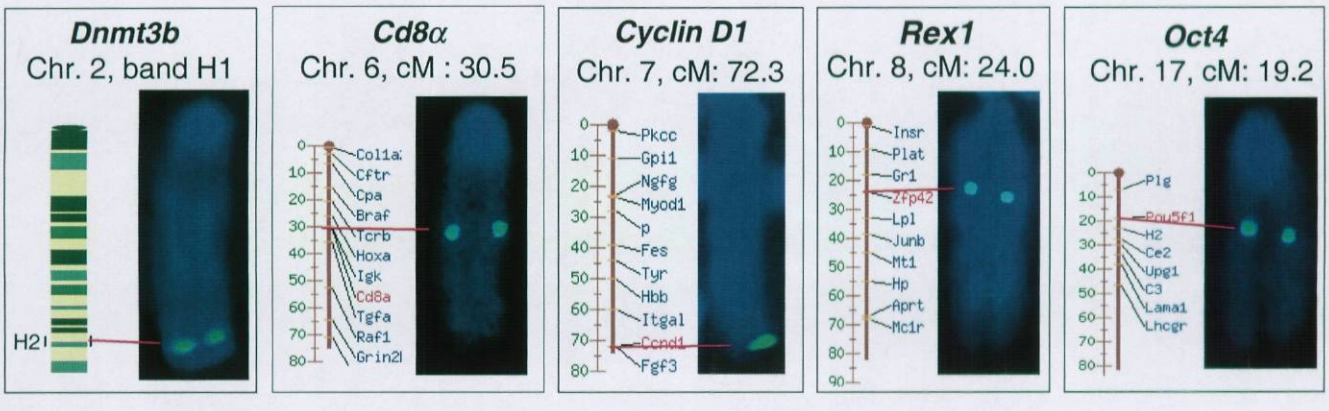


Figure 3.2.1.2- **Specificity and localisation of FISH probes on metaphase spreads.** A) *In situ* hybridisation of mouse metaphase chromosomes for *Cd8α* (green), *Rex1* (green), *Oct4* (green), γ-satellite (red), *Dnmt3b* (green) and *Cyclin D1* (green) as indicated. Note that γ-satellite probe stains chromosome centromeres, corresponding to mouse pericentric heterochromatin containing γ-satellite repeats. Note also that all locus specific probes labelled only a single locus. B) Localisation analysis of tested probes comparing chromosomal physical maps (from mouse genome informatics (MGI) research centre, 2001) and amplified labelled metaphase chromosomes. (Ccnd1 = Cyclin D1), (Zfp42 = Rex1) and (Pou5f1 = Oct4). Note consensus between physical and cytological localisation of tested probes. All preparations were counterstained with DAPI.

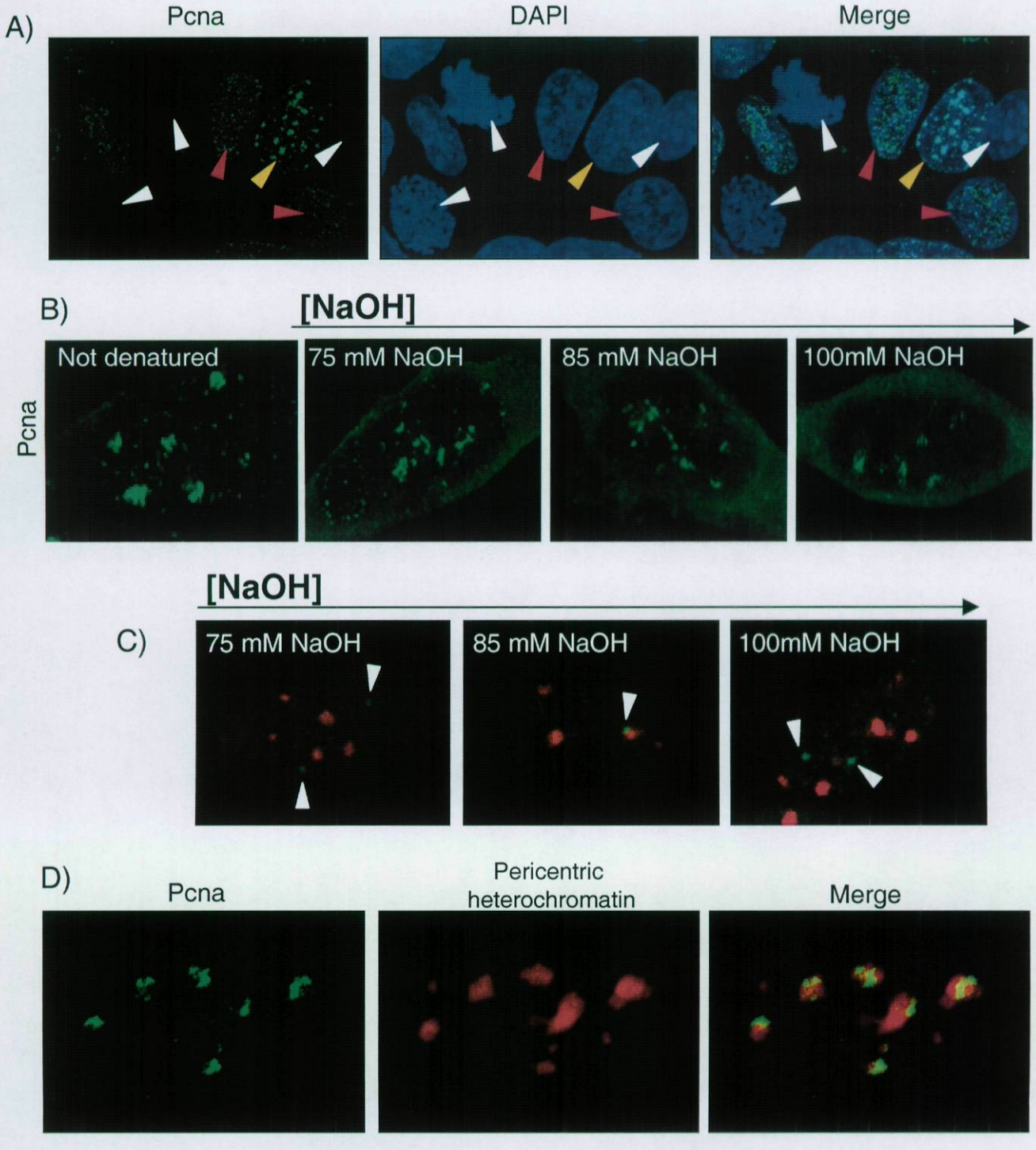


Figure 3.2.2.1- **Establishment of ImmunoDNA FISH conditions in ES cells.**
A) ES cells stained for Pcna using immunoDNA FISH fixation and permeabilization conditions. Note the presence of cells with numerous small foci (red arrows) or large clusters of Pcna protein (yellow arrow) corresponding to early and late S phase patterns respectively. Note also cells lacking Pcna staining corresponding to mitotic stages (left white arrows) and G1 or G2 stages (right white arrow). B) Effect of treating cells for two minutes at different NaOH concentrations (75, 85 and 100mM) on immunoDNA FISH for Pcna (green). Note that Pcna patterns were more severely disrupted in cells treated with 100 mM NaOH. C) Effect of treating cells for two minutes at different NaOH concentrations (75, 85 and 100mM) on immunoDNA FISH for pericentric heterochromatin (red) and Cylcin D1 (green). Note progressive increase on FISH signals intensity correlating with higher NaOH concentration treatments. D) ImmunoDNA FISH of a single ES cell nucleus stained for Pcna (green) and pericentric heterochromatin (red) treated with 85 mM NaOH. Note Pcna staining overlapping with pericentric heterochromatin. The latter is known to replicate late in S phase (Selig *et al*, 1998). All images correspond to single optical sections.

DNA denaturation prior to FISH is achieved by treating cells with sodium hydroxide (NaOH) at a determined concentration and length of time. To determine conditions that maintain integrity of nuclear structure I tested the effect on Pcna staining of treating cells for two minutes at 75, 85 and 100 mM NaOH (Figure 3.2.2.1B). Cells treated with 75 and 85 mM NaOH showed signs of Pcna pattern disruption compared to control (no denaturation) but overall Pcna staining was good. However, Pcna patterns were more severely disrupted in cells treated with 100 mM NaOH. FISH efficiency was also assessed (Figure 3.2.2.1C), and 85 mM NaOH was found to be necessary for optimal DNA denaturing conditions. In conclusion, the optimal NaOH concentration found that allows good protein staining preservation and high FISH efficiency is 85 mM NaOH treatment for 2 min.

3.2.3- Localisation analyses of active and silenced genes in relationship to heterochromatin during Embryonic Stem cell differentiation

3.2.3.1- Definition of scoring criteria

In order to determine whether localisation of silenced genes to heterochromatin is a feature of ES cells I analysed the positioning of *Dnmt3b*, *Oct4*, *Rex1* and *Cyclin D1* loci in relation to pericentric heterochromatin. ImmunoDNA FISH at 85mM NaOH was performed in two different ES cell lines, XX PGK12.1 and XY Efc1, to confirm that the results were reproducible. Cells were analysed at 0, 1.5, 3 and 11 days following differentiation *in vitro* (Efc1 cells were analysed only at 0, 3 and 11 days). In parallel, gene expression analysis was carried out for each gene using northern blotting.

Each locus specific probe was used in pairwise combination with γ-satellite probe and the proximity of target alleles to heterochromatic domains was assessed. In these analyses confocal microscopy was used to view series of 0.25 μm optical sections (Figure 3.2.3.1.1A) collected through 50 to 150 nuclei (100 to 300 scored genes) at a given differentiation time point and cell line. Apparent contact between locus specific and γ-satellite signals was scored as associated (Figure 3.2.3.1.1b).

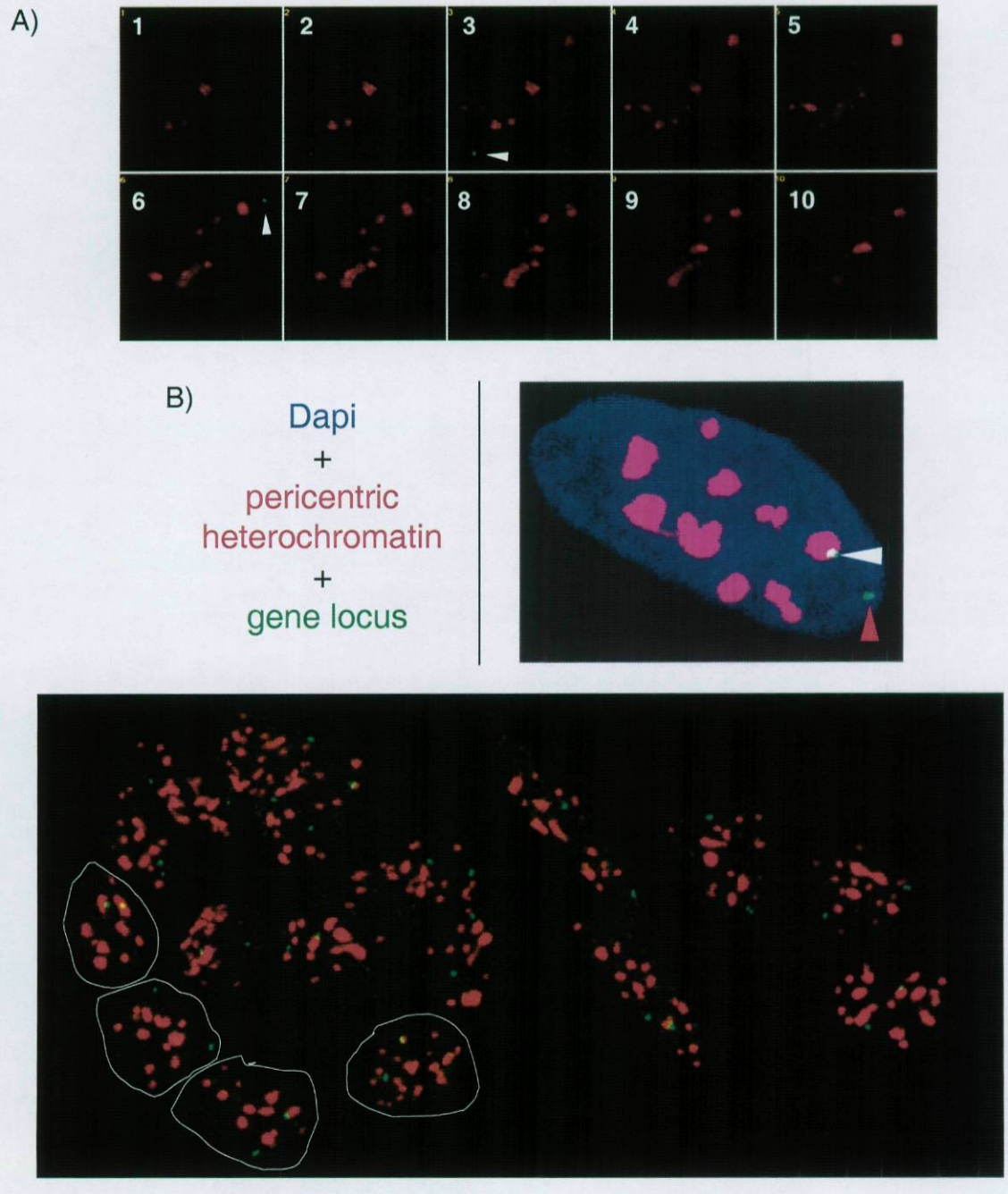


Figure 3.2.3.1- **Definition of scoring criteria**
Using locus specific probes for mouse *Oct4*, *Rex1*, *Dnmt3b* and *Cyclin D1* genes in pairwise combination with γ-satellite, the proximity of target alleles to heterochromatic domains was assessed using three-dimensional immunoDNA FISH in ES cells. (A) Example of serial 0.25 μm confocal sections taken from a nucleus labelled with Dnmt3b probe (green) and γ-satellite (red) in 3 days differentiated ES cells. Note that in this example the 2 alleles localise at different optical sections (see white arrows). (B) Single optical section of a ES cell nucleus exemplifying the two scoring categories. When a locus specific signal (here represented by Cyclin D1) is in close contact with γ-satellite signal the allele is scored as associated with pericentric heterochromatin (white arrow). When the locus specific signal is identified away from γ-satellite signal the allele is scored as non associated (red arrow). (C) Confocal image of nuclei of 1.5 day differentiated PGK12.1 cells stained for *Oct4* (green) and γ-satellite (red) exhibiting high FISH efficiency. Note that all nuclei (see encircled examples) have two identifiable loci. Each locus can be recognised as a single dot or doublet according with the cell cycle stage (G1, S phase, G2 or mitosis).

Alleles were scored as non-associated when identified as being clearly separated from γ-satellite signal. Only slides displaying high FISH efficiency (>80% of targets (alleles) labelled) were used so that almost all cells were eligible for scoring (Figure 3.2.3.1.1C). Finally, slides stained for Pcna under exactly the same immunoDNA FISH conditions were run in parallel as a control for nuclear integrity.

3.2.3.2- Analysis of the *Oct4* locus

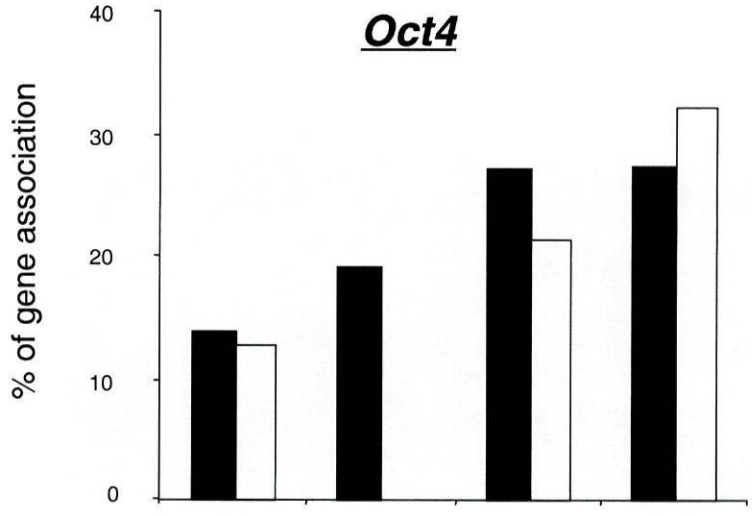
Results obtained for Oct4 are summarised in Figure 3.2.3.2.1. As expected northern blot analysis showed high levels of *Oct4* RNA in undifferentiated ES cells and markedly reduced levels following ES cell differentiation. By day 11, *Oct4* RNA was undetectable.

ImmunoDNA FISH analysis in both PGK12.1 and Efc1 cells revealed that in undifferentiated ES cells approximately 13% of Oct4 alleles are found in close contact with pericentric heterochromatin. This number steadily increased with ES cell differentiation peaking at day 11, the last time point analysed, with 27 to 32% of alleles found associated. Overall, a 2.2 fold increase of gene association with pericentric heterochromatin was observed during ES cell differentiation. This was correlated with reduced Oct4 gene expression

3.2.3.3- Analysis of the *Rex1* locus

As illustrated in Figure 3.2.3.3.1b *Rex1* is highly expressed in undifferentiated ES cells and levels decrease rapidly with ES cell differentiation. By day 11 no *Rex1* transcripts are detected. Analysis of *Rex1* locus positioning in relation to pericentric heterochromatin revealed 11 to 12.5% association in undifferentiated ES cells (Figure 3.2.3.3.1A). Concomitantly with *Rex1* downregulation, association increased to 29% at day 3, but at day 11 only 10.5% of alleles were found associated. These data show initially a correlation between increase of gene association and gene downregulation, but at day 11 percentage of gene association with pericentric heterochromatin was similar to the value found in undifferentiated ES cells.

A)



% of associated alleles in XX PGK 12.1 ES cells	13.9	19	27	27.4
N alleles	266	100	306	124
% of associated alleles in XY Efc1 ES cells	12.75	-	21.4	32
N alleles	102	-	154	100

B)

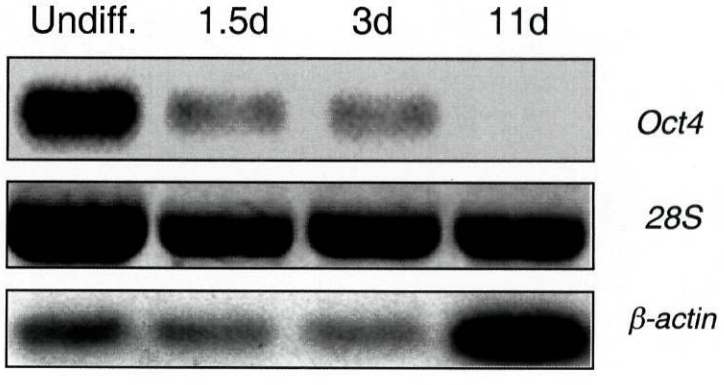
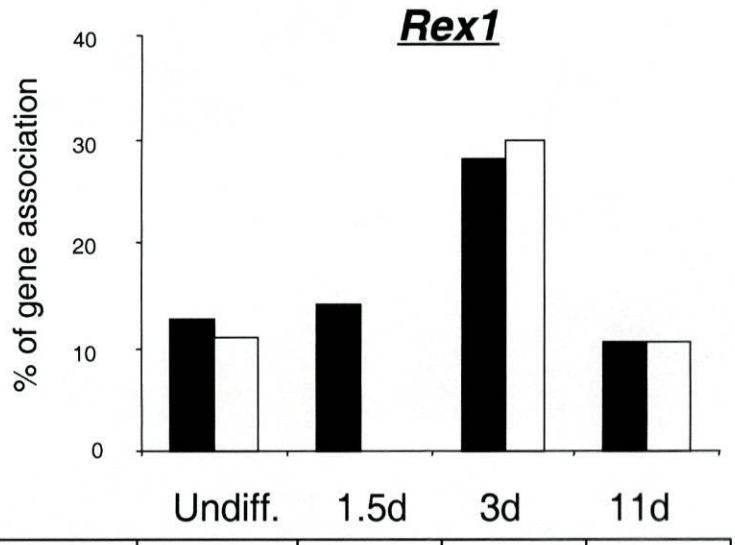


Figure 3.2.3.2.1- ***Oct4* association with pericentric heterochromatin during ES cell differentiation.**

A) Scoring data showing the percentage of gene association with pericentric heterochromatin in both XX PGK12.1 (black bars) and XY Efc1 (white bars) ES cells. Cells were analysed at 0, 1.5, 3 and 11 days (d) following differentiation *in vitro* (Efc1 cells were analysed only at 0, 3 and 11 days).B) Northern blot analysis for *Oct4* using total RNA from undifferentiated (undiff.) and differentiated XX PGK12.1 ES cells. Due to variation of *β-actin* during ES cell differentiation *28S* transcript levels were used as loading control.

A)



% of associated alleles in PGK 12.1 ES cells	12.5	14.2	28	10.4
N alleles	104	114	144	240
% of associated alleles in Efc1 ES cells	10.9	-	29.7	10.5
N alleles	110	-	128	114

B)

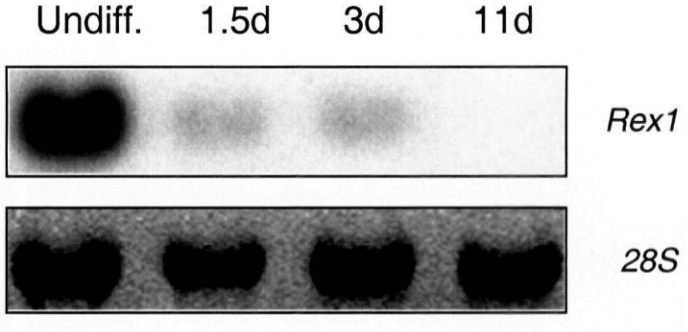


Figure 3.2.3.3.1- ***Rex1* association with pericentric heterochromatin during ES cell differentiation.**

A) Scoring data showing the percentage of gene association with pericentric heterochromatin in both XX PGK12.1 (black bars) and XY Efc1 (white bars) ES cells. Cells were analysed at 0, 1.5, 3 and 11 days (d) following differentiation *in vitro* (Efc1 cells were analysed only at 0, 3 and 11 days).B) Northern blot analysis for *Rex1* using total RNA from undifferentiated (undiff.) and differentiated XX PGK12.1 ES cells. *28S* transcript levels were used as loading control.

3.2.3.4- Analysis of the *Dnmt3b* locus

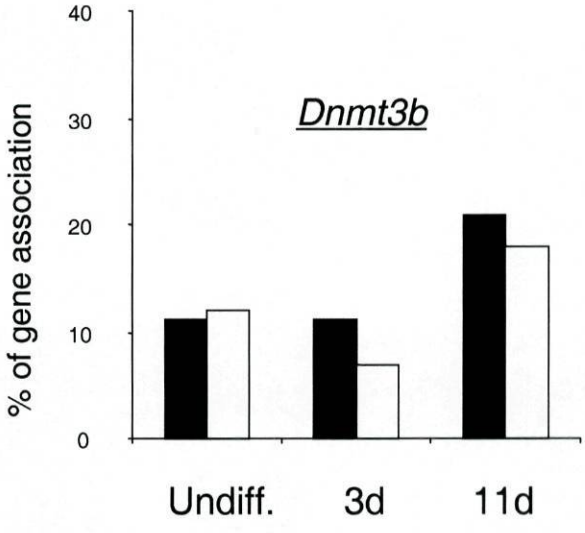
As illustrated in Figure 3.2.3.4.1B, RNA expression analysis showed that *Dnmt3*b is expressed in undifferentiated and 3 day differentiated ES cells. In fact, the latter time point showed higher levels of gene expression. In agreement with previous northern bolt analysis (Okano *et al*, 1999) no *Dnmt3b* expression is detected at day 11 (late stage of ES cell differentiation). This was also true for immunofluorescence and western blotting analysis, using Dnmt3b antisera (Dr. Ilona Zvetkova, Jose Silva and Dr. Neil Brockdorff, in preparation).

ImmunoDNA FISH analysis revealed that approximately 11% of Dnmt3b alleles are found associated with pericentric heterochromatin in undifferentiated and 3 days differentiated ES cells (Figure 3.2.3.4.1A). At day 11, association increases to approximately 20%. The small increase of *Dnmt3b* association with pericentric heterochromatin is therefore correlating with reduced gene expression.

3.2.3.5- Analysis of the *Cyclin D1* locus

Results obtained for Cyclin D1 are summarised in Figure 3.2.3.5.1. In agreement with previously published data *Cyclin D1* RNA was found to be upregulated with induction of ES cell differentiation. ImmunoDNA FISH analysis in undifferentiated ES cells revealed that 33.5 to 36% of *Cyclin D1* loci are associated with heterochromatin. This number decreased during ES cell differentiation in both Efc1 and PGK12.1 ES cells. Thus, coinciding with gene upregulation, *Cyclin D1* showed a 2.7 fold decrease in gene association to heterochromatic domains during ES cell differentiation.

A)



% of associated alleles in PGK 12.1 ES cells	11	11	20.8
N alleles	100	100	178
% of associated alleles in Efc1 ES cells	11.8	6.6	17.8
N alleles	102	106	90

B)

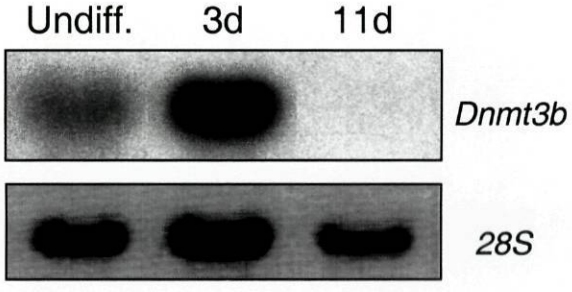
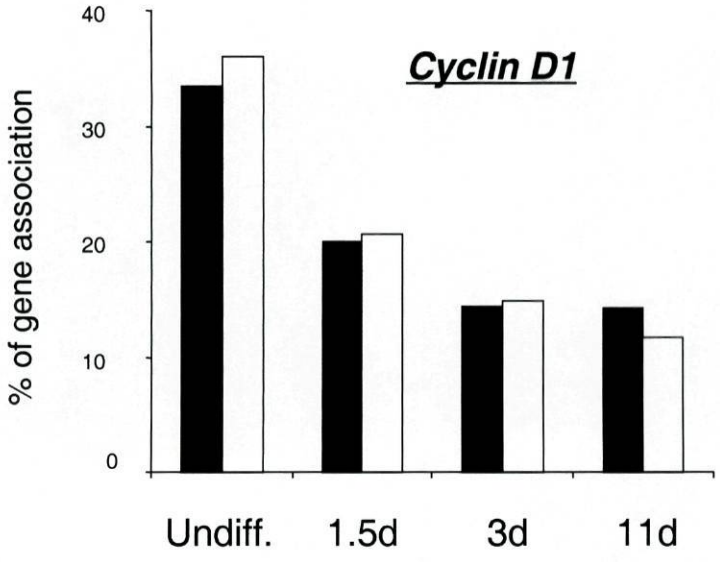


Figure 3.2.3.4.1- ***Dnmt3b* association with pericentric heterochromatin during ES cell differentiation.**

A) Scoring data showing the percentage of gene association with pericentric heterochromatin in both XX PGK12.1 (black bars) and XY Efc1 (white bars) ES cells. Cells were analysed at 0, 3 and 11 days (d) following differentiation *in vitro.* B) Northern blot analysis for *Dnmt3b* using total RNA from undifferentiated (undiff.) and differentiated XX PGK12.1 ES cells. *28S* transcript levels were used as loading control.



% of associated alleles in PGK 12.1 ES cells	33.5	20	14.4	14.2
N alleles	254	100	118	120
% of associated alleles in Efc1 ES cells	36	20.7	14.9	11.8
N alleles	114	116	114	102

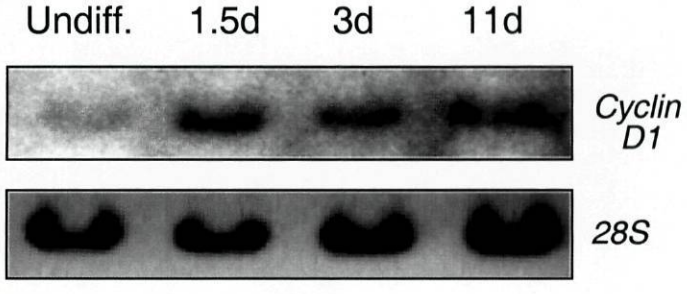


Figure 3.2.3.5.1- ***Cyclin D1*** **association with pericentric heterochromatin during ES cell differentiation.**
A) Scoring data showing the percentage of gene association with pericentric heterochromatin in both XX PGK12.1 (black bars) and XY Efc1 (white bars) ES cells. Cells were analysed at 0, 1.5, 3 and 11 days (d) following differentiation *in vitro.* B) Northern blot analysis for *Cyclin D1* using total RNA from undifferentiated (undiff.) and differentiated XX PGK12.1 ES cells. *28S* transcript levels were used as loading control.

3.2.4- Gene association with Ikaros complexes in B3 lymphocytes

To exclude the possibility that ES cell differentiation (*in vitro*) may not reflect epigenetic events occurring during development (*in vivo*) I investigated the nuclear positioning of the previously studied *Oct4* and *Cyclin D1* genes in somatic B3 cells (pre-B lymphocytes). These cells passed through embryonic development before becoming an immortalised cell line. In addition, B3 cells allow the analysis of gene positioning relative to Ikaros clusters. These domains contain pericentric heterochromatin and proteins such as Hp1 (see section 1.2.2) (Brown *et al*, 1997). Moreover, this cell system allows the inclusion in the immunoDNA FISH analysis of a control gene, *Cd8α,* which showed a high association to heterochromatic domains correlating with gene silencing (Brown *et al*, 1997; 1999). *Cd8α* is selectively expressed in Thymocyte (T) cells and was shown to be associated with pericentric heterochromatin in pre-B (B3 cell line), mature B and activated splenic B cells, but not in resting splenic B cells (Brown *et al*, 1997; 1999).

As illustrated in Figure 3.2.4.1C northern results showed *Cd8α* expression in control thymus samples but not in B3 cells. Moreover, *Oct4* and *Cyclin D1* genes are also found silenced in these cells (Figure 3.2.4.1D and E). Despite this only 11% of *Oct4* and *Cyclin D1* alleles are found associated in immunoDNA FISH analysis. These are similar levels of gene association to heterochromatin found in undifferentiated ES cells for *Oct4*, and in differentiating ES cells for *Cyclin D1*, where both alleles are actively expressed. In contrast, the majority of *Cd8α* alleles (61%) are associated with Ikaros complexes (Figure 3.2.5.1B).

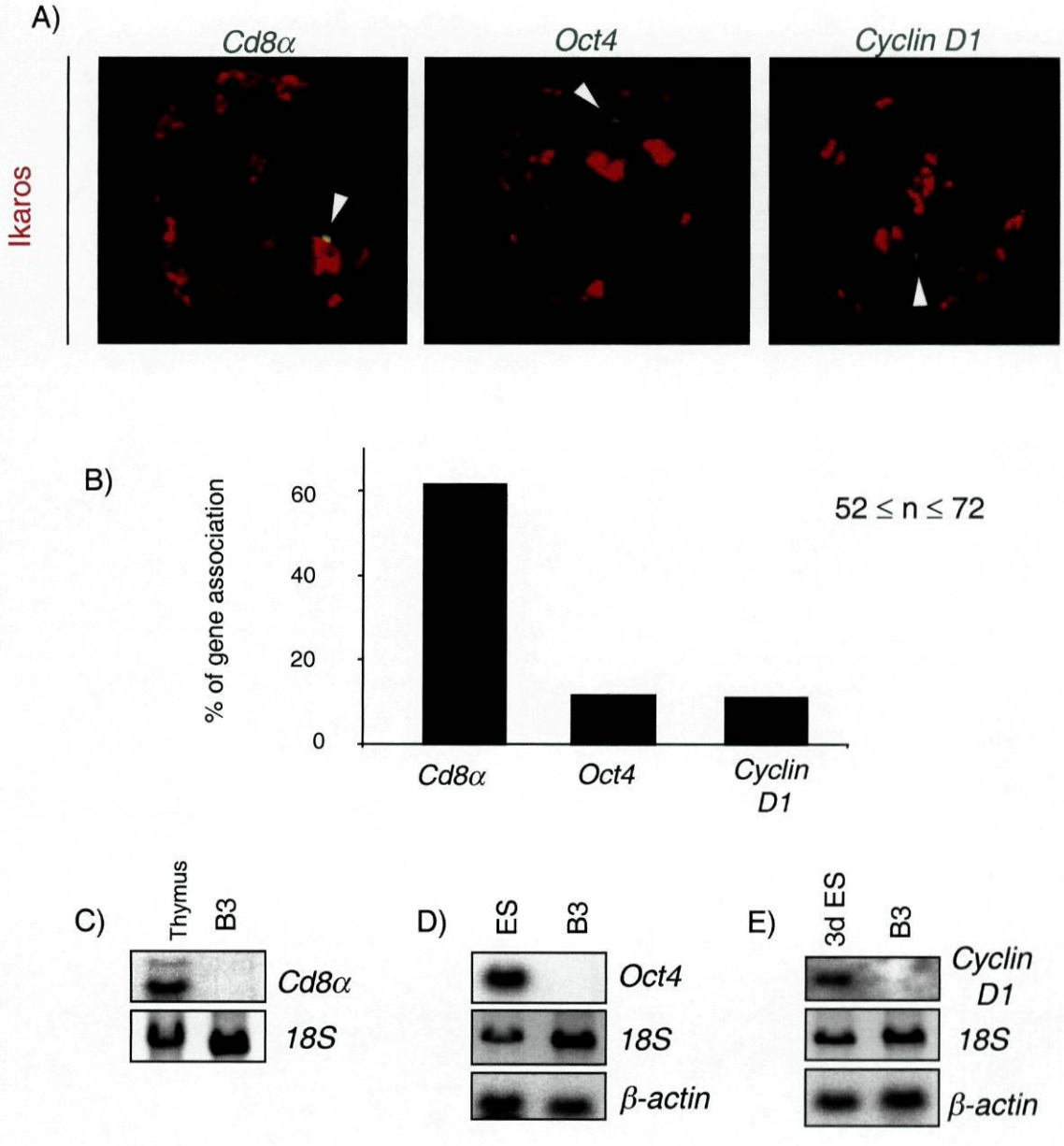


Figure 3.2.4.1- **Gene association with Ikaros complexes in B3 lymphocytes.**
A) Three-dimensional immunoDNA FISH in B3 cells (pre-B lymphocytes) for *Cd8α* (green) (left panel), *Oct4* (green) (middle panel) and *Cyclin D1* (green) (right panel) in pairwise combination with Ikaros (red). Figures show representative single optical sections. Note that *Cd8α* allele is associated with Ikaros complexes. (B) Scoring data showing the percentage of gene association with Ikaros complexes. Between 52 to 72 alleles were scored. (C to E) Northern blot analyses for *Cd8α* (C), *Oct4* (D) and *Cyclin D1* (E) using total RNA from B3 cells, thymus, undifferentiated (ES) and 3 days (3d) differentiated PGK12.1 cells as indicated. *18S* or/and *β-actin* transcript levels were used as loading control.

3.3- Discussion

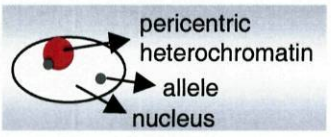
3.3.1- Nuclear positioning of developmentally regulated genes in ES cells

In contrast to the situation in lymphocytes, the correlation between gene silencing and pericentric heterochromatin association found in ES cells was relatively minor. *Oct4*, *Rex1* and *Dnmt3b* gene downregulation correlated with a small increase in gene association to heterochromatic domains equalling to approximately 2-2.5 fold. In the case of *Rex1*, association was lost at later differentiation stages. *Cyclin D1* association with heterochromatin diminished (3.2 fold) from day 0 to 11 of ES cell differentiation, coincidentally with gene upregulation. These results were substantiated for two different ES cell lines, XX PGK12.1 and XY Efc1, demonstrating a high degree of reproducibility. The maximal percentage values of 32% for *Oct4*, 29.7% for *Rex1*, 20.9% for *Dnmt3b* and 36% for *Cyclin D1* do not support, in these examples, heterochromatin gene recruitment as a requirement for maintaining gene silencing. There are a number of possible reasons for this, which are discussed below.

It is unlikely that technical reasons are responsible for the relatively minor association found in ES cells. First, the immunoDNA FISH procedure used was identical to that used by Brown *et al* (1997), who first reported the association of silent genes with heterochromatic domains. Second, experiments were carried out in two different ES cell lines and repeated more than once for most time points with reproducible results.

ES cell differentiation is heterogeneous and it is possible that only certain lineages show association, possibly accounting for the low level of association seen. However, no sub-populations of cells were found, i.e., cells exhibiting one, both or neither alleles associated with heterochromatin had an expected distribution for the given association frequency, at all time points analysed (Figure 3.3.1.1).

		Both alleles associated	One allele associated	Neither alleles associated	Statistical analysis
Oct4 allele distribution per cell in undifferentiated Efc1 ES cells	Observed distribution	2	9	40	p- 0.1275 q- 0.8725 N=51
	Expected distribution	0.8 (P^2)	11.3 (2pq)	38.8 (q^2)	chi-square = 2.53 0.20>p>0.10
Oct4 allele distribution per cell in 11 day differentiated Efc1 ES cells	Observed distribution	5	22	23	p- 0.32 q- 0.68 N=50
	Expected distribution	5.1 (P^2)	21.8 (2pq)	23.1 (q^2)	chi-square = 0.004 0.99>p>0.95



p- frequency of associated alleles
q- frequency of non associated alleles

Figure 3.3.1.1 - Statistical analysis of Oct4 alleles distribution
The table shows Oct4 alleles distribution within the 3 possible classes in undifferentiated and 11 day differentiated ES cells. These are representative examples of the alleles distribution in the generality of scores. Note that observed distributions do not differ significantly from expected distributions for the given frequencies. This result rules out the existence of cell sub-populations, for the classes above described, within undifferentiated and differentiated ES cells.

This suggests that in ES cells a random allele (among those studied genes) has 2-2.5 fold increased chance to associate with heterochromatic domains during gene downregulation in comparison to the active counterpart.

The ES cell system may lack certain epigenetic mechanisms or factors that are only present later in development. Such factors could be responsible for the higher association with heterochromatic domains observed for example during gene downregulation in lymphocytes. Examples of such factors include the lymphoid lineage transcriptional regulators Ikaros and Helios, and the PcG proteins (see chapter 4). These factors, when present at high levels, could increase the chance of sequestering genetic domains into the close proximity of repressive nuclear compartments (pericentric heterochromatin). Such compartments contain histone hypoacetylation, histone methylation and DNA methylation, which are marks of heritable gene silencing (reviewed in Lachner and Jenuwein, 2002). Supporting this idea Brown *et al* (1999), comparing resting with activated splenic B cells, found Ikaros absent in resting B cells and present at heterochromatic domains following activation. Correlating with this, Ikaros target silenced genes showed a selective repositioning to heterochromatic domains.

Another possibility is that centromeric association of silenced genes is a dynamic process and that factors regulating nuclear dynamics are developmentally regulated. Studies in yeast have correlated telomere silencing with compartmentalisation to the nuclear periphery (reviewed in Henikoff, 1992). Recently, Heun *et al* (2001) demonstrated in living yeast cells that indeed telomeres have a higher chance to localise to the nuclear periphery in comparison to other chromatin domains. However, this was not a static association, telomeres were found to have continuous oscillatory movements. The implications of this could be that movement might help regulating macromolecular interactions such as chromatin modifying complexes in chromatin dynamics.

The chromosomal context of loci was suggested to influence whether or not heterochromatin association of silent alleles occurs in lymphocytes (see section 3.1.1) (Brown *et al*, 2001). In these studies the human *β-globin* locus, which is localised at a cytological heterochromatic chromosomal region, is associated with pericentric heterochromatin when silenced. In contrast, the human *α-globin* locus, which is localised at a cytological euchromatic (Giensa light staining) chromosomal region, is never associated with pericentric heterochromatin, independently of the transcriptional status. According to information available in Ensenbl database (2003) *Oct4* (chromosome 17, band B3) and *Cyclin D1* (chromosome 7, band F5) are localised in cytological euchromatic bands. *Dnmt3b* is localised at an intermediate cytological band (weak Giensa staining). No information on *Rex1* chromosomal localisation is available. In contrast, *Cd8α* (chromosome 6, band C3) localises at a heterochromatic cytological band (Giensa dark staining). Taken together, these observations may indicate that the low degree of gene association for *Oct4*, *Cyclin D1* and *Dnmt3b* relates to their immediate chromosomal environment. However, more analyses would be required to determine if other genes for which nuclear localisation has been studied also fit this pattern. For this it will be important to develop new tools for defining the chromosome environment of genes. Cytological maps are relatively crude and in the case of the mouse genome sequence available on ensembl, cytogenetic coordinates are extrapolated from syntenic human genome maps.

In conclusion, the data presented in this section does not show a striking correlation between association of genes with pericentric heterochromatin and gene silencing. However, the data is limited and somewhat inconclusive. Further analyses looking at more genes regulated during ES cell differentiation and in other cell types are required to clarify whether heterochromatin association of silent genes is a feature of all cell lineages.

Chapter 4: Developmentally regulated localisation of the Eed-Enx1 Polycomb complex to the inactive X chromosome

4.1- Introduction

4.1.1- The search for proteins effecting X inactivation

X;autosome translocation studies revealed that inactivation on the X chromosome can spread into autosomal genes (reviewed in Russell, 1983). The probability of silencing was found to correlate with proximity to the breakpoint. This observation suggested parallels with the position effect variegation (PEV) in *Drosophila* (see section 1.1 for further detail). However, evidence that mammalian homologues of PEV modifiers play a role in X inactivation has not been reported. In fact, analysis of cells null for *Suv39h1* and *Suv39h2*, the mammalian homologues of *Drosophila Su(var)39*, revealed that these proteins are responsible for the methylation of histone H3 at lysine 9 (H3-K9) in pericentric heterochromatin but not on the inactive X chromosome (see section 1.1.3 for further detail) (Peters *et al*, 2002). One possible explanation is that PEV modifier proteins are primarily involved in the structure of constitutive heterochromatin (always heterochromatic), and not in facultative (developmentally regulated) heterochromatin. Candidate proteins for facultative heterochromatin include the Polycomb Group (PcG) proteins (reviewed in Pirrotta, 1995) (see sections 1.1.6 to 1.1.8 for further detail), and recently, a specific PcG protein has been implicated in the X inactivation process. In a study to characterise the potential role of the Polycomb group protein embryonic ectoderm development (Eed) in extraembryonic lineages, it was found that this protein is required for the maintenance of imprinted X inactivation (Wang *et al*, 2001). *In situ* hybridisation of whole mount 8.5 dpc homozygous *eed* null ($eed^{-/-}$) embryos, with a probe for *Pl1*, a marker for terminally differentiated trophoblast giant cells (extraembryonic tissue), revealed that these embryos were poorly stained when compared to wild-type 8.5 dpc $eed^{+/+}$. Moreover, the authors also noticed that female $eed^{-/-}$ embryos had far less Pl1 staining than $eed^{-/-}$ male. Genetically,

the only difference between male and female *eed*$^{-/-}$ embryos was the dosage of X chromosomes, so, the most likely explanation for this phenotype was that the *eed* mutation somehow affected X chromosome inactivation.

To address this issue the authors crossed *eed*$^{+/-}$;X^{GFP}/Y with *eed*$^{+/-}$;X/X mice. From this cross all females should be GFP positive in the embryo proper, and GFP negative in extraembryonic tissues as a result of imprinted X inactivation of the paternal inherited X^{GFP}. At stages 4.5 and 5.0 dpc *eed*$^{-/-}$;X^{GFP}/X embryos were seen to be phenotypically similar to wild type, with extraembryonic endoderm and trophoblast cells being GFP negative. This indicated that imprinted X inactivation had occurred in a proportion of the cells. However, *eed*$^{-/-}$;X^{GFP}/X showed increased fluorescence in extraembryonic tissues as development progressed up until 7.5 dpc, the latest time point analysed. These results implied that X inactivation had occurred, but there was a subsequent failure to maintain silencing, leading to GFP expression from the inactive X. In addition, 7.5 dpc female *eed*$^{-/-}$ embryos showed apparently normal mosaicism for GFP expression in the embryo proper. Since the phenotype was confined to extraembryonic tissues, the authors proposed that *eed* was involved in imprinted but not random X inactivation.

Because homozygous mutant embryos, both male and female die by 8.5 dpc, Wang *et al* (2001) further assessed the role of Eed in the maintenance of imprinted X inactivation by crossing *eed*$^{+/-}$;X^{GFP}/Y males with females heterozygous for a hypomorphic mutation (*eed*$^{hypom/+}$;X/X). Compound heterozygous (*eed*$^{hypom/-}$) have a less severe phenotype than *eed*$^{-/-}$ embryos, allowing survival to late prenatal stages. At 6.5 dpc no difference was detectable between heterozygous *eed*$^{hypom/-}$ and wild type female embryos. In both cases, the paternally derived X^{GFP} remained inactive. However, with further embryonic development female *eed*$^{hypom/-}$ GFP reactivation was observed in extraembryonic tissues.

Based on these findings Mak *et al* (2002) investigated localisation of Eed protein, and also of Enx1, a second PcG protein known to interact with Eed, in trophoblast stem cells (TS) (Mak *et al,* 2002). TS cells are derived from the trophectoderm of mouse blastocysts and represent a good model system to study imprinted X inactivation. Mak *et al* (2002) observed Eed/Enx1 staining at the inactive X in interphase and throughout mitosis. Mitotic chromosomal analysis showed these complexes to be distributed in a banded manner resembling the previously described *Xist* RNA distribution pattern (Duthie *et al*, 1999). Interestingly, immunofluorescence analysis of XX somatic cells, for both Eed and Enx1, did not show localisation with the inactive X, reinforcing the idea that Eed/Enx1 mediated silencing is restricted to imprinted X inactivation in the trophectoderm (Mak *et al*, 2002).

Together, the studies by Wang *et al* (2001) and Mak *et al* (2002) suggest that there may be lineage specific pathways for establishing X inactivation. Here, I further investigated this hypothesis by analysing localisation of PcG proteins in XX ES cells, which undergo random X inactivation upon differentiation.

4.2- Results

4.2.1- Eed and Enx1 associate with the inactive X in differentiating embryonic stem cells

To investigate localisation of Eed and Enx1 in differentiated ES cells immunofluorescence analysis was performed using XX PGK12.1 ES cells. Localisation was examined at days 0, 3 and 10 of differentiation. Nuclear staining for both Eed and Enx1 was observed as a diffuse signal at all time points (Figure 4.2.1.1), however, the immunofluorescence signal was much fainter at day 10. Interestingly, at day 3, Eed and Enx1 could be seen forming a large focus (henceforth named Eed/Enx1 body) in addition to the general diffuse staining (Figure 4.2.1.1B). This pattern resembled that described by Mak *et al* (2002) for XX TS cells. On mitotic Figures Eed and Enx1 were seen to

localise in a banded pattern on a single chromosome (Figure 4.2.1.1C). Surprisingly, in day 10 differentiated ES cells the Eed/Enx1 body was not detectable (Figure 4.2.1.1D).

In order to determine if this Eed/Enx1 body was the inactive X, I first examined an XY ES cell line, Efc1 (Figure 4.2.1.2A). Whilst high levels of Eed and Enx1 signal could be detected in the nuclei, in contrast to 3 days differentiated XX PGK12.1 cells, 3 days differentiated XY Efc1 cells never exhibit the presence of the Eed/Enx1 body, showing only diffuse staining. This result provided evidence of an association of Eed and Enx1 with the inactive X. To confirm this directly specific markers for the inactive X were used. At this stage of ES cell differentiation only two markers are known: the accumulated *Xist* RNA (Panning *et al*, 1997; Sheardown *et al*, 1997), and methylation of histone H3 at lysine 9 (H3-K9) which marks the X chromosome in mitotic Figures (Mermoud *et al*, 2002). Methylated H3-K9 staining also occurs at pericentromeric heterochromatin (Lachner *et al*, 2001). Thus, immunoRNA FISH for Eed and *Xist* RNA and Immunofluorescence for Eed and methyl H3-K9 were carried out in 3 days differentiated PGK12.1 cells. ImmunoRNA FISH analysis clearly shows that accumulated *Xist* RNA signal overlaps with the Eed/Enx1 body (Figure 4.2.1.2B). Similarly, immunofluorescence detection of methylated H3-K9 on mitotic figures demonstrated colocalisation with the Eed/Enx1 body (Figure 4.2.1.2C). Methylated H3-K9 staining was also present at the pericentromeric heterochromatin (DAPI dense regions). Together, these results demonstrate that Eed-Enx1 complexes associate with the inactive X in random X inactivation.

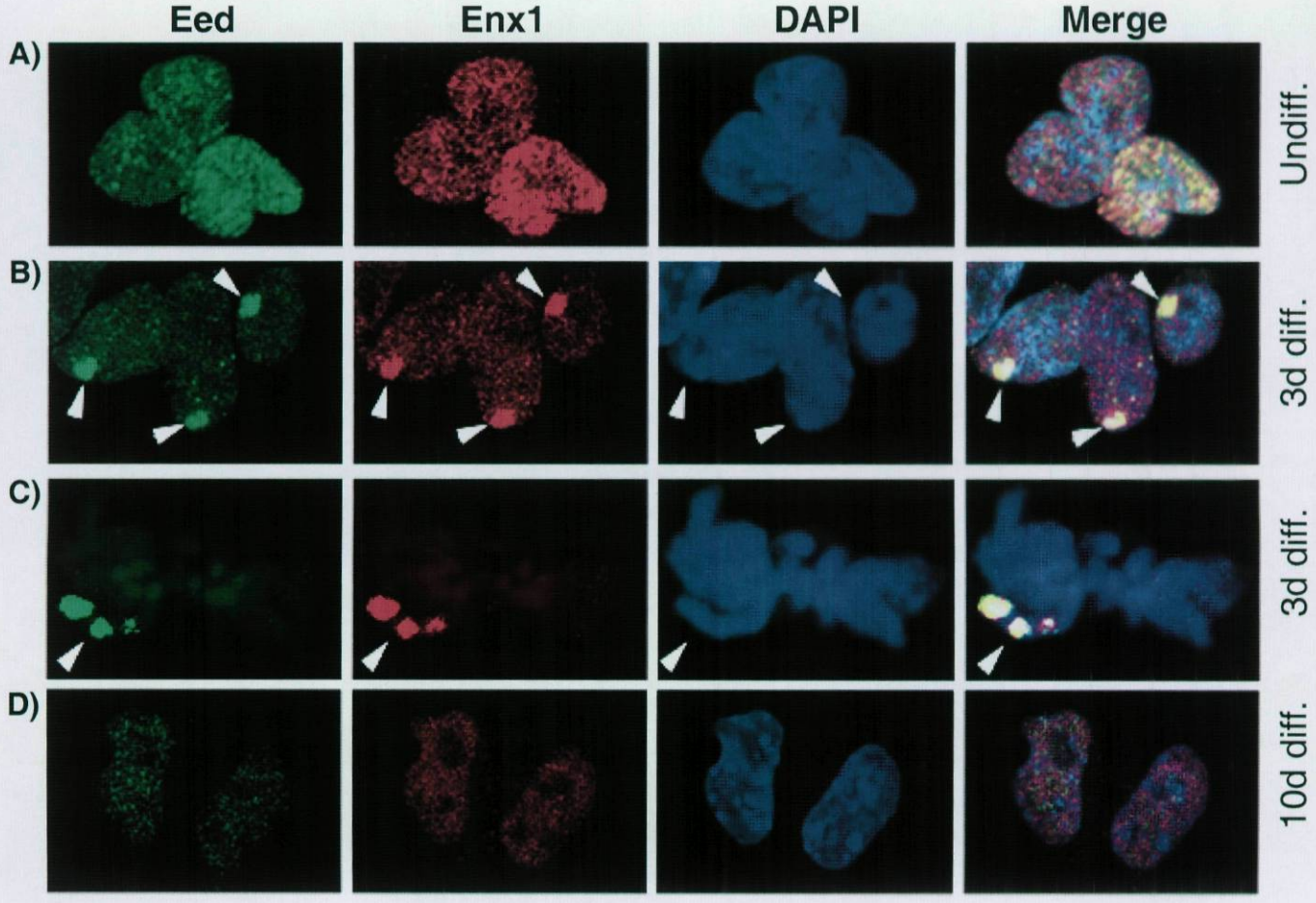


Figure 4.2.1.1- **Eed and Enx1 form a chromosomal body in differentiated XX ES cells.** (A-D) Immunofluorescence for Eed (green) and Enx1 (red) in undifferentiated (undiff.), 3 and 10 days (d) differentiated (diff.) PGK12.1 XX ES cells as indicated. Representative fields of view are illustrated (B) Arrowheads indicate Eed/Enx1 bodies. (C) Arrowhead indicates a single chromosome with Eed/Enx1 localisation in a cell undergoing mitosis. (D) Note weak general staining and the absence of Eed/Enx1 body. All merged panels include DAPI DNA counterstain (blue).

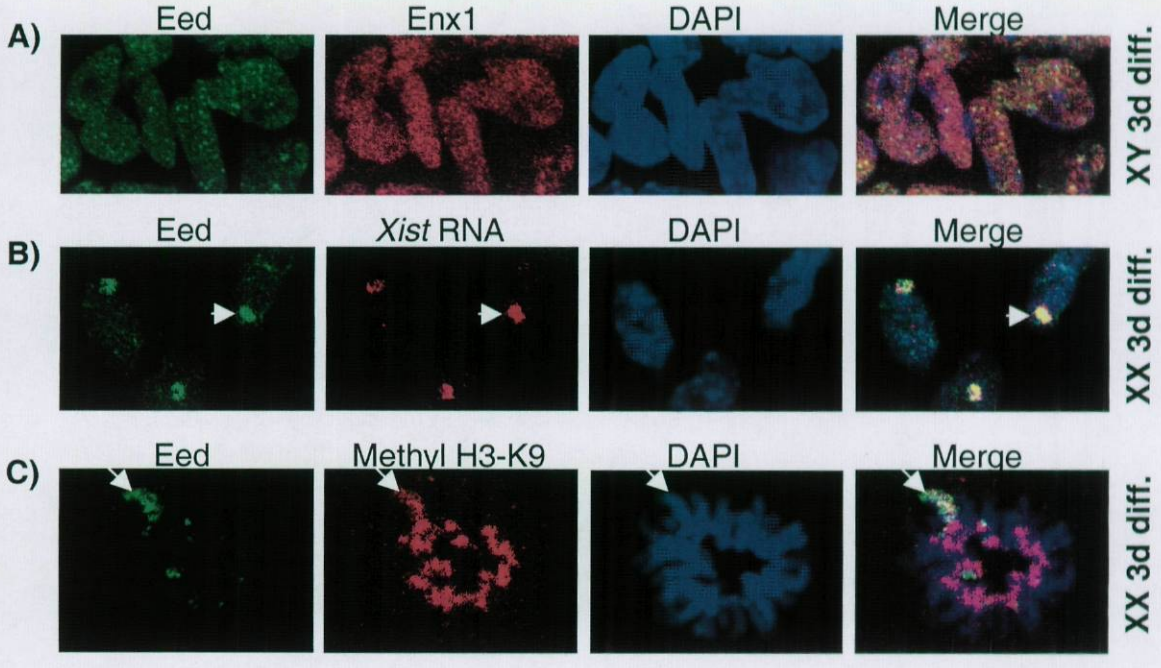
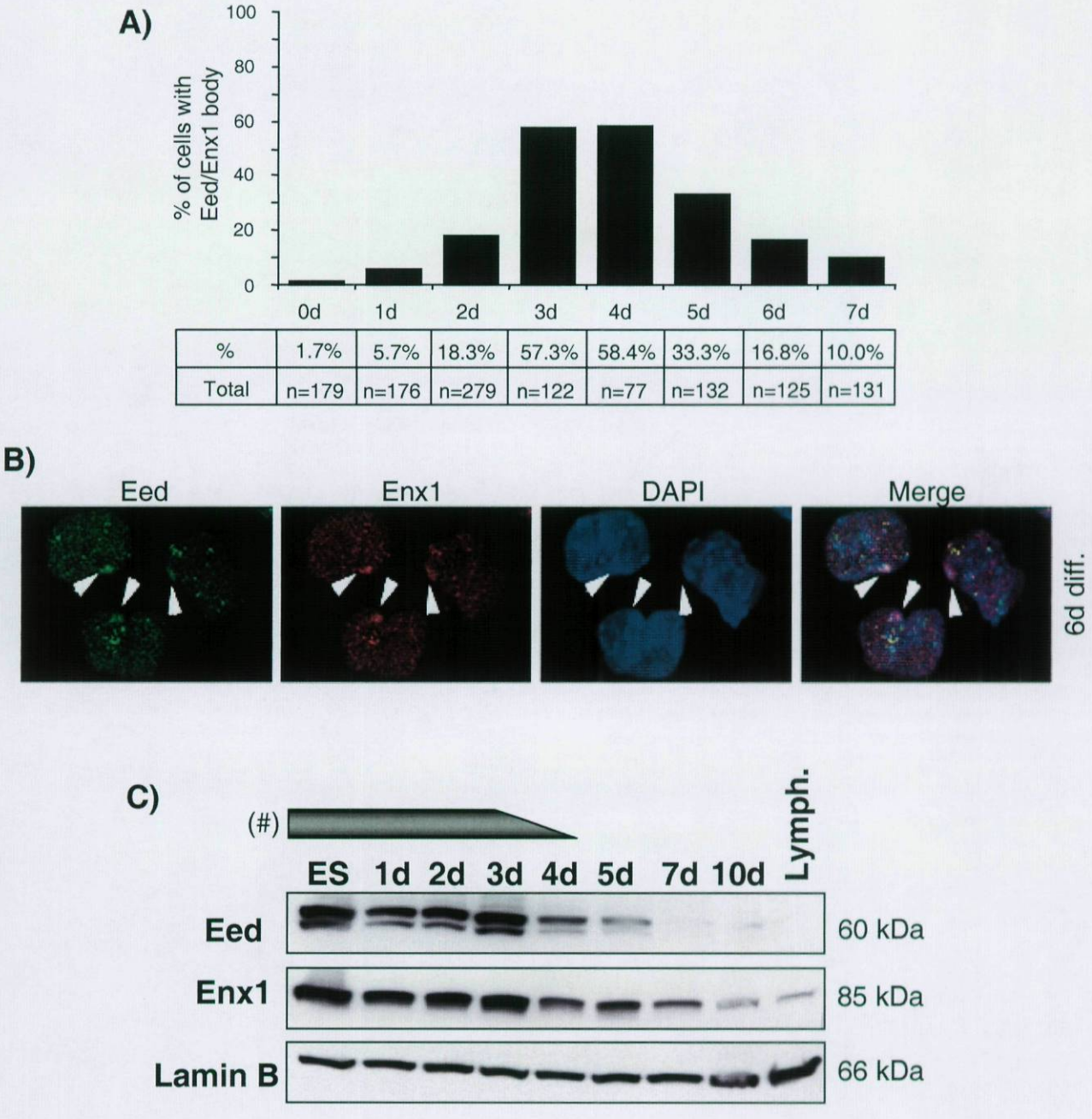


Figure 4.2.1.2 - **Eed and Enx1 are associated with the inactive X in differentiated XX ES cells**.
(A) Immunofluorescence for Eed (green) and Enx1(red) in 3 days (d) differentiated (diff.) XY ES Efc1 cells. Note that cells lack Eed/Enx1 bodies. (B) ImmunoRNA FISH for Eed (green) and *Xist* (red) in 3 days differentiated XX ES PGK12.1 cells. Note colocalisation of Eed/Enx1 bodies and accumulated *Xist* RNA (arrow). (C) Immunofluorescence for Eed (green) and methylated histone H3 lysine 9 (methyl H3-K9) (red) in 3 days differentiated XX ES PGK12.1 cells. Arrow points to a chromosome labelled by Eed and methyl H3-K9 antisera. The latter is considered to be a mitotic marker of the X chromosome (Mermoud *et al*, 2002; Peters *et al*, 2002) (see text). In addition methyl H3-K9 staining is observed at pericentric regions. Nuclei were counterstained with DAPI.

4.2.2- Eed and Enx1 association with the inactive X is transient

The previous results demonstrated that Eed/Enx1 proteins accumulate on the inactive X in 3 day differentiated XX PGK12.1 cells but not in undifferentiated, nor 10 day differentiated cells. To investigate the apparent transient nature of Eed and Enx1 accumulation in more detail a range of time points were scored for colocalisation in XX PGK12.1. The results are summarised in table 4.2.2.1A. The Eed/Enx1 body is detectable in ES cells, but at very low frequency. The small number of positive cells may correspond to ES cells that have begun to differentiate spontaneously. Following differentiation the proportion of cells with Eed/Enx1 body increases, peaking at days 3 and 4, with numbers close to 60%. From day 4 onwards percentile values decrease to a minimum of 10% at 7 days of differentiation. To verify these results a second XX ES cell line, LF2, was analysed at 0, 3 and 7 days of differentiation. The Eed/Enx1 body could be observed in 57% (71/124) of 3 day differentiated cells, whereas at 7 days of differentiation no cells (0/129) displayed it.

In general, Eed/Enx1 staining appeared weaker at later time points. This was true for general nuclear staining as well as for the inactive X. Examples are shown in Figure 4.2.2.1B. These observations were verified by western blot analysis carried out by a colleague, Dr. Ilona Zvetkova (Figure 4.2.2.1C). Levels of both Eed and Enx1 are high in undifferentiated ES cells and during days 1-3 of differentiation, relative to lymphocyte nuclear extract control. As differentiation progressed however, levels fell markedly, and by day 10 of differentiation were similar to those seen in the lymphocyte control. Enx1 signal was in fact maintained at a slightly higher level than Eed in more differentiated cell types. This observation suggests that the disappearance of Eed/Enx1 bodies is attributable to a reduction in overall levels of Eed and Enx1 proteins during differentiation.



%	1.7%	5.7%	18.3%	57.3%	58.4%	33.3%	16.8%	10.0%
Total	n=179	n=176	n=279	n=122	n=77	n=132	n=125	n=131

Figure 4.2.2.1- **Transient recruitment of Eed-Enx1 complexes to the inactive X correlates with high protein levels.**
(A) Scoring data showing the percentage of cells with Eed/Enx1 bodies during days (d) 1-7 of PGK12.1 XX ES cells differentiation. (B) Immunofluorescence for Eed (green) and Enx1 (red) in 6 days differentiated (diff.) XX ES cells. Note weak Eed/Enx1 body staining (see arrowheads). Cells are counterstained with DAPI. C) Western blot analysis of cell extracts from PGK12.1 XX undifferentiated and differentiated ES cell cultures as indicated. Lymphocyte (Lymph.) extracts provide a somatic cell control. Proteins analysed were Eed, Enx1 and Lamin B (loading control). Molecular sizes are indicated. Note that doublet for Eed protein may be attributable to alternative RNA transcript (Denisenko *et al*, 1998), or post-translational modification (Ng *et al*, 2000). (#) Window of opportunity for *Xist* RNA to effect gene silencing (Wutz and Jaenisch, 2000)

As shown in Figure 4.2.2.1A no more than 60% of cells exhibited simultaneously the presence of Eed/Enx1 body. There are two possible reasons for this. First, Eed/Enx1 localisation may occur in ES cells differentiating down specific lineage pathways. Second, if differentiation occurs asynchronously and Eed/Enx1 localisation is only transient, Eed/Enx1 localisation on the inactive X would not be seen in all cells at a given time point. To determine which of these is true, analysis by immunoRNA FISH for Eed and *Xist* RNA were performed in 3 days differentiated XX PGK12.1. The results demonstrated that cells with accumulated *Xist* RNA generally had colocalising Eed protein (66/71). The remaining cells had punctate *Xist* signal on both alleles indicating that they are undifferentiated or at a very early stage of differentiation (Panning, *et al* 1997; Sheardown *et al*, 1997). There was no concentration of Eed protein seen in association with this punctate signal (0/50). (Figure 4.2.2.2A).

As shown above, from day 4, the percentage of cells with an Eed/Enx1 body starts to decrease, and the signal becomes fainter. To address whether or not this was a consequence of cell differentiation another marker, the Oct4 protein, was used. Oct4 is a transcription factor expressed in undifferentiated and early differentiated ES cells (Scholer *et al*, 1989). When double staining of Eed and Oct4 was made in 6 days differentiated XX PGK12.1 cells, a clear pattern emerged for cells presenting the Eed body. Generally, cells with strong staining for Eed/Enx1 body were Oct4 positives (43/43), while cells with weak Eed/Enx1 body signal were Oct4 negative (90/91) (Figure 4.2.2.2B). It is important to point out that the majority of cells at this stage are Oct4 negative with poor Eed staining and no body present. Together with the above results this data suggests that Eed/Enx1 association with the inactive X is a function of the differentiation state.

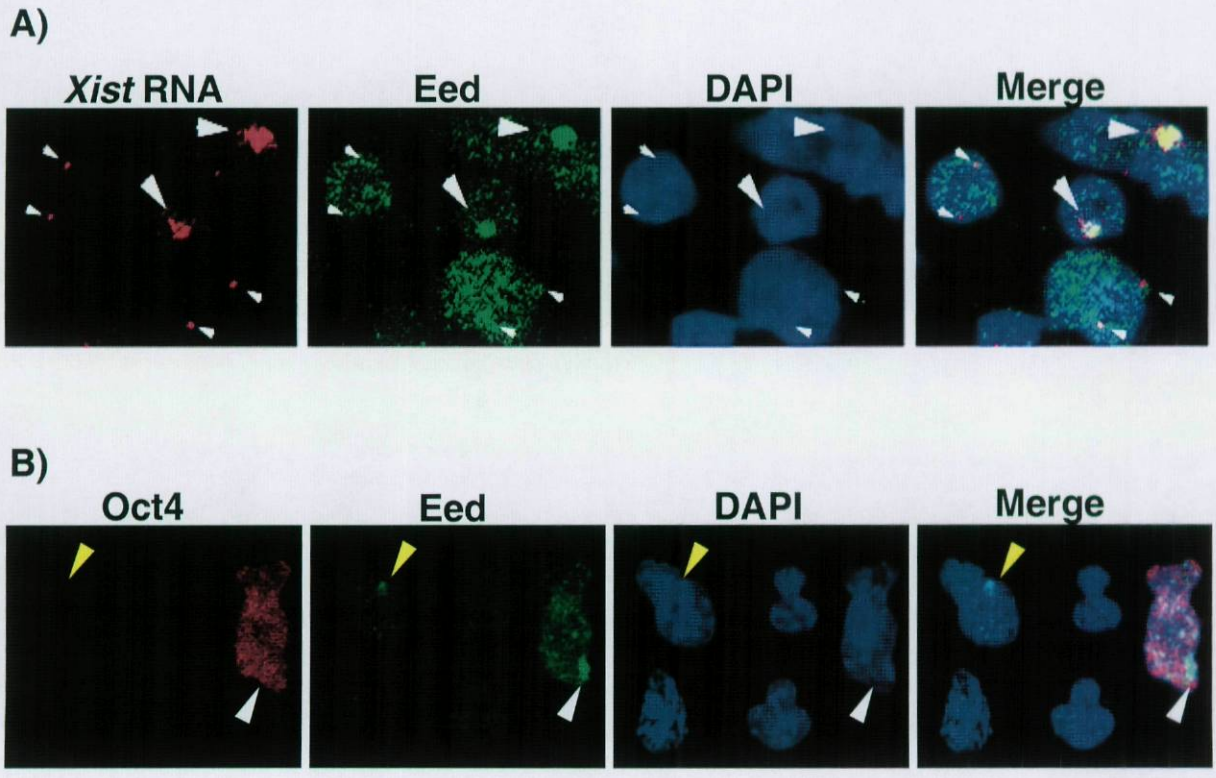


Figure 4.2.2.2 - **Eed and Enx1 transient association with the inactive X occurs in all differentiating XX ES cells.**
(A) ImmunoRNAFISH for *Xist* (red) and Eed (green) in 3 days differentiated PGK12.1 XX ES cells. Note that cells lacking Eed/Enx1 bodies show expression of two punctate *Xist* signals (small arrows), characteristic of undifferentiated XX ES cells. Note also that Eed does not accumulate with *Xist* punctate signal. (B) Immunofluorescence for Eed (green) and Oct4 (red) in 6 days differentiated PGK12.1 XX ES cells. Note yellow arrow pointing to a cell that is Oct4 negative and has weak Eed staining, characteristic of late differentiated XX ES cells. White arrow indicates a cell with strong Eed signal that is Oct4 positive, characteristic of undifferentiated and early differentiated XX ES cells. See text for scores and additional information. Nuclei were counterstained with DAPI.

4.2.3- Localisation analysis of Hdac1 and Hdac2

Histone deacetylases (HDACs) remove the acetyl group of lysine residues of histone N-terminal tails (Kuo and Allis, 1998). This event is thought to have a causative role in regulating gene transcription. In order to determine whether PcG proteins interact with HDACs Van der Vlag and Otte (1999) cloned several human cDNAs encoding PcG proteins into two-hybrid vectors and screened these for potential interaction with the human histone deacetylase 2 (HDAC2). A positive interaction was found for EED and further analysis demonstrated that the WD40 domain was essential for such interaction. Interestingly the Eed null and hypomorphic mutated proteins showed no interaction with HDAC2. In similar assays EED, was also shown to interact with HDAC1 and HDAC3. Co-immunoprecipitations, using nuclear extracts showed EED in a complex with HDAC1, HDAC2 and ENX1.

To investigate whether Hdac1 and Hdac2 (henceforth Hdac1/2) associate with the inactive X, their localisation was studied by immunofluorescence. Analyses were carried out for 2, 3 and 6 days differentiated PGK12.1 XX ES cells. Eed staining was used to detect the inactive X. Nuclear staining for both Hdac1/2 was observed at all time points and was generally diffuse. Small Hdac1 foci were sometimes seen to overlap with Eed foci, however, no specific localisation to the inactive X was observed (Figure 4.2.3.1A). This was true for both Hdac1/2 at all time points analysed. Western blot analysis carried out by Dr. Ilona Zvetkova for both Hdac1 and Hdac2 revealed that protein levels remained constant over ES cell differentiation, although they were lower in lymphocytes (Figure 4.2.3.1G).

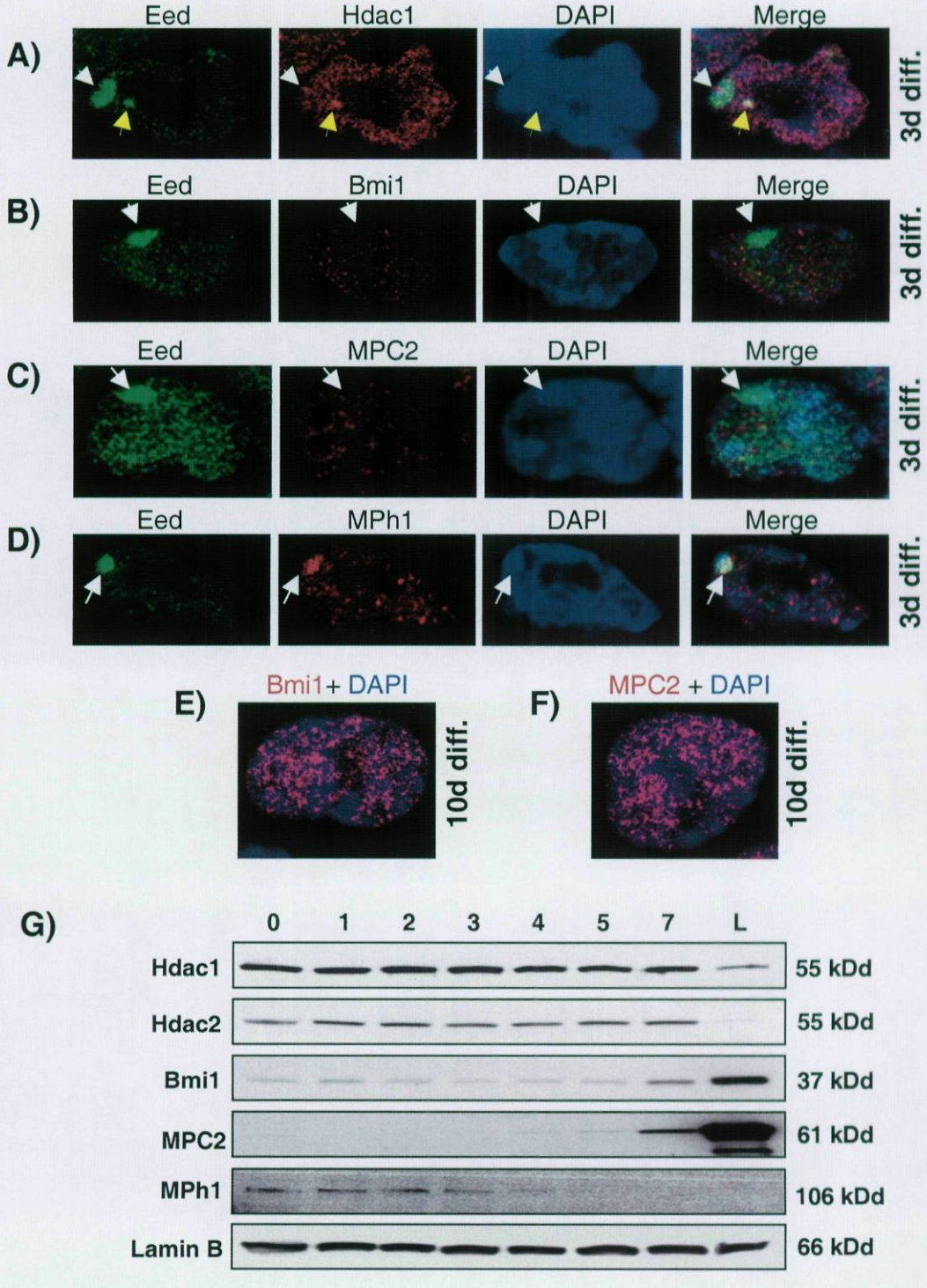


Figure 4.2.3.1- **Analysis of chromatin modifiers for potential involvement in X inactivation**.
(A-F) Immunofluorescence analysis carried out on 3 and 10 days (d) differentiated (diff.) PGK12.1 XX ES cells as indicated. Illustrated are examples of single cells stained for: (A) Eed (green) and Hdac1 (red). Note absence of Hdac1 enrichment underlying the Eed/Enx1 body (white arrow). In the same figure see example of Eed and Hdac1 overlapping small focus (yellow arrow). (B and C) Immunofluorescence for Eed (green) and Bmi1 (red) (B) or MPC2 (red) (C). Note general weak Bmi1 and MPC2 signal. Note also lack of Bmi1 and MPC2 staining underlying Eed/Enx1 body. (D) Eed (green) and MPh1 (red). Note association of MPh1 with Eed/Enx1 body (arrow). (E) Bmi1 (red) and (F) MPC2 (red). Note increase of Bmi1 and MPC2 immunofluorescence signal in comparison to 3 days differentiated ES cells (B and C respectively). Note also absence of any large nuclear focus. All cells were counterstained with DAPI. (G) Western blot analysis of cell extracts from PGK12.1 XX undifferentiated and differentiated ES cell cultures as indicated. Lymphocyte (Lymph.) extracts provide a somatic cell control. Molecular sizes as indicated. Proteins analysed were Hdac1, Hdac2, Bmi1, MPC2. MPh1 and Lamin B (loading control).

4.2.4- Localisation analysis of PRC1 complex proteins

An important consideration is whether or not other PcG proteins could also be involved in X inactivation. Both PcG complexes, E(Z)-ESC and Polycomb repressive complex 1 (PRC1), are involved in the maintenance of gene repression of overlapping developmental regulated genes (Simon *et al*, 1992). Mutational analyses of *Drosophila E(z)* gene has implicated it in the binding of PRC1 PcG proteins to polytene chromosomes (Rastelli *et al*, 1993). Recently, studies in early *Drosophila* development (preblastoderm stage) showed that LexA fused with ESC co–immunoprecipitates with E(Z) and with the PRC1 complex proteins Polycomb (Pc) and Polyhomeotic (Ph) (Poux *et al*, 2001). However, LexA-ESC interaction with PRC1 components was absent from later stages.

Biochemical and immunofluorescence analyses have demonstrated that mammalian homologues of *Drosophila* Polyhomeotic (Ph) (mouse MPh1 and MPh2), Polycomb (Pc) (mouse M33 and MPc2) and Posterior sex combs (Psc) (mouse Mel18 and Bmi1), which are components of the *Drosophila* PRC1 complex, are also part of a mammalian PcG complex (Shao *et al*; 1999; reviewed in Satjin and Otte 1999; reviewed in Francis and Kingston, 2001). To investigate whether these other PcG proteins localise to the inactive X, the localisation of Bmi1, MPc2 and MPh1 was studied by immunofluorescence. This was performed for 3 day differentiated XX ES PGK12.1 cells using specific antisera against the above mentioned proteins in combination with Eed antisera. Nuclear staining was weak and diffusely distributed throughout the nucleus for both Bmi1 and MPc2. In addition, both Bmi1 and MPc2 did not reveal specific localisation to Eed body (n=100) (Figures 4.2.3.1B and C). In contrast Mph1 staining was strong and exhibited multiple foci. Interestingly, 32% (45/145) of the cells, had underlying Mph1 at the Eed body (Figure 4.2.3.1D).

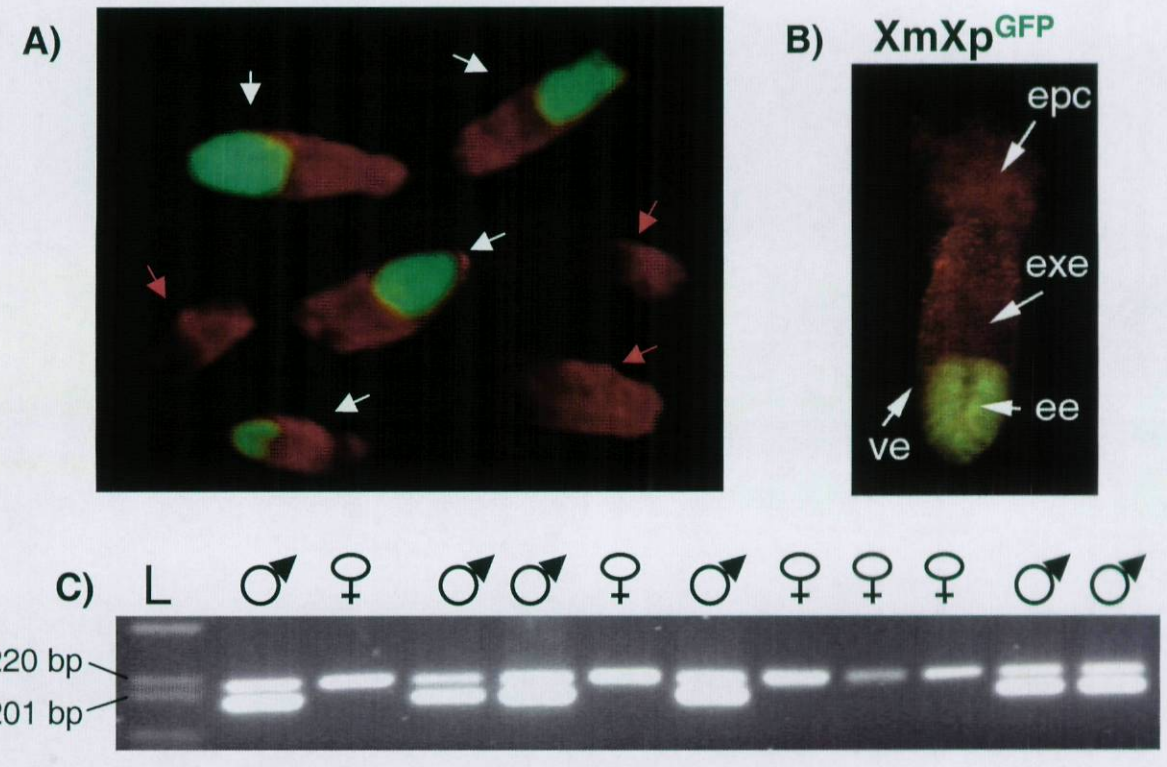
Bmi1 and MPc2 staining appeared weaker in comparison to Eed and Mph1. This may imply a role of Bmi1 and MPc2 at other stages of differentiation. Consistent with this western blot analysis carried out by Dr. Ilona Zvetkova revealed that levels increase at late differentiation stages (Figure

4.2.3.1G). In contrast, the expression pattern for MPh1 resembled that of Eed, with highest levels being detectable in undifferentiated ES cells and up to 3 days of differentiation.

The increased levels of Bmi1 and MPc2 with ES cell differentiation suggested a role for these proteins late in the differentiation process. To investigate this, immunofluorescence analyses for Bmi1 and MPc2 were performed at days 6 and 10 of XX ES cell differentiation. As in day 3, both proteins exhibited a general nuclear staining but at these later stages the signals were stronger (Figures 4.2.3.1E and F). However, there was no large nuclear focus, indicating that PRC1 complex proteins in general don't localise to the inactive X.

4.2.5- Eed and Enx1 localise to the inactive X during random X inactivation *in vivo*

To verify that my observations demonstrating transient localisation of Eed/Enx1 to the inactive X in differentiating ES cells reflects events that occur *in vivo* I examined cells of embryos undergoing random X inactivation. Random X inactivation is initiated in cells of the pluripotent epiblast between 5.5 and 6.5 days post coitum (dpc) (Figure 1.3.3.1) (Monk and Harper, 1979; Takagi *et al*, 1982). To examine the involvement of Eed and Enx1 in random X inactivation *in vivo*, immunofluorescence analysis was carried out on cells from dissociated embryos. At 6.5 dpc, the earliest stage examined, approximately 50% of the embryos had a single Eed/Enx1 body detectable in the majority of cells. These embryos were confirmed as being XX by PCR analysis of ectoplacental cones (Figure 4.2.5.1C), or by expression of the transgenic green fluorescence protein (GFP) inherited on the paternal X (Figure 4.2.5.1B). Eed/Enx1 bodies were not observed in XY male embryos (not shown). To ensure that Eed/Enx1 bodies are present in cells of the embryo proper, both GFP positive cells (random X inactivation), or GFP negative (random and imprinted X inactivation) were scored (Figure 4.2.5.1D). No significant difference was observed, demonstrating that Eed/Enx1 body formation occurs in embryonic ectoderm cells undergoing random X inactivation.



E)

	GFP+	GFP-
%Eed/Enx1 body	86%	92%
N	43	61

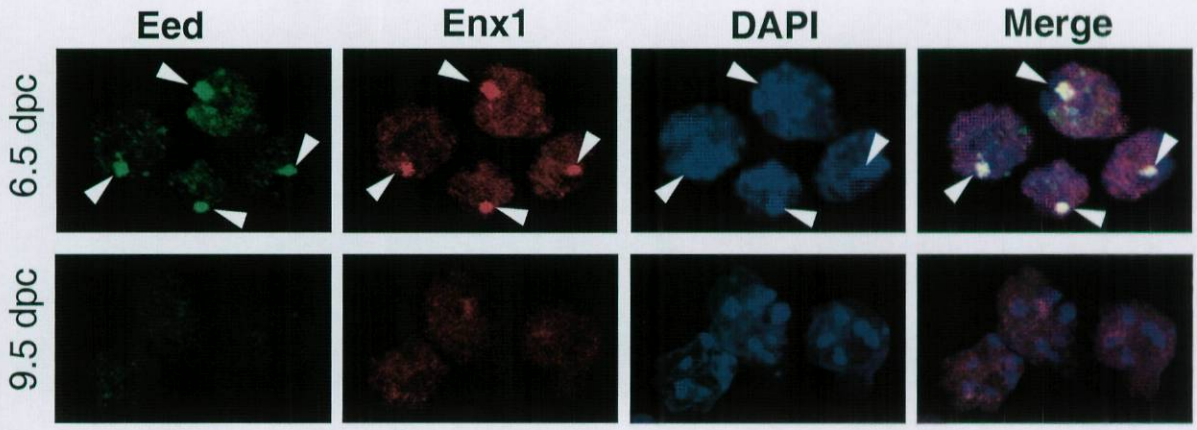
Figure 4.2.5.1- **Eed and Enx1 localise with the inactive X *in vivo*.**
(**A**) Image showing XmXp^{GFP} (white arrows) and XmYp (red arrows) embryos. (**B**) Image of a 6.5 dpc XmXp^{GFP} embryo exhibiting GFP (green) staining as a result of random X inactivation in embryonic ectoderm cells (ee). Note absence of GFP staining in visceral endoderm (ve), extraembryonic ectoderm (exe) and ectoplacental cone (epc). These tissues have undergone imprinted X inactivation of Xp. (**C**) PCR analysis of 6.5 dpc embryos to determine gender. The 2% agarose gel shows the products of UBE1XA and UBE1XB primers (see material and methods), which amplify a 198 bp and a 217 bp band from the Y and X chromosome respectively. (♀) XX and (♂) XY embryos. Ladder (L). (**D**) Illustration of mosaic GFP (green) expression as a result of random X inactivation in embryonic ectoderm cells from dissociated 6.5 dpc XmXp^{GFPX} embryos. Note the presence of Enx1 bodies in either GFP positive (+) and negative (-) cells. Cells are couterstained with DAPI (blue). (**E**) Scores of GFP + and - cells for the presence of Eed/Enx1 bodies. Note that these occur at similar frequency in GFP positive and negative cells. Xp - paternal inherited X; Xm- maternal inherited X.

Eed/Enx1 bodies are not observed in primary or transformed XX somatic cells (Mak *et al*, 2002), indicating that a transient localisation, as observed in XX ES cells, also occurs *in vivo*. Therefore, embryos at stages between 6.5 and 11.5 dpc were examined (Figure 4.2.5.2B). Disappearance of Eed/Enx1 bodies was seen to occur progressively. As noted in differentiating XX ES cells, reduced frequency of Eed/Enx1 bodies was accompanied by a reduction in the strength of signal, both over the inactive X chromosome, and in the nucleus generally (Figure 4.2.5.2A). These results demonstrate that transient recruitment of Eed-Enx1 complexes occurs in random X inactivation *in vivo*.

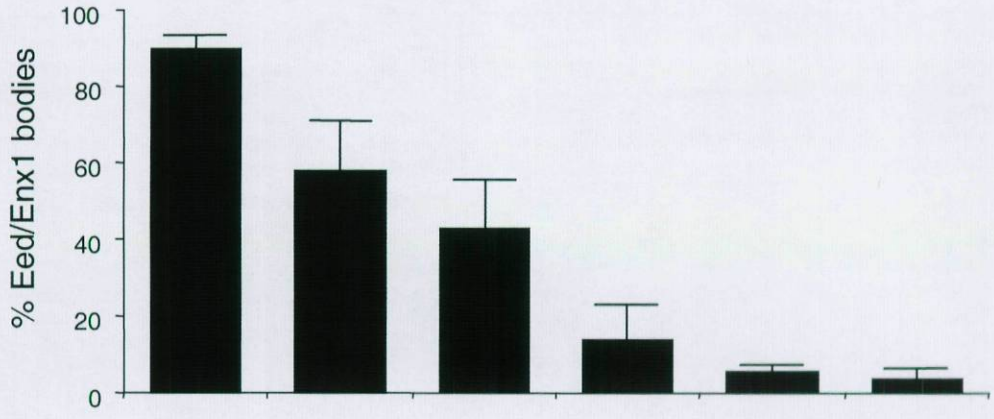
4.3- Discussion

Previous studies have shown that the Eed-Enx1 PcG complex has a role in imprinted X inactivation specifically in trophectoderm. My findings demonstrate that the Eed-Enx1 complex is also involved in random X inactivation in ES cells and in the embryo proper. In addition, the data presented show that Eed-Enx1 complexes are transiently recruited to the inactive X at the onset of random X inactivation *in vivo* and in the embryonic stem cell system. Transient localisation was seen in a maximum of 60% of differentiating XX ES cells. As mentioned previously my data suggests that this is because XX ES cells differentiate asynchronously and therefore indicate that localisation of Eed-Enx1 occurs in all cells. Support for this conclusion comes from analysis of embryos. At 6.5 dpc, immediately after the onset of X inactivation in the embryo proper, nearly all the cells showed Eed-Enx1 bodies. The asynchronicity of X inactivation in differentiating XX ES cells has been seen previously in studies on *Xist* expression (Sheardown *et al*, 1997), and on H3-K9 methylation (Mermoud *et al*, 2002). It should be stated that I cannot rule out that low levels of the complex are present on the inactive X at later stages and that immunofluorescence is not sensitive enough to detect this.

A)



B)



%Eed/Enx1 bodies	89.9 ± 2.6	57.9 ± 13	42.9 ± 12.8	13.7 ± 9.2	5.6 ± 1.9	3.6 ± 2.9
Total XX embryos	8	14	8	7	10	6
Total cells	519	1378	928	765	1089	663

Figure 4.2.5.2- **Transient association of Eed-Enx1 complexes with the inactive X during random X inactivation *in vivo*.**
(A) Immunofluorescence analysis of Eed (green) and Enx1 (red) in XX embryos isolated at 6.5 and 9.5 dpc. Note the presence (see arrows) and the absence of Eed/Enx1 bodies in 6.5 and 9.5 dpc embryos respectively. All merged panels include DAPI DNA counterstain. (B) Scoring data showing percentage (%) of cells with Eed/Enx1 bodies in XX embryos harvested at developmental stages between 6.5 and 11.5 dpc.

Transient recruitment appears to be related to the fact that both Eed and Enx1 are downregulated, as shown by western blot analysis. This was seen most dramatically for Eed protein. The fact that Eed and Enx1 proteins are present at high levels in undifferentiated ES cells and during early stages of differentiation suggests that this may be important for the *Xist* RNA mediated chromosomal silencing that occurs at early stages only (compare Figure 1.3.10.1 and Figure 4.2.2.1C). In Support of this the ESC protein is also required in early *Drosophila* development to establish silencing (Struhl and Brower 1982; Simon *et al*, 1995). At the end of embryogenesis the expression of *Esc* mRNA reduces and it is not required for the viability of adults (Ng *et al*, 2000).

The decrease of Eed and Enx1 protein levels found during ES cell differentiation and embryo development mirrors the *Drosophila* ESC and E(z) developmental expression profile (Ng *et al*, 2000). This developmental regulation is not unique among chromatin proteins, in fact this has been shown to be functionally relevant. The murine *de novo* methyltransferase 3a (Dnmt3a) and 3b (Dnmt3b) are also highly expressed at early embryonic developmental stages and downregulated at later stages (Okano *et al*, 1999). These proteins are thought to establish *de novo* DNA methylation patterns (Okano *et al*, 1999). The *de novo* methylated DNA is then maintained during DNA replication by Dnmt1 (reviewed in Bestor, 2000). Thus, it is possible that, later in embryonic development, a different complex replaces Eed-Enx1 function in X inactivation.

Previous studies have demonstrated that EED binds both *in vitro* and *in vivo* to HDAC1 and HDAC2 (van der Vlag and Otte, 1999). However, localisation of either Hdac1 or Hdac2 was not specifically observed at the inactive X. High levels of both proteins were however seen in undifferentiated and differentiated ES cells. One possible interpretation is that, because the two Hdacs are ubiquitously present in many different complexes, Hdac enrichment at specific sites could be masked by general staining throughout the nucleus. It is also possible that an as yet untested or undiscovered Hdac can interact with Eed and that this factor is involved in X inactivation. Finally, it

is possible that the interaction between Eed and Hdacs is weak and transient, and for this reason enrichment at the inactive X sites does not occur.

Recent data indicates that the PRC1 and ESC-E(Z) complexes interact transiently early in embryogenesis in *Drosophila* (Poux *et al*, 2001). However, mammalian PcG proteins Bmi1 and MPc2, which are part of the PRC1 complex, have shown contrasting protein expressions patterns to Eed and Enx1 and were not found enriched at the inactive X during ES cell differentiation. However, Mph1, which is also a component of the PRC1 complex, showed a similar expression profile to Eed and Enx1. Interestingly, Mph1 colocalised with the Eed/Enx1 body in a proportion of cells (32%) differentiated for 3 days. The fact that only a small proportion of cells shows colocalisation may suggest the existence of specific cell types within differentiated ES cell populations where this event occurs. This could possibly be investigated using lineage specific markers. The significance of this particular finding is not known. It is possible that MPh1 interacts with protein complexes other than PRC1 in a developmentally regulated manner. Therefore, it may have a role in the X inactivation process outside the PRC1 complex. However, because *Drosophila* Ph, Pc and posterior sex combs (Psc) have 2 mammalian homologues Mph1/Mph2, M33/MPc2 and Mel18/Bmi1 respectively, another possibility is that an early acting developmental PRC1 complex may exist, formed for example with Mph1, M33 and Mel18.

Chapter 5: Functional analysis of the Eed-Enx1 complex in X inactivation

5.1- Introduction

X inactivation is initiated by the accumulation of *Xist* RNA in cis over the length of the chromosome. RNAFISH analysis of XX ES cells revealed that at around 1.5 days of differentiation approximately 25% of cells exhibit accumulated *Xist* RNA signal (Sheardown *et al*, 1997; Panning *et al*, 1997). As differentiation progresses the number of cells exhibiting such a pattern increases and is seen in the majority of cells by day 6 (Sheardown *et al*, 1997). The early occurrence of the Eed/Enx1 body formation during ES cell differentiation (see section 4.2.2) and its closely related banding pattern to *Xist* RNA, first described by Mak *et al* (2002), suggested a possible interaction between the *Xist* RNA and the Eed-Enx1 PcG complex. Thus, Eed and Enx1 are candidates for proteins recruited by *Xist* RNA. To address this issue I studied the timing of Eed/Enx1 body formation in comparison to *Xist* accumulation at the inactivating X. To gain additional insight into the relationship between these two events I also analysed an XY ES cell line carrying a multicopy *Xist* transgene.

Mak *et al* (2002) have shown that the Eed-Enx1 complex remains tightly associated with the inactive X throughout the cell cycle. This led to the suggestion that these proteins may maintain the X inactive state by a direct mechanism. The presence of a SET domain in the Enx1 protein, the catalytic site in histone methyltransferases (HMTases) (Rea *et al*, 2000), in addition to the *in vitro* and *in vivo* association of Eed with histone deacetylases (van der Vlag and Otte, 1999), indicates that the Eed-Enx1 complex may have a role in histone modification in X inactivation.

To characterise the potential involvement of Eed in gene repression Denisenko and Bomsztyk (1997) used a Gal4-Eed fusion protein and an appropriate reporter gene driven by a promoter that contains Gal4 binding DNA elements. They demonstrated that Eed inhibits transcription activity and in addition, two domains within Eed are responsible for the repression. One domain is located in the

N-terminus and the other in the C-terminus, which comprises the highly conserved WD40 domain. Analysis with both Gal4-Eed null and Gal4-Eed hypomorphic fusion proteins, which comprise mutations within the WD40 domain, failed to induce any silencing on the reporter gene.

Subsequent experiments demonstrated that EED interacts with Hdacs both *in vitro* and *in vivo* (van der Vlag and Otte, 1999). Importantly, EED and ENX1 immunoprecipitates from a human tumorogenic cell line, were able to deacetylate histone H4 *in vitro*, a hallmark of gene silencing (van der Vlag and Otte, 1999). In addition, they showed that repression by LexA-EED fusion protein was relieved in the presence of the histone deacetylase inhibitor trichostatin A (TSA). Together, these data suggested that gene repression activity by EED-ENX1 is mediated through histone deacetylation.

Studies to investigate whether mammalian homologues of E(z) were involved in gene silencing have shown that transgenic flies (*Drosophila*) for Enx1 exhibit enhancing of position effect variegation (PEV) (Laible *et al*, 1997). Also, *Saccharomyces cerevisiae* (yeast) defective in telomeric silencing due to mutation of a gene (*Δset1*) that contains a SET domain, were shown to have gene repression restored with the addition of Enx1 and Ezh1 transgenes (Laible *et al*, 1997).

Studies with proteins containing the SET domain, Suv39h1/SUV39H1, have shown that this motif has histone methyltransferase (HMTase) activity specific for histone H3 (Rea *et al*, 2000) (see section 1.1.3 for additional information). More detailed experiments showed that Lysine 9 (K9) of the histone H3 N-terminus is the preferred residue for Suv39h1 HMTase activity. However, ENX1 transgenic proteins, containing the SET domain, have undetectable HMTase activity towards free histones (Rea *et al*, 2000).

In light of the results showing that Eed-Enx1 complexes are recruited transiently at the onset of random X inactivation it was imperative to determine their function. To achieve this I studied the

timing of Eed/Enx1 body formation in relation to the appearance of chromatin changes at the inactivating X in differentiating XX ES cells. In addition, I analysed *eed* mutant embryos to determine the requirement of Eed-Enx1 complexes in X chromosome inactivation.

5.2-Results

5.2.1- Eed-Enx1 recruitment to the inactive X occurs rapidly in response to *Xist* RNA expression

5.2.1.1- Analysis of differentiating XX ES cells

The previously described studies suggested that the association of Eed and Enx1 proteins with the inactive X is a very early event in both XX ES cell differentiation and *in vivo* (see sections 4.2.2 and 4.2.5 for additional information). To determine the relationship of *Xist* RNA accumulation and Eed/Enx1 localisation, detailed analysis was carried out at early XX ES cell differentiation time points. ImmunoRNA FISH for Enx1 and *Xist* RNA was performed in 2, 3 and 6 days differentiated XX PGK12.1. The results are summarised in Table 5.2.1.1.1A. At 2 days of differentiation 80% of cells with accumulated *Xist* RNA signal also showed Enx1 localisation. This number increased to 93% at day 3 of differentiation. Conversely, more than 97% of cells with an Enx1 body, at days 2 and 3, also exhibited accumulated *Xist*. The fact that a higher proportion of cells displayed *Xist* accumulation but no Enx1 body at day 2 may indicates that there is a short lag between *Xist* RNA accumulation and Eed/Enx1 recruitment. However, Eed and Enx1 protein detection is less efficient using immunoRNA FISH conditions compared with immunofluorescence, and this may account for the absence of Eed/Enx1 signal in cells that just have initiated stable *Xist* RNA expression (Figure 5.2.1.1.1B). Importantly, this result indicates that Eed and Enx1 are candidates for proteins directly recruited by *Xist* RNA. Consistent with previous results, the number of cells exhibiting Enx1 bodies underlying accumulated *Xist* decreased to 36% at day 6.

A)

	Sequence of analysis	N	Only *Xist* accumulation	Only Enx1 accumulation	Both accumulated
2d diff.	*Xist* cloud versus presence of Eed/Enx1 body	100	**20%**	—	**80%**
	Eed/Enx1 body versus presence of *Xist* cloud	100	—	**3%**	**97%**
3d diff.	*Xist* cloud versus presence of Eed/Enx1 body	100	**7%**	—	**93%**
	Eed/Enx1 body versus presence of *Xist* cloud	102	—	**2%**	**98%**
6d diff.	*Xist* cloud versus presence of Eed/Enx1 body	100	**64%**	—	**36%**
	Eed/Enx1 body versus presence of *Xist* cloud	100	—	**0%**	**100%**

Xist accumulation Enx1 accumulation Enx1 + *Xist* accumulation

B)

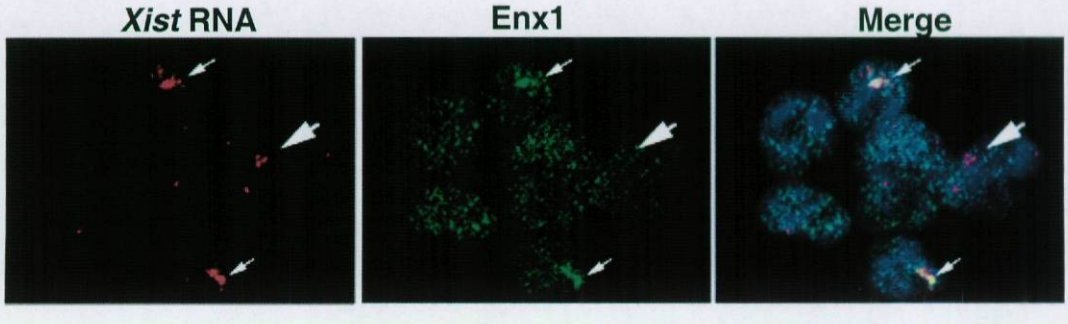


Figure 5.2.1.1.1 - **Recruitment of Eed-Enx1 complexes correlates with accumulation of stable *Xist* RNA.** (A) Scoring data showing the correlation between presence of Eed/Enx1 body and accumulated *Xist* RNA in PGK12.1 XX ES cells differentiated (diff.) for 2,3 and 6 days (d). (B) ImmunoRNA FISH for *Xist* RNA (red) and Enx1 protein (green) in 2 days differentiated PGK12.1 XX ES cells. Illustrated are examples of cells exhibiting colocalisation of accumulated *Xist* and Enx1 (small arrows) and a single cell with accumulated *Xist* RNA only (large arrow). Merged panel includes DAPI DNA counterstain (blue).

5.2.1.2- Recruitment of Eed/Enx1 by *Xist* RNA in transgenic LH1 XY ES cells

To further address whether *Xist* RNA recruits Eed-Enx1 complexes, analyses were carried out in XY LH1 ES cells, which contain an autosomally integrated multicopy Xist transgene (Figure 5.2.1.2.1B) (Herzing *et al*, 1997). Characterisation of this cell line has revealed the presence of accumulated *Xist* RNA in undifferentiated ES cells, indicating that the observed accumulated *Xist* RNA signal may originate from the transgene (Dr. Allistair Newall and Dr. Neil Brockdorff, personal communication). To address this RNAFISH analysis was performed on undifferentiated LH1 ES cells. In wild type undifferentiated XY ES cells both *Tsix* and *Xist* are expressed and these can be visualised by RNAFISH as a single punctate signal (Lee *et al*, 1999). I used the pTsix5' probe, which only detects *Tsix* RNA, in combination with the pEFB probe, which recognises both endogenous and transgene *Xist* RNA (Figure 5.2.1.2.1C). The proportion of cells exhibiting accumulated *Xist* signal was approximately 30% (n=100). More than 80% (n=43) of these cells showed also one *Tsix* and one *Xist* punctate RNA signal located elsewhere in the nucleus (Figure 5.2.1.2.1D). Control slides, containing wild type XY ES cells, never exhibited accumulated *Xist* RNA (n=100). This result demonstrated that the expression of accumulated *Xist* RNA is at least in a majority of the cases, if not all, originated from the transgene.

Subsequently, immunoRNA FISH analysis was carried out for Enx1 and *Xist* RNA. This demonstrated that Eed/Enx1 bodies are present and colocalise with ectopic *Xist* RNA (Figure 5.2.1.2.1E). Thus, Eed and Enx1 recruitment can occur in response to stable *Xist* RNA expression, even in undifferentiated XY cells, which normally do not show accumulated *Xist* RNA. Scoring of this experiment demonstrated the presence of Enx1 body in 58% (n=111) of cells which simultaneously exhibited accumulated *Xist* RNA. This is slightly lower than in differentiating XX ES cells, probably reflecting the relatively low level and diffuse *Xist* accumulation observed in undifferentiated LH1 cells.

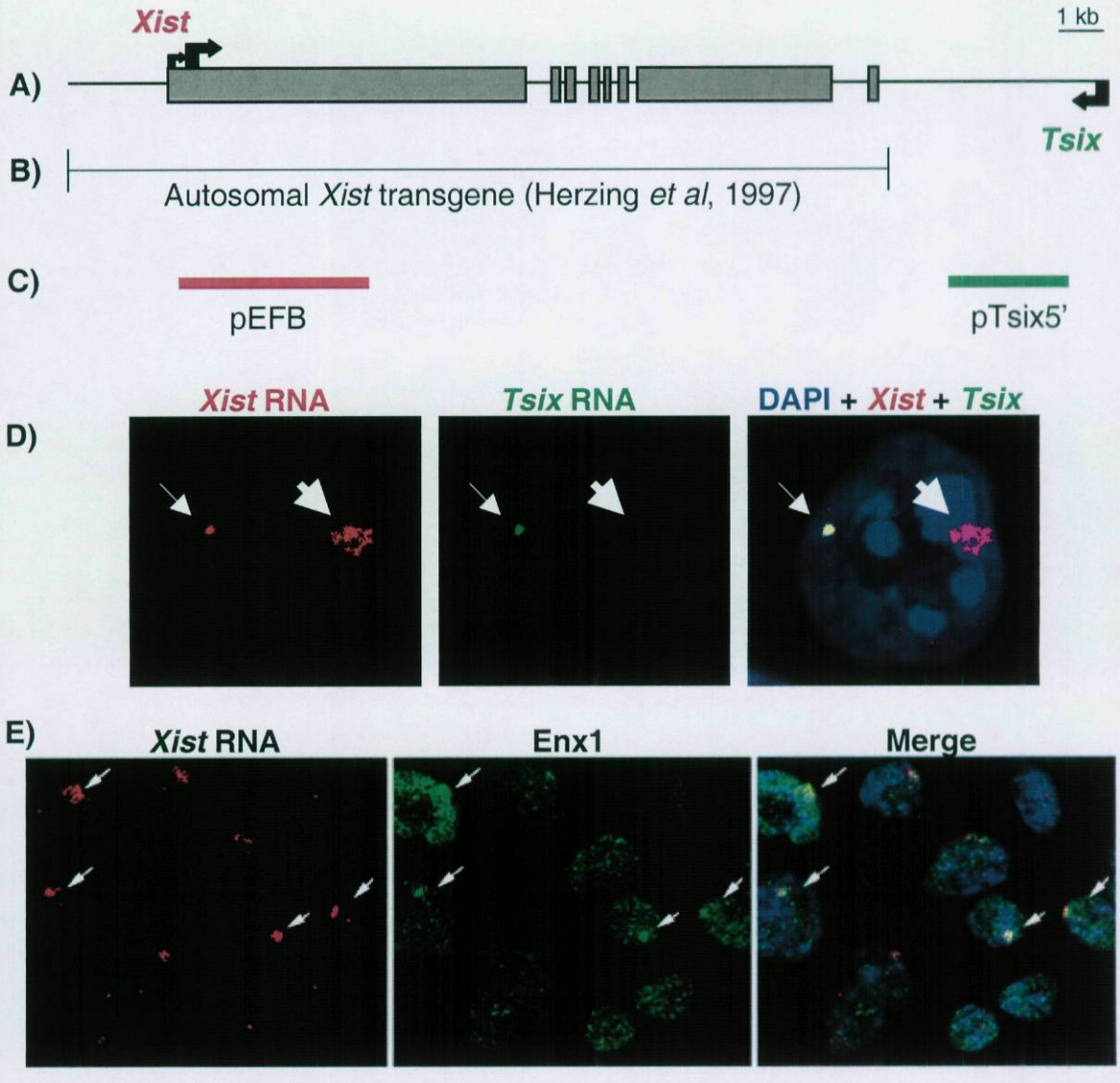


Figure 5.2.1.2.1 - ***Xist* RNA is sufficient to recruit Eed-Enx1 complexes.** (A-E) Analysis of undifferentiated XY LH1 ES cells, which carry a multicopy Xist RNA transgene (see text for additional information). (A) Schematic representation of the *Xist* locus structure. Arrows indicate *Xist* (left) and *Tsix* (right) promoters. (B) Portion of the *Xist* locus included in the multicopy transgene. (C) Probes used in the detection of endogenous *Xist* and transgene transcript (red), and *Tsix* transcript (green). (D) Double RNAFISH analysis for *Xist* (red) and *Tsix* (green). Figure shows one example of a cell exhibiting punctate *Xist* and *Tsix* RNA signal, characteristic of undifferentiated XY ES cells (Lee *et al*, 1999) corresponding to the endogenous locus (small arrow), and one accumulated *Xist* RNA signal corresponding to multicopy transgene expression (large arrow). (E) ImmunoRNA FISH analysis for *Xist* (red) and Enx1 (green). As illustrated a group of cells exhibit ectopic *Xist* RNA that contain underlying Enx1 signal. Examples with colocalising signal have arrowhead. All cells are counterstained with DAPI. See text for scores and additional information.

5.2.2- Recruitment of Eed-Enx1 complexes precedes histone H3 N-terminal modifications

5.2.2.1- Analysis of differentiating XX ES cells

The timing of Eed/Enx1 body formation (at day 2 of ES cell differentiation) suggested a very early role in X inactivation. At day 2 of ES cell differentiation the inactive X is also known to undergo chromatin changes, namely methylation of histone H3 at lysine 9 (H3-K9) (Heard *et al*, 2002; Mermoud *et al*, 2002), deacetylation of H3-K9 and demethylation of H3-K4 (Heard et al, 2002). To investigate the relationship of these modifications with formation of the Eed/Enx1 body, XX PGK12.1 ES cells at early and late X inactivation stages (2, 3 and 6 days of ES cell differentiation respectively) were analysed by immunofluorescence for colocalisation of histone N-terminal modifications with Eed/Enx1 bodies.

Detection of methylated H3-K9 was performed using antisera raised to a branched H3 peptide dimethylated at H3-K9. Previously, this antibody was shown to distinctly stain pericentric heterochromatin and the inactive X (Peters *et al*, 2001, 2002; Mermoud *et al*, 2002). The analysis was made scoring first for the presence of a single Eed/Enx1 body, and then for the presence or absence of underlying H3-K9 methylation. Methylated H3-K9 staining of pericentromeric heterochromatin, as assessed by colocalisation with DAPI dense staining regions of the nucleus, was observed at all stages analysed (Figure 5.2.2.1.1A). A clear H3-K9 methylated focus underlying Eed/Enx1 bodies was seen in 40% of the cells (n=100) at day 2. A weaker methylated H3-K9 staining was seen in a further 33% of cells. However, 27% of cells at this stage showed no detectable H3-K9 methylation at the Eed/Enx1 body. In contrast, 88.1% of 3 days differentiated cells (n=101) had a clear H3-K9 methylated focus underlying the Eed body, and by day 6 almost all cells exhibited the modification. Similarly, deacetylation of H3-K9 and demethylation of H3-K4 were seen to occur rapidly at the time of Eed-Enx1 complexes recruitment (Figures 5.2.2.1.1B and C). Together, these results are consistent with Eed-Enx1 complexes catalysing histone H3 N-terminal modifications.

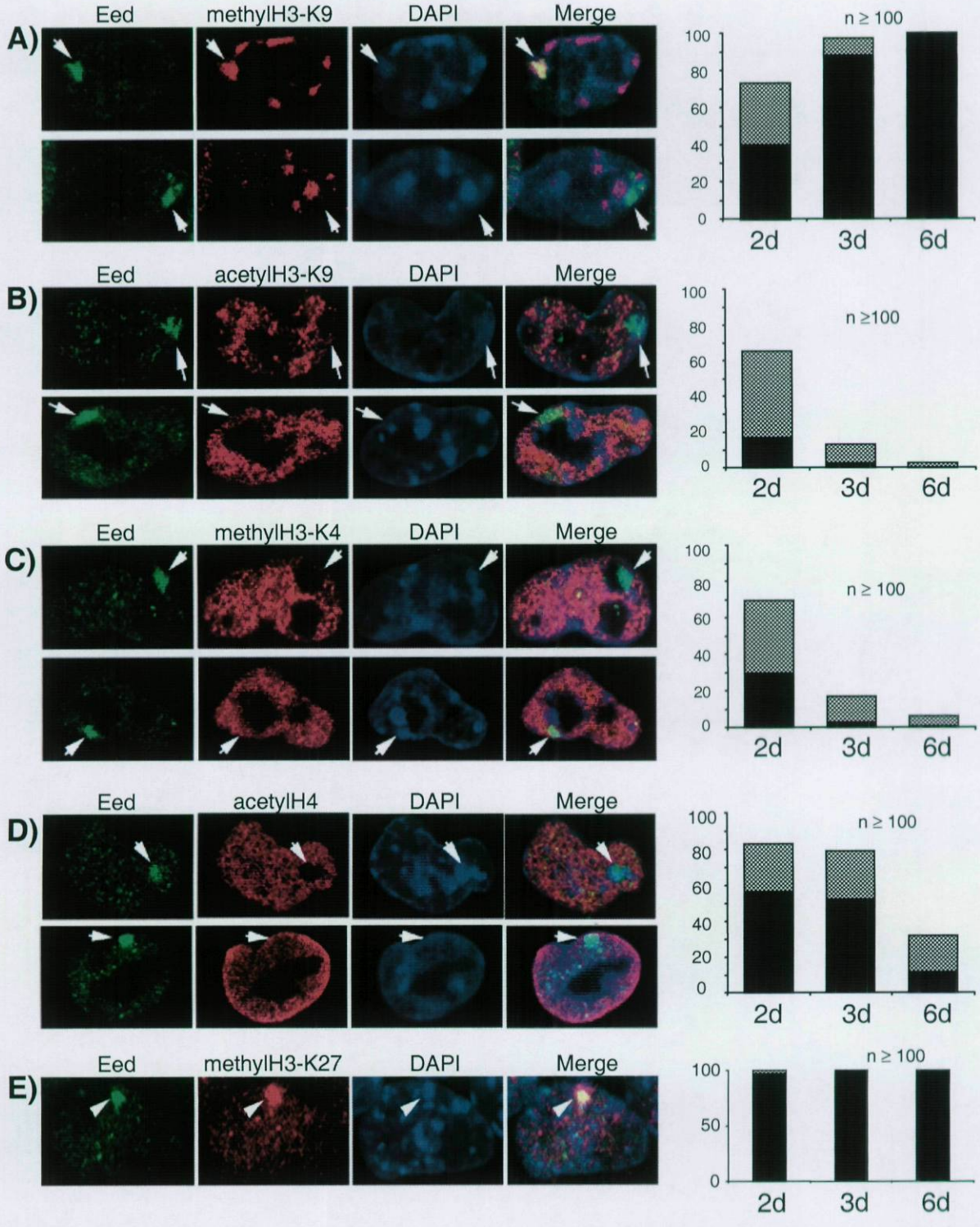


Figure 5.2.2.1.1 - **Recruitment of Eed-Enx1 complexes on the inactive X chromosome correlates with establishment of deacetylation and methylation of histone H3-K9, methylation of H3-K27, and loss of methylation at H3-K4.** (**A-D**) Immunofluorescence analysis of ES cells differentiated for 2, 3 and 6 days (d) was used to assess correlation of Eed bodies and histone H3-K9 methylation (methyl) (**A**), H3-K9 acetylation (acetyl) (**B**), H3-K4 methylation (**C**), H4 acetylation (**D**), and H3-K27 methylation (**E**). (**A**) Illustrated are examples of individual cells showing Eed (green) and methyl H3-K9 (red), with upper panels illustrating colocalisation of Eed body and methyl H3-K9 and the lower panels illustrating an Eed body without associated methyl H3-K9 (arrow); (**B**) examples of Eed (green) and acetyl H3-K9 (red), illustrating absence (upper panel), and presence (lower panel) of acetyl H3-K9 associated with an Eed body (arrow); (**C**) examples of Eed (green) and methyl H3-K4 (red), illustrating absence (upper panel), and presence (lower panel) of methyl H3-K4 associated with an Eed body (arrow); (**D**) examples of Eed (green) and acetylated H4 (red), illustrating absence (upper panel), and presence (lower panel) of acetylated H4 associated with an Eed body (arrow); (**E**) Example of a single cell showing Eed (green) and methyl H3-K27 (red), with panels illustrating colocalisation of Eed body and methyl H3-K27. Scoring data showing the proportion of cells (n ≥ 100) which have methylated H3-K9, acetylated H3-K9, methylated H3-K4, acetylated H4 and methylated H3-K27 associated with Eed bodies is shown to the right. Filled areas represent the proportion of cells where histone was clearly detectable. Shaded areas represent cells where an intermediate level of histone was detected. DNA is counterstained with DAPI. All images are single optical sections.

As a control for timing of events of the X inactivation process in these experiments I also analysed global deacetylation of histone H4 (Figure 5.2.2.1.1D). Previously this was shown to occur later in XX ES cell differentiation, after H3-K9 and H3-K4 modifications (Keohane *et al*, 1996; Heard *et al*, 2001). In agreement with these studies, hypoacetylation of histone H4 in association with Eed/Enx1 bodies was only detected in a small proportion of cells after 2 and 3 days of differentiation, respectively 17 and 21.4%, but had occurred in the majority of cells after 6 days of differentiation.

Concomitantly with this thesis work it was demonstrated *in vitro* that purified and recombinant *Drosophila* ESC-E(Z) and mammalian EED-ENX1 complexes, but not E(Z)/ENX1 alone, have histone methyltransferase (HMTase) activity specific to histone H3 (Cao *et al*, 2002; Czermin *et al*, 2002; Muller *et al*, 2002; Kuzmichev *et al*, 2002). In addition, the histone H3 lysine 27 (H3-K27) was identified as the preferred substrate of HMTase activity (Cao *et al*, 2002; Muller *et al*, 2002). Moreover, recent studies have demonstrated that an antiserum for tri-methylated (tri-methyl) H3-K27 highlights specifically the inactive X chromosome in XX somatic cells (Antoine Peters and Thomas Jenuwein, personal communication). Using the latter antibody I went on to investigate the occurrence of H3-K27 tri-methylation on the inactive X in relation to the Eed/Enx1 bodies in differentiating XX PGK12.1 ES cells. Interestingly, strong tri-methyl H3-K27 staining underlying the Eed/Enx1 body was observed in nearly all cells at all differentiation stages tested (Figure 5.2.2.1.1E). This result is also consistent with Eed-Enx1 complexes tri-methylating H3-K27 on the inactive X.

5.2.2.2- Analysis of LH1 XY ES cells

To further investigate the relationship of Eed/Enx1 recruitment and histone H3/H4 N-terminal modifications analyses were carried out on undifferentiated LH1 XY cells, which misexpress *Xist* transgene RNA (see section 5.2.1.2 for additional information). Deacetylation and methylation of H3-K9, loss of methylation at H3-K4, deacetylation of global histone H4 and methylation of H3-K27 were all seen in association with Eed/Enx1 bodies (Figure 5.2.2.2.1). Scoring data shows that H3 N-terminal modifications and histone H4 deacetylation are detectable approximately 75% of the time with the exception of tri-methylation of H3-K27, which is detected in nearly all cells.

Taken together these results demonstrate that recruitment of the Eed-Enx1 complex precedes or coincides with establishment of histone H3 N-terminal modifications on the inactive X.

5.2.3- Effect of trichostatin A on the Eed-Enx1 complex stability

The results obtained in the previous section are consistent with Eed-Enx1 complexes being involved in the deacetylation of H3-K9 residue in the inactive X. To investigate this further, analysis was carried out on differentiating XX ES PGK12.1 cells treated with trichostatin A (TSA). The fungal antibiotic TSA is an effective inhibitor of histone deacetylases at nanomolar concentrations and induces a rapid increase of histone acetylation when added to cell cultures (Yoshida *et al*, 1990). Previous work performed by O'Neil *et al* (1999), using also XX ES PGK12.1 cells, found that culturing cells in the presence of 5ng/ml TSA (5ng/ml) increased histone H4 acetylation levels, while having a minimum effect on cell growth (higher levels of TSA are lethal to ES cells). Metaphase analysis of differentiated ES cells, grown in the presence of 5ng/ml TSA, labelled with acetylated histone H4 antisera, never revealed a hypoacetylated X (cells were monitored from days 0 to 12 of ES cell differentiation).

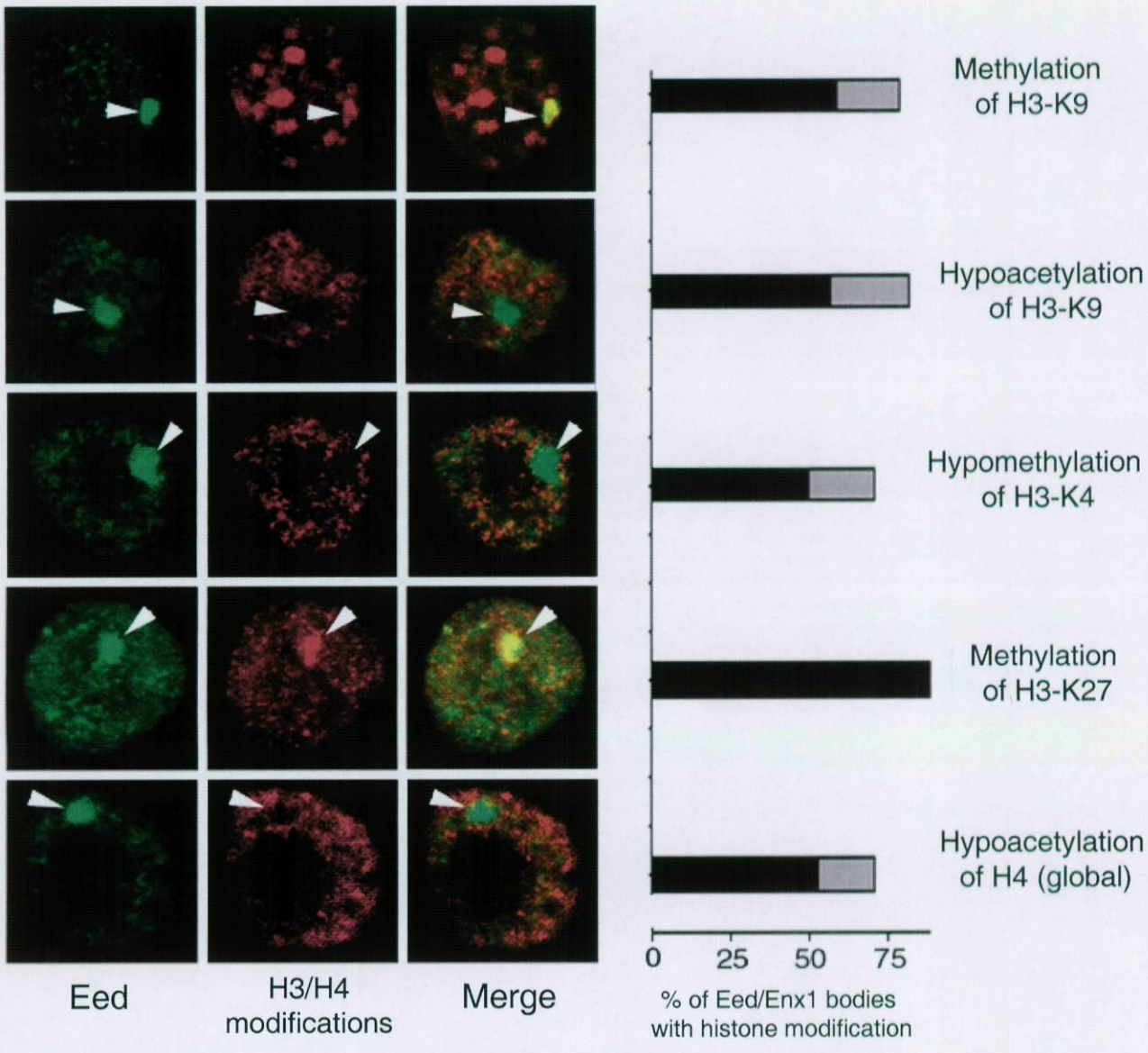


Figure 5.2.2.2.1 - **Recruitment of Eed-Enx1 complexes by ectopic *Xist* RNA cause chromosomal deacetylation and methylation of histones H3/H4.**
Immunofluorescence for Eed (green) and either methylated H3-K9 (red), acetylated H3-K9 (red), methylated H3-K4 (red), methylated H3-K27 (red) or acetylated H4 (red) in undifferentiated LH1 XY ES cells expressing ectopic *Xist* RNA. Individual cells illustrate examples with colocalisation of Eed bodies and either methylated H3-K9, hypoacetylated H3-K9, hypomethylated H3-K4, methylated H3-K27 or hypoacetylated H4 (see arrows). Right panels indicate scoring data showing the proportion of cells with methylated H3-K9, hypoacetylated H3-K9, hypomethylated H3-K4, methylated H3-K27 and hypoacetylated H4 associated with Eed bodies. All images are of single optical sections.

PGK12.1 XX ES cells were differentiated for 3 days in the continuous presence of 5ng/ml TSA or in the presence (for just 6 hours prior to fixation) of 150ng/ml TSA. Immunofluorescence analysis was performed using Eed and Enx1 antisera. As a control, plates containing untreated 3 days differentiated PGK12.1 were used. In cells treated with 5ng/ml TSA for 3 days there was general diffuse Eed/Enx1 staining and, in contrast to control slides, no Eed/Enx1 bodies (0/150) were observed (Figure 5.2.3.1A). Analysis of cells treated for 6 hours prior to fixation with 150ng/ml TSA, revealed that Eed/Enx1 bodies were still present (Figure 5.2.3.1B). However, in 15% (15/100) of the cells Eed and Enx1 were observed at DAPI dense regions. Untreated cells never showed this pattern (0/100). This result suggests that TSA interferes with the Eed-Enx1 complex nuclear distribution. Subsequent immunofluorescence analysis in these cells revealed that the Eed/Enx1 body remained methylated and hypoacetylated at H3-K9 (not shown).

In order to investigate absence of the Eed/Enx1 body in cells treated with 5ng/ml TSA I performed RNAFISH analysis for *Xist*. These cells showed no accumulation of *Xist* RNA, but instead exhibited one or two punctate *Xist* RNA signals (Figure 5.2.3.1C). A proportion of cells (12/100), exhibited also scattered *Xist* RNA signal. This result suggests that TSA interferes with the *Xist* gene regulation by preventing the accumulation of *Xist* RNA. Consequently, this will cause total failure of X inactivation.

5.2.4- Eed-Enx1 complexes are required to establish H3-K9/K27 methylation on the inactive X

The observation that Eed-Enx1 complex recruitment precedes appearance of histone H3-K9/K27 methylation on the inactive X, together with the fact that Enx1 contains a SET domain, suggests that this complex could be responsible for catalysing these modifications. Investigation of the functional role of the Eed-Enx1 complex can be approached in at least two ways: *in vitro* assays assessing the potential of Eed-Enx1 complex to induce histone modifications, or analysis of murine *Enx1* and *eed* mutants. I have used the latter strategy, analysing the eed^{3354SB} mutant mice. eed^{3354SB} carries a point mutation (null) located in the WD40 protein domain (Figure 1.1.7.1.1A) (Shumacher *et al*, 1996).

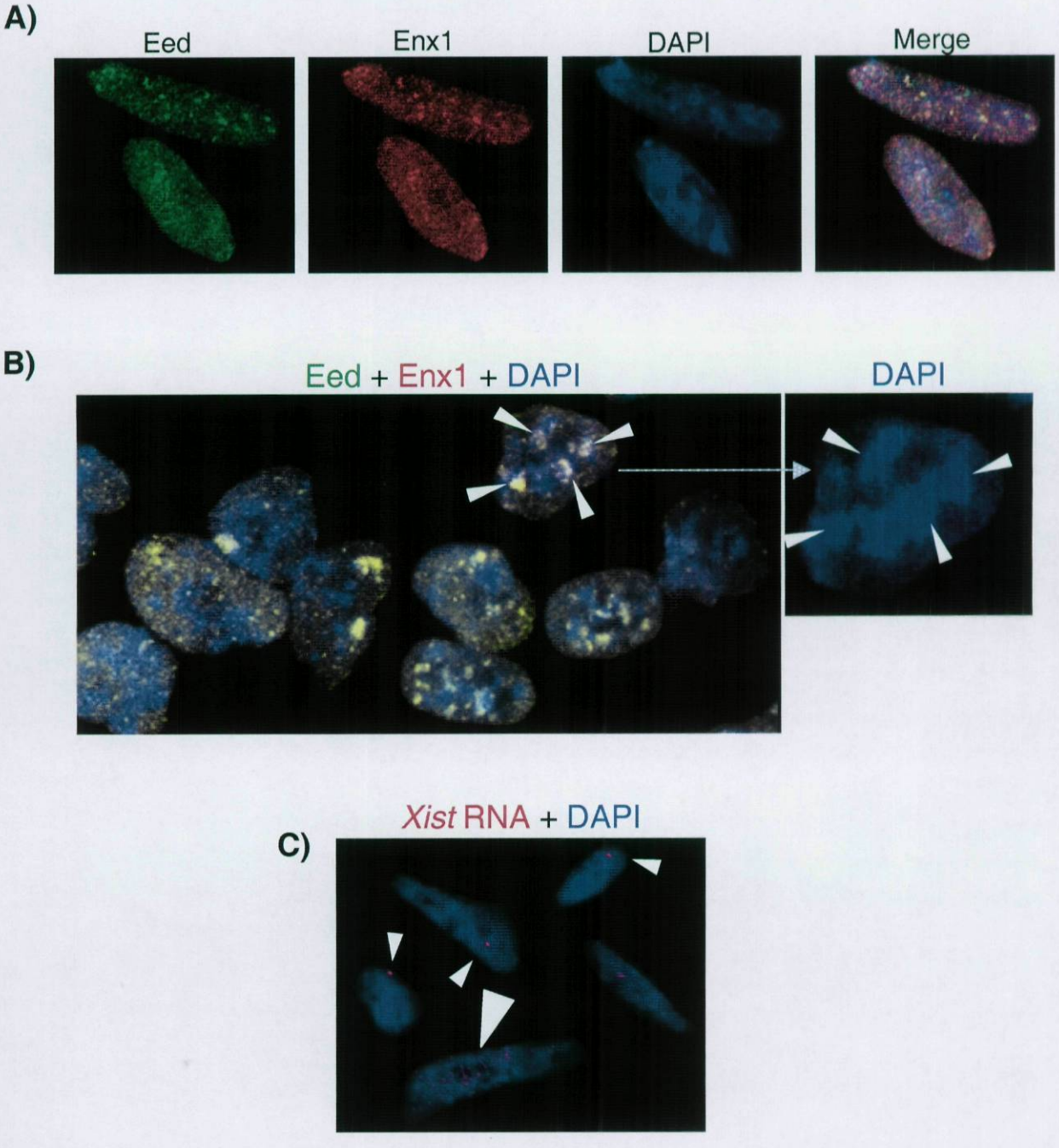


Figure 5.2.3.1 - **Effect of trichostatin A treatment on stability of the Eed-Enx1 complex.** (A and B) Immunofluorescence for Eed (green) and Enx1 (red) in 3 days differentiated XX ES cells treated in the continuous presence of 5ng/ml trichostatin A (TSA) (A), or in the presence (for just 6 hours prior to fixation) of 150ng/ml TSA (B). (A) Note the absence of Eed/Enx1 body. (B) Note the presence of some cells exhibiting multiple Eed/Enx1 bodies, as a result of the TSA treatment, which overlap with DAPI dense domains (see right panel). (C) RNAFISH for *Xist* (red) in 3 days differentiated XX ES cells treated in the continuous presence of 5ng/ml TSA . Note that cells lack accumulated *Xist* signal. Instead, cells exhibit one punctate *Xist* signal (small arrows) or (in a smaller proportion of cells) scattered *Xist* signal (large arrowhead). All cells were counterstained with DAPI (blue).

Previous *in vitro* and *in vivo* analysis using Eed constructs that expressed the null, hypomorphic and C-terminal deleted proteins, showed that these do not interact with Enx1 (Denisenko *et al*, 1998). In contrast, constructs for wild type and N-terminal deleted Eed proteins were found to bind well to Enx1. These results demonstrated that the Eed-Enx1 binding is mediated by an intact WD40 domain. eed^{3354SB} allele is therefore expected to disrupt Enx1 binding *in vivo*.

Homozygous eed^{3354SB} mutants ($eed^{-/-}$) die at around 8.5 dpc (Shumacher *et al*, 1996; Wang *et al*, 2001). To study X inactivation in the embryo proper, of *eed* mutants, analyses were carried out on the embryonic ectoderm of 7.5 dpc embryos, which is prior to the stage at which homozygous mutants die, but after the onset of random X inactivation. Immunofluorescense localisation analysis for Eed and Enx1 proteins in female $eed^{-/-}$ mutant embryos revealed that Eed protein is not detected at all, suggesting that it is destabilised as a consequence of the mutation, or alternatively that the epitope recognised by the antibody has been destroyed (Figure 5.2.4.1B). Enx1 is detected in $eed^{-/-}$ mutant cells, however, these show an abnormal localisation, being diffusely distributed both in the nucleus and cytoplasm. An identical pattern was obtained in male $eed^{-/-}$ embryos. As illustrated in the Figure 5.2.4.1B, Eed/Enx1 bodies were not detected in XX mutant embryos (n=4 mutant embryos). Moreover, heterozygous embryos showed overall reduced levels of Eed and weaker Eed/Enx1 body staining in comparison to wild type embryos.

In order to investigate the *Xist* RNA pattern and whether, in the absence of Eed/Enx1 body, low levels of Enx1 protein localise to the inactive X, immunoRNA FISH analysis was performed in XX mutant embryos. As illustrated in Figure 5.2.4.1C *Xist* appears localised to the inactive X, however, no Enx1 is detected. In fact Enx1 staining in $eed^{-/-}$ embryos was nearly completely lost under these experimental conditions. As immunoRNA FISH involves a cell permeabilisation step prior to fixation (see material and methods), and Enx1 may not be associated in a complex or with chromatin, the likely explanation for this result is that the protein probably diffuses out of the cells.

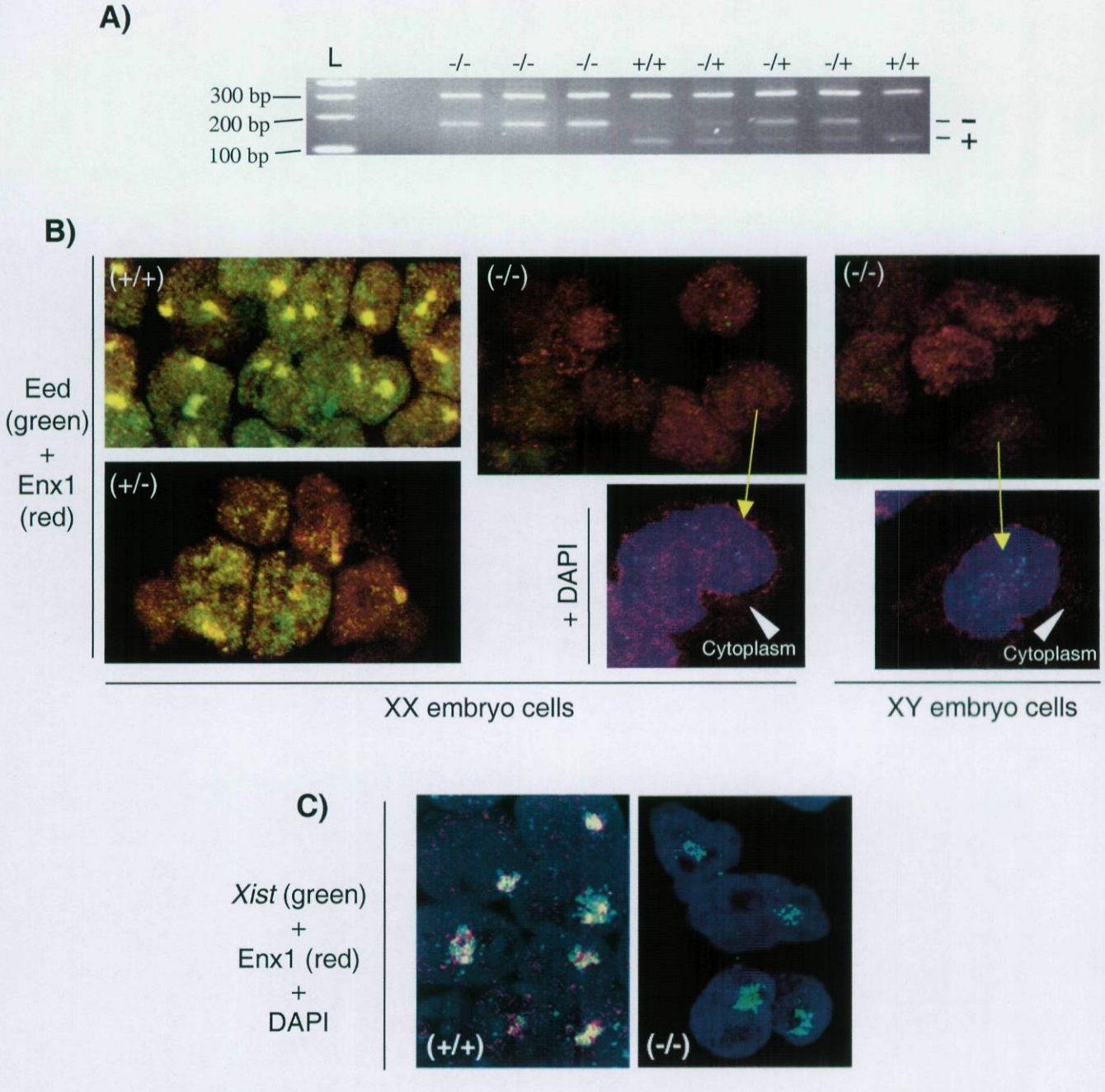


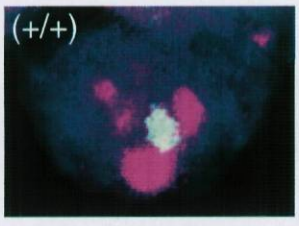
Figure 5.2.4.1- **Mutant XX *eed* embryos cells do not form Eed/Enx1 bodies.**
A) Genotyping analysis. This was assessed by digesting the 570 bp PCR product of E3 and E4 pair of primers with AluI. Wild type (+/+) embryos show a band at approximately 100 bp and *eed* mutant (-/-) embryos show a band at approximately 200 bp on a 2.5% agarose gel. *eed* heterozygous (+/-) show both bands. B) Immunofluorescence for Eed (green) and Enx1 (red) in 7.5 dpc *eed* mutant, heterozygous and wild type embryos. Note that Eed/Enx1 bodies stain more weakly in heterozygous than in wild type cells. In homozygous mutant cells Eed signal is nearly undetectable. In addition, Enx1 protein is found in the nucleus and cytoplasm, and there are no Eed/Enx1 bodies detectable. C) ImmunoRNA FISH analysis for *Xist* (green) and Enx1 (red) in 7.5 dpc XX *eed* mutant and wild type embryos. Note the absence of underlying Enx1 staining at the accumulated *Xist* RNA signal in XX *eed* mutant cells (absence of yellow signal).

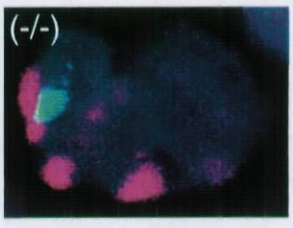
To further analyse the consequences of disruption of Eed-Enx1 complexes on X inactivation in female *eed*$^{-/-}$ embryos, immunoRNA FISH analyses were carried out using either antisera to methylated H3-K9 or tri-methylated H3-K27. As illustrated (Figures 5.2.4.2A and B), wild type embryo controls showed the previously characterised pattern in differentiated ES cells (section 5.2.2.1), with the inactive X staining for methylated H3-K9 and tri-methylated H3-K27. In contrast, the *Xist* RNA labelled X of female *eed*$^{-/-}$ embryo cells showed absence of H3–K9 methylation in the majority of cells and absence of tri-methylated H3-K27 in all cells. Pericentric heterochromatin and general nuclear staining for methylated H3-K9 was however unaffected. This latter observation is expected as H3-K9 methylation at these sites is catalysed by the Suv39h1 and Suv39h2 proteins (see section 1.1.3) (Peters *et al*, 2001). The tri-methylated H3-K27 antibody also failed to detect signal underlying *Xist* RNA and general staining for tri-methylated H3-K27 was much reduced (Figure 5.2.4.3). Therefore, the Eed-Enx1 complex is required for H3-K27 methylation on the inactive X and in addition appears to be the major H3-K27 HMTase present at this developmental stage. Together, these results demonstrate that Eed-Enx1 complexes establish H3-K9/K27 methylation early in development, both on the inactive X and elsewhere in the genome.

5.2.5- H3-K9/K27 methylation is required for stable maintenance of histone modifications and gene silencing

As female *eed*$^{-/-}$ embryos were previously shown to initiate but not maintain imprinted X inactivation in trophectoderm (Wang *et al*, 2001), analyses were also carried to determine if deacetylation of H3-K9 and demethylation of H3-K4 occur on the inactive X in the mutant embryonic ectoderm (embryo proper). In a large proportion of cells hypoacetylation of H3-K9 was seen underlying *Xist* signal (Figure 5.2.5.1). However, in 21.9% ± 0.3 of *eed*$^{-/-}$ cells (n=104) compared to 6.15% ± 2.8 in wild type cells, H3-K9 hypoacetylation was not detected. Similarly, H3-K4 hypomethylation occurs on the inactive X of *eed*$^{-/-}$ cells, but in a large proportion of these (21.6% ± 3.7) the inactive X was methylated at this site. In addition, general nuclear staining for H3-K9 acetylation appeared to be stronger in mutant embryos compared to wild type littermates.

A) *Xist* (green) + methyl H3-K9 (red) + DAPI

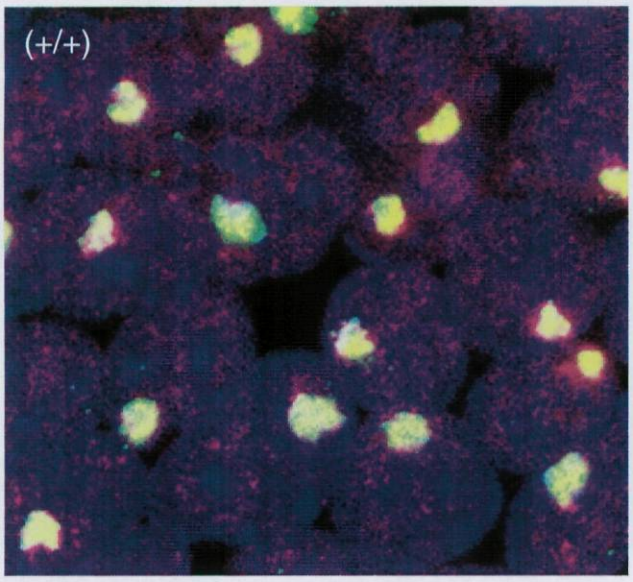


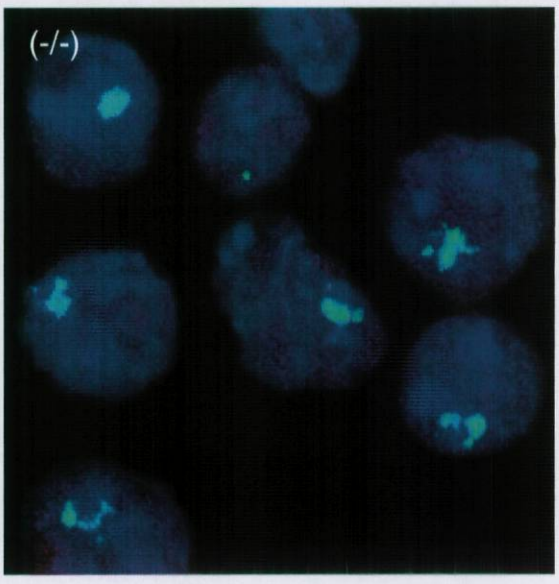


	Xi methylated	Xi not methylated	N (cells)
Embryo 1	77	2	79
Embryo 2	56	1	57
Total	**133**	**3**	**136**

	Xi methylated	Xi not methylated	N (cells)
Embryo 1	4	42	46
Embryo 2	6	56	62
Total	**10**	**98**	**108**

B) *Xist* (green) + Methyl H3-K27 (red) + DAPI





	Xi methylated	Xi not methylated	N (cells)
Embryo 1	60	0	60
Embryo 2	60	0	60
Embryo 3	40	0	40
Total	**160**	**0**	**160**

	Xi methylated	Xi not methylated	N (cells)
Embryo 1	0	42	42
Embryo 2	0	31	31
Embryo 3	0	60	60
Total	**0**	**133**	**133**

Figure 5.2.4.2- **Eed-Enx1 complexes are required to establish H3-K9/K27 methylation on the inactive X chromosome.**
(A and B) Examples of immunoRNA FISH analysis assessing association of methylated (methyl) H3-K9 (A), and methylated K27 (B), with *Xist* RNA domains (inactive X (Xi)) in homozygous *eed* mutants (-/-) compared to wild type (+/+) embryos. Scoring data recording the percentage of cells with colocalising signals are shown below the panels. All cells are counterstained with DAPI.

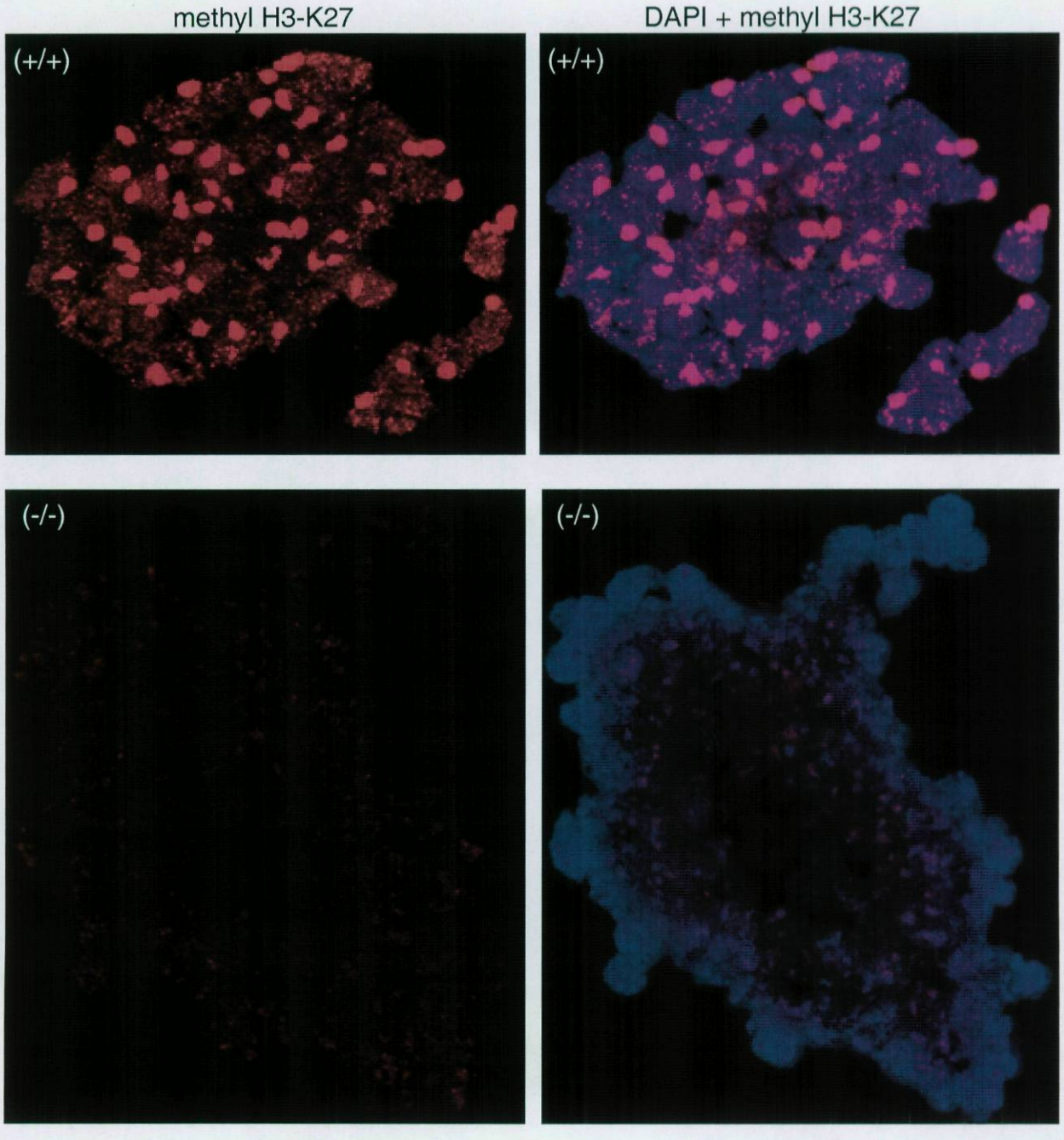
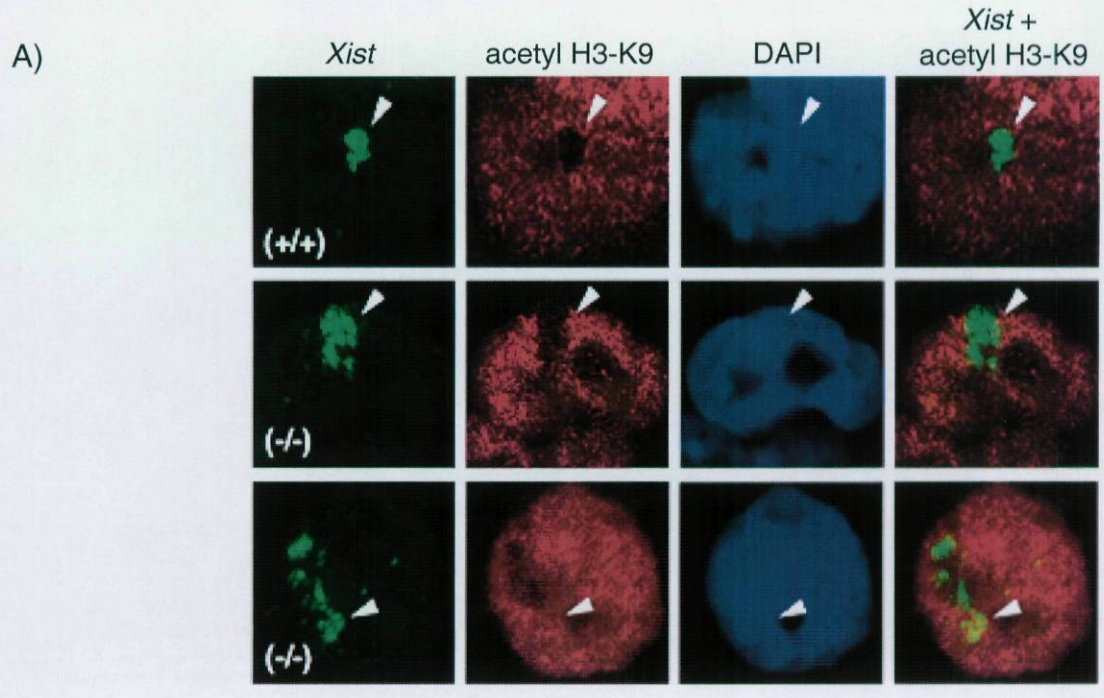
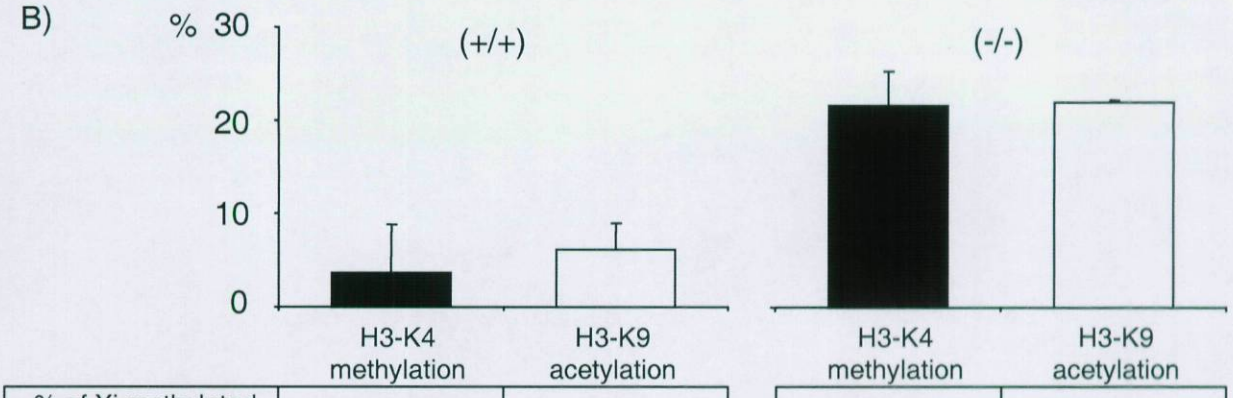


Figure 5.2.4.3 - **Eed-Enx1 complexes establish H3-K27 methylation genome wide.** Examples of immunofluorescence analysis assessing H3-K27 methylation (methyl) in XX 7.5 dpc *eed* mutant (-/-) and wild type (+/+) embryo cells. Illustrations represent represent true differences between *eed* mutant and wild type methyl H3-K27 staining as images were collected using exactly the same settings. Note that *eed* mutant cells lack H3-K27 body. In addition, *eed* mutant cells exhibit low levels of H3-K27 methylation relative to wild type cells.





% of Xi methylated for H3-K4 or acetylated for H3-K9	3.65 ± 5.2	6.15 ± 2.8	21.6 ± 3.7	21.9 ± 0.3
N (embryos)	2	2	3	2
N (cells)	152	84	154	112

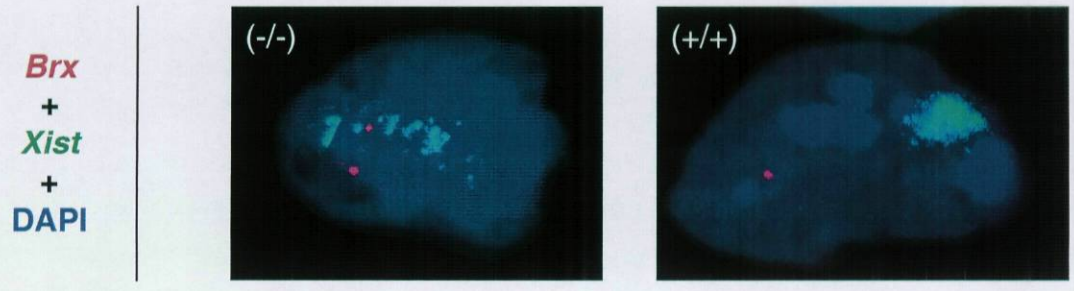
Figure 5.2.5.1 - **H3-K9/K27 methylation is required for stable maintenance of silent chromatin.**

A) Examples of immunoRNA FISH analysis assessing association of *Xist* RNA (green) and acetylated (acetyl) H3-K9 (red) in XX *eed* wild type (+/+) and mutant (-/-) embryo cells. In wild type embryos, the majority of cells show hypoacetylation of H3-K9 underlying *Xist* RNA (top panel). This is true in many cells from homozygous mutant embryos (middle panel). However, a significant proportion of homozygous mutant cells exhibit association of acetyl H3-K9 signal and *Xist* RNA (lower panels). Note also that in the middle and bottom panels *Xist* RNA appears to be more diffusely distributed suggesting that the inactive X in *eed*$^{-/-}$ cells is relatively decondensed. All images are single optical sections. B) Scoring data recording the percentage of cells exhibiting acetylated H3-K9 or methylated H3-K4 signal underlying the accumulated *Xist* RNA signal (inactive X (Xi)).

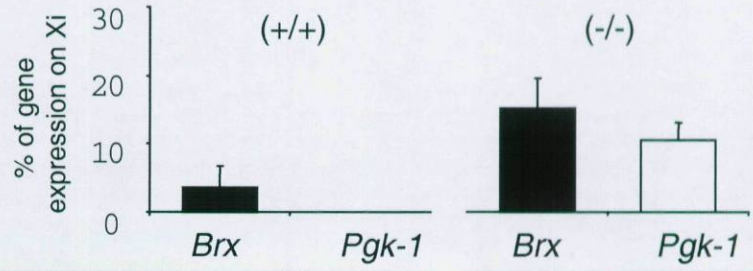
In order to study whether absence of H3-K9/K27 methylation in the embryo proper affects silencing at the inactive X, I analysed nascent transcripts for two X linked genes, *Brx* and *Pgk-1*. The *Brx* transcript was detected using λIIIF9 and λIVC3 probe. Expression was detected in approximately 60% of 7.5 dpc embryonic ectoderm cells (n=105), in close agreement with previous studies (Clerc and Avner, 1998). The *Brx* punctate signal was located away from the domain coated by the *Xist* RNA, in the majority of cells of both mutant and wild type embryos (Figures 5.2.5.2A and B). However, in 15.4% ± 4.4 of cells in mutant embryos, a second *Brx* punctate signal was found co-localising with accumulated *Xist* RNA (inactive X) (Figure 5.2.5.2A). This contrasted with the situation in wild type embryos where only 3.2% ± 3.5 of cells had a second punctate signal co-localising with accumulated *Xist* RNA.

Nascent transcripts for *Pgk-1* were detected using λPgk-1 probe and were seen in 36% of the cells (n=102), again in close agreement with previous studies (Sheardown *et al*, 1997; Panning and Jaenisch, 1996). In mutant embryo cells *Pgk-1* expression from the inactive X allele was detected in 10.6% ± 2.6 of the cases. In contrast, no *Pgk-1* expression was detected from the inactive X in wild type embryo cells. Together, these results suggest that H3-K9/K27 methylation is required for stable maintenance of silent chromatin and gene silencing

A)



B)



% of	3.2 ± 3.5	0	15.4 ± 4.4	10.6 ± 2.6
N embryos	4	3	3	6
N cells	184	171	153	139

Figure 5.2.5.2- **Expression of X-linked genes in XX *eed* mutant 7.5 dpc embryos.** A) Examples show a cell from an XX wild type embryo (+/+) with a *Brx* (red) transcript from the active X chromosome (right panel), and a cell from an XX mutant (-/-) embryo with *Brx* expression from both active and inactive X (Xi) (left panel). The inactive X is marked by *Xist* RNA (green). B) Scoring data illustrates the percentage of cells expressing *Brx* or *Pgk-1* from both active and inactive X in wild type or mutant embryo cells.

5.3- Discussion

5.3.1- Interaction between *Xist* RNA and Eed/Enx1 complexes

The data presented here are consistent with the idea that *XIST* RNA recruits Eed-Enx1 complexes. This was first proposed by Mak *et al* (2002), based on observations that in TS cells, Eed and Enx1 localise in a banded manner on the mitotic inactive X chromosome, resembling the previously described *Xist* RNA pattern (Duthie *et al*, 1999). Here, this was further substantiated with the observation that recruitment of Eed-Enx1 complexes occurred extremely rapidly after the onset of X inactivation, in parallel with the accumulating *Xist* RNA, in differentiating XX ES cells. In addition ectopic expression of *Xist* RNA in undifferentiated cells is sufficient to recruit Eed-Enx1 complexes. Thus, it is possible that the *Xist* RNA directly recruits the Eed-Enx1 complexes. Previous work from Denisenko and Bomsztik (1997), using yeast two hybrid screens, have shown an interaction between Eed and the heterogeneous nuclear ribonucleoprotein K (hnRNP K), a classic RNA-binding protein (Burd and Dreyfuss, 1994). This interaction was shown to be important to silence a reporter gene (Denisenko and Bomsztik, 1997). Subsequently, Eed and Enx1 proteins were found to bind RNA (homopolyribonucleotides) *in vitro* (Denisenko *et al*, 1998). These observations also support a model of direct recruitment by *Xist* RNA. Arguing against this however the recruitment of Eed-Enx1 in early mouse embryos occurs at the morula stage (Silva *et al*, 2003) whereas *Xist* RNA accumulation starts earlier, at the 2-4 cell stage (Nesterova *et al*, 2001). Further studies will be required to investigate this.

Another relevant observation was that no detectable Eed or Enx1 concentration was associated with punctate *Xist* signal. This may be attributable to the low level of *Xist* RNA, or alternatively to antisense RNA (*Tsix*) interference, which may block recruitment of the complex. Taken together, these data indicate for the first time that RNA may work in PcG repression function by specifically directing PcG proteins to their target loci.

5.3.2- Functional role of the Eed-Enx1 complex

In female *eed*$^{-/-}$ embryos Enx1 protein does not localise to the inactive X. This result is consistent with *in vitro* analysis demonstrating that the *eed*$^{-}$ mutation disrupts the WD40 domain required for the interaction with Enx1 (Denisenko *et al*, 1998). Thus, in the absence of functional Eed protein, the Enx1 putative histone methyltransferase (HMTase) activity cannot be directed towards specific targets. Therefore, failure to localise Enx1 accounts for the absence of H3-K9/K27 methylation on the inactive X in *eed*$^{-/-}$ embryos. These results are consistent with the finding that *in vitro* purified and recombinant *Drosophila* ESC-E(Z) and mammalian EED-ENX1 complexes, but not E(Z)/ENX1 alone, have histone methyltransferase (HMTase) activity specific to histone H3 (Cao *et al*, 2002; Czermin *et al*, 2002; Muller *et al*, 2002; Kuzmichev *et al*, 2002).

A point of contention concerns the preferred lysine(s) substrate of ESC-E(Z)/Eed-Enx1 complex HMTase activity. In the experiments described above I used an antibody raised against a branched peptide containing a dimethylated lysine (-TAR**K(Me)$_2$**ST-) described on Peters *et al*, (2001) and a highly specific antibody against tri-methylated K27 (Antoine Peters and Thomas Jenuwein, personal communication). Recent data by Dr. Peters and Dr. Jenuwein (personal communication) demonstrates that the former antibody also recognises tri-methylated K9 and tri-methylated K27 while the latter antibody is relatively specific towards tri-methylated K27. Therefore, Eed-Enx1 HMTase activity on the inactive X occurs at the H3-K27 residue but is still unclear whether it also occurs at the H3-K9 residue.

In vitro assays with recombinant ESC-E(Z)/EED-ENX1 complexes and polynucleosomes identified H3-K27 as the preferred substrate of HMTase activity (Cao *et al*, 2002; Muller *et al*, 2002). However, similar assays, using purified ESC-E(Z)/EED-ENX1, showed HMTase activity for both H3-K9 and H3-K27 (Czermin *et al*, 2002; Kuzmichev *et al*, 2002). The differences in the results may be attributed to the usage of distinct ESC-E(Z)/EED-ENX1 complexes (recombinant and purified) or due to unspecific HMTase activity towards H3-K9 in the *in vitro* assays. Together, these

results suggest that Enx1 catalyses tri-methylation (me_3) of H3-K27 *in vivo*, but whether it is also involved in H3-K9 methylation remains an open question.

The fact that *eed* mutant embryos show hypoacetylation of H3-K9 on the inactive X in the majority of cells indicates that Eed-Enx1 does not play a role in targeting Hdacs to specific sites. Consistent with this TSA treatment of cells (150ng/ml) differentiated for 3 days did not disrupt localisation of Eed-Enx1 on the inactive X. It was however striking that TSA treatment results in mislocalisation of Eed-Enx1 to pericentric heterochromatin. Other studies have found that TSA treatment can lead to delocalisation of HP1 protein from pericentric heterochromatin (Tadei *et al*, 2001). This may be important in terms of making centric heterochromatin available for binding of Eed-Enx1 complexes.

5.3.3- Role of H3-K9/K27 methylation in X inactivation

The $eed^{-/-}$ embryo phenotype suggests that H3-K9/K27 methylation on the inactive is required, in the embryo proper, for the stable maintenance of gene silencing and of chromatin modifications, namely H3-K9 hypoacetylation and H3-K4 hypomethylation.

Previous data had already reported the importance of Eed in maintaining the inactive X state (Wang *et al*, 2001). In these studies 7.5 dpc female $eed^{-/-}$ embryos carrying a GFP transgene located on the paternal inherited X (X_p^{GFP}) and a normal inherited maternal X (X_m), exhibit fluorescence in extraembryonic tissues (imprinted X inactivation), in contrast to wild type $eed^{+/+};X_mX_p^{GFP}$. However, 7.5 dpc female $X_mX_p^{GFP}$ $eed^{-/-}$ embryos were found to exhibit an apparently normal mosaic pattern for GFP expression in the embryo proper. This apparent discrepancy with my results for the embryo proper can be accounted for by the assay used. In $eed^{-/-};X_mX_p^{GFP}$ detection of GFP fluorescence cells is facilitated in extraembryonic tissues by the fact that in normal conditions the transgene is always silenced. However, analysis in the embryo proper is complicated because to start with there is mosaic expression of GFP positive and negative cells due to random X inactivation. The fact that in my analysis only a proportion of cells (10-15%) in the $eed^{-/-}$ embryo proper show

failure of maintaining X-linked genes silenced would account for the apparent normal mosaic pattern reported by Wang *et al* (2001). In addition X inactivation is initiated earlier in trophectoderm than in the embryo (3.5 dpc compared with 6.5 dpc), so, reactivation in *eed*$^{-/-}$ trophectoderm has had more time to manifest. If *eed*$^{-/-}$ embryos survived longer, reactivation may be seen in a greater proportion of embryo cells. However, it should also be noted that other mechanisms, for example DNA methylation, may help to stabilise X inactivation to a greater extent in the embryo compared with extraembryonic lineages. This could be consistent with a number of studies demonstrating that DNA methylation of the inactive X does not occur to the same degree in trophectoderm (Sado *et al*, 2000).

Chapter 6: Conclusions

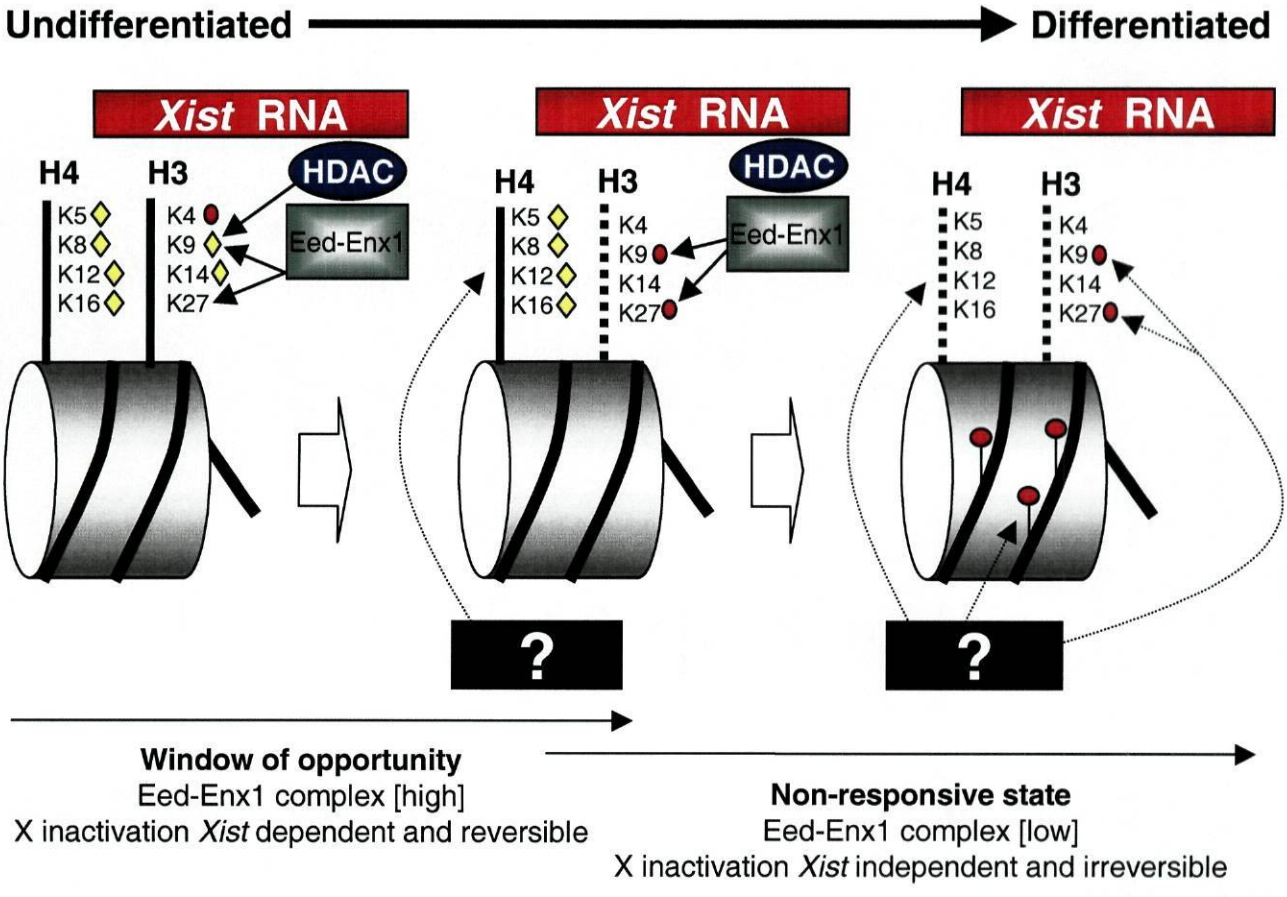
6.1- Summary

This thesis investigates heritable gene silencing mechanisms in embryonic stem cells and differentiated derivatives as a model for embryonic development. First, I determined whether compartmentalisation of repressed genes to heterochromatic domains is a feature of heritable gene silencing in ES cells. The nuclear localisation of the developmentally regulated genes *Oct4*, *Rex1*, and *Dnmt3b* (active in undifferentiated ES cells), and *Cyclin D1* (activated upon differentiation) during ES cell differentiation, showed that whilst silenced alleles have an increased tendency to be associated with pericentric heterochromatin, the effect is relatively weak compared with results reported for lymphocytes. Second, I studied the role of PcG proteins in early stages of the X chromosome inactivation process. This followed previous work where Eed-Enx1 polycomb group complexes were found to associate with the imprinted inactive X in trophoblast stem cells (Mak *et al*, 2002). Here, I demonstrated that association of Eed-Enx1 complexes with the inactive X also occurs in random X inactivation. In both cultured female ES cells and female embryos Eed-Enx1 complexes associate transiently with the inactive X. This occurs at an early stage of the X inactivation process, peaking at days 3 and 4 of ES cell differentiation, but disappearing by day 7. Loss of Eed-Enx1 localisation on the inactive X is accompanied by a diminution of Eed and Enx1 protein levels. The association of Eed-Enx1 complexes with the inactive X is coincident with *Xist* RNA coating and precedes early N-terminal histone modifications. Analysis of an XY ES cell line carrying an *Xist* transgene demonstrated that ectopic *Xist* RNA expression is sufficient to recruit Eed-Enx1 complexes. Analyses of *eed* mutant mice demonstrated that Eed-Enx1 complexes are required to establish H3-K9/K27 methylation on the inactive X. Eed-Enx1 complexes were also implicated in the establishment of H3-K27 methylation genome wide. In addition, H3-K9/K27 methylation on the inactive X is required, in the embryo proper, for the stable maintenance of gene silencing and chromatin modifications, namely H3-K9 hypoacetylation and H3-K4 hypomethylation.

6.2- General discussion

6.2.1- Progress in understanding X inactivation

Figure 6.2.1.1 illustrates a model for X inactivation that is developed based on findings in this thesis. My data clearly support the idea that Eed-Enx1 recruitment is *Xist* dependent. First my results demonstrate that *Xist* RNA is sufficient to recruit Eed-Enx1 complex. Moreover, analysis of female blastocysts carrying a mutant *Xist* RNA allele ($Xist^{inv}$) inherited from the paternal X, which is able to express mutant RNA but fails to induce X inactivation, failed to recruit Eed-Enx1 complexes (Silva *et al*, 2003) (see appendix). In addition, recent studies in the lab have shown that when *Xist* expression from the paternally inherited X switches off in the inner cell mass (prior to random X inactivation), Eed association is rapidly lost (Winifred Mak, personal communication). Despite this evidence for *Xist* dependence the available data probably favour indirect recruitment. First, *Xist* RNA accumulation *in vivo* starts at the 2-4 cell stage (Nesterova *et al*, 2001), whereas recruitment of Eed-Enx1 occurs at the morula stage (Silva *et al*, 2003), despite the presence of high levels of both Eed and Enx1 at the 2-4 cell stage (Winifred Mak, personal communication). Second, Eed-Enx1 complexes are not required to establish X inactivation in $eed^{-/-}$ embryos, suggesting that other factors are primarily responsible for setting up chromosome silencing. These factors are probably the direct target of the A-repeat silencing element located at the 5' end of *Xist* RNA (Wutz *et al*, 2002). However, I cannot rule out a direct association of Eed-Enx1 with *Xist*, mediated either by the A repeats or some other region of *Xist* RNA. This can be further investigated by *in vitro* analysis, looking at whether *Xist* RNA domains can bind to Eed-Enx1 complexes purified from cell extracts.



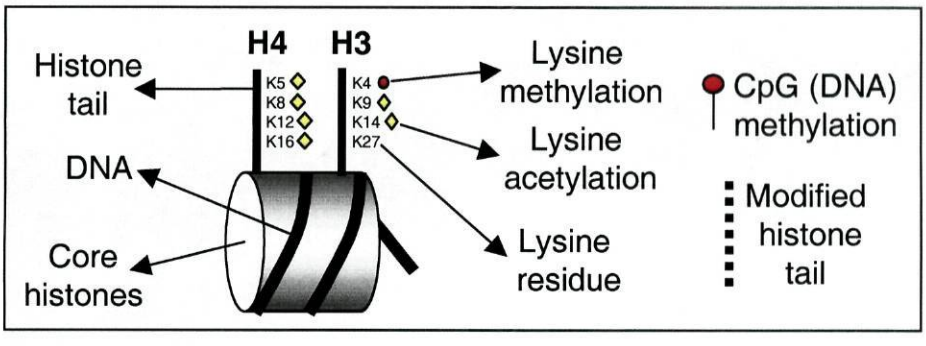


Figure 6.2.1.1 - **Model to explain the role of Eed-Enx1 complexes in X inactivation**
Figure illustrates a nucleosome on the inactivating X chromosome. *Xist* RNA recruits Hdac and Eed-Enx1 complexes, leading to H3-K9 deacetylation, H3-K9/K27 methylation and loss of methylation at H3-K4. The resultant change in histone H3 tail is proposed to represent a primary level of chromatin silencing that is *Xist* dependent. Subsequent silencing factors (indicated by question mark black box), probably in response to the primary changes, induce further chromatin modifications in a *Xist* RNA independent manner. These are represented by hypoacetylation of histone H4 tails and DNA methylation at CpG islands. Taken into account the sequence of events described for ES cells, the latter modification should participate in the irreversible chromosomal silencing. Subsequent silencing factors may also be involved in the maintenance of H3-K9/K27 methylation. See text for further details.

Analysis of *eed*$^{-/-}$ mice showed that the inactive X is hypoacetylated at H3-K9, which implies that Hdac(s) proteins are in place at the X chromosome without the presence of Eed and Enx1 proteins. Based on this I suggest that the primary target for *Xist* RNA is a histone deacetylase complex with activity towards H3-K9 (Figure 6.2.1.1). It is known that only cells representative of early developmental stages can induce silencing in response to *Xist* (Wutz and Jaenisch, 2000). It follows that the putative Hdac complex is either unavailable later in development, or alternatively that a developmentally regulated cofactor is required for the interaction with *Xist*.

An interesting possibility, illustrated in Figure 6.2.1.1, is that the Eed-Enx1 complex is a module of this complex such that recruitment of the Hdac by *Xist* RNA leads then to recruitment of Eed-Enx1. In support of this histone deacetylases have been reported to be associated with ESC-E(Z)/EED-ENX1 complexes (Tie *et al*, 2001; Czermin *et al*, 2002; Kuzmichev *et al*, 2002; Furuyama *et al*, 2003). Interestingly, Furuyama *et al* (2003) suggested that the entire ESC-E(Z) complex is likely to be a subset of a larger complex distinct from PRC1. This was based on the fact that freshly prepared *Drosophila* embryonic extracts compared to the same extracts stored at 4^0C overnight, have increased high molecular weight ESC-E(Z) complex detected compared to the aged extracts. Moreover, the presence of histone deacetylase (RPD3) was disproportionately represented in the larger complex. In future studies it will be important to analyse early mouse embryos/ES cells to determine if such a supercomplex may be present.

To summarise so far, the model proposes that during the *Xist* responsive stage of development, *Xist* RNA recruits a supercomplex capable of deacetylating H3-K9 (initiating chromosome silencing), and methylating H3-K9/K27 (stabilising inactive X chromatin). Taking into account the work of Wutz and Jaenisch (2000), in undifferentiated and early differentiated ES cells the *Xist* mediated chromosomal silencing is reversible. Assuming that my analysis of Lh1 cells (XY ES cell line carrying an *Xist* transgene) reflect the same situation described by Wutz and Jaenisch (2000), it can be said that H3-K9 hypoacetylation, H3-K9/K27 methylation and histone H4 hypoacetylation are all

Xist dependent and reversible. The presence of chromosomal histone H4 hypoacetylation is at variance with the results of Wutz and Jaenisch (2000). This can be accounted for by the use of different antisera; Wutz and Jaenisch (2000) used antisera specific to histone H4 acetylated at lysine 12 and I used antisera raised against a conjugated peptide acetylated at lysines 5, 8, 12 and 16 that specifically recognises acetylated histone H4. It is therefore possible that the latter antisera have increased sensitivity in relation to the former. Alternatively, because *Xist* expression in Lh1 cells is constitutive and this cell line has been kept for a number of passages it is possible that X inactivation may become gradually irreversible, and that Wutz and Jaenisch (2000) did not express their inducible *Xist* transgene for long enough to see this. This could be tested by expressing an inducible *Xist* transgene for several passages, i.e., for longer than 6 days as described in Wutz and Jaenisch studies (2000), and analysing after cessation of transgene expression whether the inactive chromosome still revert to an active state. Assuming that X inactivation in Lh1 cells is reversible, it can be said that the described histone modifications are not sufficient to effect *Xist* independent and irreversible gene silencing.

At late differentiation stages the Eed-Enx1 localisation to the inactive X is undetectable, raising the question of how histone H3-K9/K27 methylation is maintained. Similarly maintenance of H3-K9 methylation and other modifications is at later developmental stages no longer *Xist* dependent and is largely irreversible. To explain this I suggest that there are different complexes required for the maintenance of these modifications and that they are recruited by the underlying chromatin modifications established early in development, rather than on *Xist* RNA per se (Figure 6.2.1.1). A key requirement of this model, to account for the fact that X inactivation becomes *Xist* independent, is that chromatin silencing catalysed by these complexes is self-maintaining through successive cell divisions. Also, the maintenance machinery would have to be developmentally regulated to explain the fact that *Xist* independent and irreversible chromosomal silencing only occurs after differentiation (Wutz and Jaenisch, 2000). An interesting candidate for a role in this process is DNA methylation. DNA methylation is a relatively late event in the in the X inactivation process

(Keohane *et al*, 1996). Targeted mutagenesis of the DNA methyltransferase I (Dnmt1) enzyme demonstrated that it is necessary for the maintenance of the inactive X in random X inactivation (Sado *et al*, 2000). 8.5 dpc *Dnmt1* mutant female embryos, carrying an X linked lacZ transgene were found to have lacZ expression in around 50% of the cells, due to random X inactivation, and silencing of the lacZ transgene on the inactive X. However, 9.5 dpc *Dnmt1* mutant embryos exhibited a considerable increase in the number of lacZ positive cells. This dependence on Dnmt1 interestingly becomes apparent when Eed-Enx1 association with the inactive X is being lost. Thus, DNA methylation may function in the maintenance of X inactivation by recruiting enzyme complexes with Hdac and HMTase activity. Indeed it has been shown that Mecp2, a methylcytosine binding protein, recruits the NuRD complex, containing type I Hdacs, to methylated DNA (Zhang *et al*, 1999). Also Dnmt1 can interact with Hdacs (Fuks *et al*, 2000) and HMTases (Fuks *et al*, 2003; Datta *et al*, 2003). As in the presence of Dnmt1 DNA methylation is self-maintaining through successive cell divisions, this could explain maintenance of X inactivation late in development.

H3-K9/K27 methylation on the inactive X is maintained throughout development, despite loss of Eed-Enx1 localisation (AHFP Peters, T. Jenuwein, personal communication). There are two possible explanations. First, the Eed-Enx1 complexes might still localise to the inactive X but at a level that is not detectable by immunofluorescence. This seems unlikely in light of the dramatic disappearance of Eed protein detected by western blotting during ES cell development. Similarly in *Drosophila* ESC protein is only required early in development to establish silencing (Struhl and Brower 1982; Simon *et al*, 1995). Second, maintenance of H3-K9/K27 methylation patterns might be carried out by another HMTase complex. The protein G9a is a good candidate as *in vitro* studies showed that it has HMTase activity towards H3-K9 and K27 (Tachibana *et al*, 2001). In addition, *G9a* deficient mice stop developing at 9.5 dpc exhibiting reduced H3-K9 methylation compared to wild type embryos (Tachibana *et al*, 2002). Alternatively maintenance of methylated H3-K9/K27 may be carried out by an unidentified HMTase. A final possibility is that Enx1 protein is responsible for maintenance of H3-K9/K27 methylation but as part of a different complex. This idea

comes from a number of observations. In *Drosophila*, E(Z) (Enx1) protein is required for maintaining silencing in adult stages (Jones and Gelbart, 1990), contrasting with ESC (Eed). Moreover, a recent study has shown that in adult stages E(Z) is part of a different complex that does not include ESC (Furuyama *et al*, 2003). Also, unlike Eed, Enx1 protein is only partially shut down during ES cell differentiation (Silva *et al*, 2003). Finally, a recent study has shown that K27 methylation is markedly reduced following conditional deletion of Enx1 in mouse pre-B cells (Su *et al*, 2002).

6.2.2- Parallel with constitutive heterochromatin silencing

Constitutive heterochromatin (pericentric heterochromatin) and facultative heterochromatin (developmentally regulated heterochromatin) have many parallels. Higher order structure of pericentric heterochromatin requires the action of HMTases (SET domain containing proteins), Suv39h1 and Suv39h2, which methylate histone H3 at lysine 9 (Peters *et al*, 2001). This mark was shown to provide a binding site for the chromodomain protein HP1 (Bannister *et al*, 2001; Jacobs *et al*, 2001; Lachner *et al*, 2001, Nielsen *et al*, 2001). This pathway is highly conserved, occurring in *S. Pombe* (yeast), *Drosophila* (fly) and mammals (Bannister *et al*, 2001; Jacobs *et al*, 2001; Lachner *et al*, 2001, Nielsen *et al*, 2001). This is thought to confer stability to the modified chromatin state. Given the occurrence of K9/K27 methylation on the inactive X it is interesting to consider if a chromodomain containing protein may also be involved in the X inactivation process. Recent *in vitro* data demonstrated that E(Z)/ENX1 HMTase activity, methylation of H3-K9/K27, provides a binding mark for *Drosophila* PC homologues, which are chromodomain containing proteins (Cao *et al*, 2002; Czermin *et al*, 2002; Kuzmichev *et al*, 2002). This in turn could provide a way to target the PRC1 complex to ESC-E(Z)/Eed-Enx1 targets. However, there is no evidence that the same occurs in X inactivation. In fact, the murine PC homologue MPc2 and other PRC1 proteins failed to localise to the inactive X in differentiated female ES cells (see section 3.2.4). However, it is possible that under the conditions used antisera to MPc2 did not work efficiently. Also, there is other PC homologues in mammalian cells, like M33, which was not analysed. Thus, the putative involvement

of PRC1 in X inactivation should not be ruled out. Further analyses of localisation of PRC1 proteins on the inactive X will be important, especially because of the observed localisation of MPh1, a PRC1 protein, to the inactive X in a proportion of cells. Related to this it will be interesting to look at X inactivation in mice with PRC1 protein mutations, in particular for MPh1.

In a further parallel with X inactivation, pericentric heterochromatin also has and RNA component (Maison *et al*, 2002). Moreover, the RNA interference (RNAi) machinery, was recently demonstrated to be involved in the initiation of H3-K9 methylation and pericentric heterochromatin formation in yeast (*S. pombe*) and plants (*Tetrahymena thermophila*) (Hall *et al*, 2002; Mochizuki *et al*, 2002; Reinhart and Bartel, 2002; Volpe *et al*, 2002). Previous work showed that the RNA machinery is composed of a group of proteins that in response to double-stranded RNA (dsRNA) trigger the nuclear production of small RNAs, which in turn destroys target mRNA (reviewed in Jenuwein 2002). Interestingly, the *C. elegans* MES-6 protein, ESC/Eed homologue, was identified as being part of the RNAi machinery (Dudley *et al*, 2002). Because MES-6 localises to chromatin domains, it is likely that the RNAi machinery both degrades mRNA and silences the targeted locus (Dudley *et al*, 2002). The latter is also supported by the fact that the RNAi machinery, as described above, is involved in chromatin modification. Since this is a widespread silencing mechanism (from yeast to mammals), it is conceivable that the RNAi machinery, mediated by PcG proteins, may also participate in the X inactivation process. However, this remains to be demonstrated.

6.2.3- Implications for understanding gene regulation in development

Stem cells are thought to have a more open chromatin state in comparison to its differentiated derivatives in order to retain the capacity to differentiate along many distinct lineage pathways. One idea tested here was whether gene silencing, accompanied with ES cell differentiation, resulted in the increased recruitment of silent genes to heterochromatic compartments (pericentric heterochromatin). Only a relatively weak effect was seen for the genes studied. This could reflect a difference in stem cells of the early embryo where the mechanisms of silencing would be more

relaxed to allow for different cell fate decisions to be executed. However, as stated earlier, this could also reflect the chromosome environment of the selected genes and clearly more work is needed to investigate this.

Relating to the issue of silencing mechanisms in ES cells it is very interesting that high levels of Eed-Enx1 complexes, which are associated with K27 methylation, are seen in ES cells. In this context it is interesting to note that high levels of Eed and Enx1 fall during embryonic development, trophoblast stem cell differentiation (Silva *et al*, 2003) and also during B cell lineage differentiation and maturation (Su *et al*, 2003). This developmental regulation is also mirrored in other organisms: *Drosophila* ESC and E(Z) proteins are present at high levels in embryos and at a much reduced level in larvae and pupae (later developmental stages) (Ng *et al*, 2000). The presence of *C. elegans* (Worm) MES-6 protein, which is the ESC homologue, is restricted to early embryos and to the germline of larvae and adults (Korf *et al*, 1998). The *Arabidopsis* (plant) fertilization-independent-endosperm (FIE) and Medea (MEA), homologues of ESC and E(Z) respectively, control seed development (Yadegari *et al*, 2000) and are expressed in the female gametophyte before pollination and in the developing endosperm after pollination (Luo *et al*, 2000). These observations therefore present a paradox in that high levels of a mark associated with gene silencing are found in cells that supposedly have an open chromatin configuration. A possible explanation is that the Eed-Enx1 complex mediated repression induces reversible gene silencing that only becomes stable and self-maintaining upon subsequent development. This would provide totipotent or pluripotent cells with a relatively plastic system with which to define cell identity commitment but prior to determination. Cell determination may be carried out by factors like MPC2 and Bmi1 PcG proteins, which showed highest levels at later differentiation time points (Figures 3.2.3.1E and F). This of course resembles the model for X inactivation where silencing early in development is *Xist* dependent and reversible, and later in development becomes irreversible and self-maintaining.

It should be noted that in contrast to ESC, E(Z) is still required late in *Drosophila* development (Jones and Gelbart, 1990). This is consistent with the expression profile found for E(Z) in *Drosophila* (Ng *et al*, 2000) and for Enx1 in this thesis studies, where E(Z)/Enx1 expression at later developmental stages is much reduced but not completely abolished. Moreover, E(Z) is found with a novel complex, distinct from the ESC-E(Z), at later *Drosophila* developmental stages (larval) (Furuyama *et al*, 2003). Thus, the transition from embryonic ESC-E(Z) complexes to the larval E(Z) complex may reflect developmentally important functional differences. Possibly related to this, the E(Z)/Enx1/ENX1 protein was shown to have a role in cell proliferation, in addition to homeotic gene regulation, although, the connection with cell cycle genes is not clear (Gatti and Bake, 1989; O'Carrol *et al*, 2001; Visser *et al*, 2001 Varambally *et al*, 2002).

In conclusion, high levels of Eed-Enx1 complexes are inversely correlated with the progress of development and cell differentiation. The role of these complexes is consistent with the definition, but not with the determination, of pluripotent cell fates. This makes genes and genomes still amenable to changes (more plastic), to respond to developmental signals and to possess a high degree of pluripotency. Thus, the identification of the Eed-Enx1 complex targets may shed light on genes regulating the pluripotent state *versus* differentiation and, cell lineage commitment prior to determination *versus* determination and maintenance. One way to identify such candidate genes could be through microarray technology, comparing the expression profiles of undifferentiated or early differentiated ES cells treated by RNAi against components of the Eed-Enx1 complex (Eed or Enx1) with the same cells treated with unrelated control RNAi oligonucleotides. The choice of these cell stages is crucial as the Eed-Enx1 complex is likely not to have a functional role in late differentiated and in somatic cells.

6.2.4- Relation between the EED-ENX1 complex and disease

During the course of this work a report has demonstrated, for the first time, that *XIST* mediated silencing can also occur in somatic cells (XY human fibrosarcoma HT-180 cell line) (Hall *et al*,

2002). A cell clone derived from HT-180 cells carrying an XIST transgene was shown to express ectopic *XIST* which displayed proper chromosomal localisation. Importantly, these cells exhibited a Barr body (large intense DAPI staining focus), which was co-localising with ectopic *XIST* RNA. Moreover, the chromosome was found to exhibit H4 hypoacetylation, late replication and transcription silencing. Consequently, this finding challenges the previously defined window of time where stable *Xist* RNA is able to trigger chromosome silencing (Brown and Willard, 1994; Clemson *et al*, 1998; Wutz and Jaenisch, 2000). Two possible interpretations can be drawn from this: First, some somatic cell lines may retain *Xist* mediated silencing capacity. Second, factors recruited by *Xist*, including the Eed-Enx1 complex, may be abnormally expressed in somatic tumourogenic cells.

Supporting the latter idea ENX1 protein was shown to be overexpressed in metastic human prostate cancer cells (Varambally *et al*, 2002). Small interfering RNA (siRNA) duplexes targeted against the *ENX1* transcript reduced the amounts of ENX1 protein and also inhibited cell proliferation *in vitro* (Varambally *et al*, 2002). Transfection of RWPE prostate cells with wild type ENX1 was shown to induce the repression of a group of genes. These included transcriptional regulators, solute carriers, a cell cycle regulator (*Cdc27*) and also a PcG gene (*Enhancer of polycomb 1* (*EPC1*)). Moreover, the ENX1 mediated gene silencing was dependent on HDAC activity, because the HDAC inhibitor trichostatin A (TSA) abolished the effects of ENX1. Since HDAC interacts with the EED-ENX1 complex through EED (Van der Vlag and Otte, 1999) it is likely that the ENX1 role in prostate cancer is mediated by the EED-ENX1 complex.

A further link between Eed-Enx1 and tumourogenesis is that the recently identified PcG group gene *SU(Z)12* (Birve *et al*, 2001), which was also shown to be a component of the ESC-E(Z)/EED-ENX1 complex (Cao *et al*, 2002; Czermin *et al*, 2002; Muller *et al*, 2002; Kuzmichev *et al*, 2002), was found to be up-regulated in a number of different human tumours, including tumours of the colon, breast, and liver (Kirmizis *et al*, 2003). Together, these data suggest that the EED-ENX1 complex, which is overexpressed in cancer cells, is likely to participate in a crucial checkpoint that controls

cell proliferation. Speculating further, the EED-ENX1 complex may induce tumourogenicity by reprogramming somatic cells into pluripotent like cells, which are capable of self-perpetuation and long term proliferation. To investigate this, analysis of transgenic non-tumourogenic somatic cells carrying constitutively expressing high levels of Eed-Enx1 complex components would be of great interest. In addition, microarray analysis comparing transgenic somatic cells carrying constitutively expressing Eed-Enx1 components with the same cells carrying mutated versions of Eed-Enx1 complex components, would identify genes that are targeted by the complex. However, the exact composition of the Eed-Enx1 complex is not known yet. So, efforts should be placed on the full characterisation of the Eed-Enx1 complex components before proceeding to transgenic studies in somatic cells.

In conclusion these advances in tackling the memory system open fascinating possibilities which could lead to the better understanding of disease and perhaps to the efficient reprogram of terminally differentiated cells. If that becomes a reality the impact for medical purposes would be enormous.

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Mitotically Stable Association of Polycomb Group Proteins Eed and Enx1 with the Inactive X Chromosome in Trophoblast Stem Cells

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Summary

X inactivation in female mammals is one of the best studied examples of heritable gene silencing and provides an important model for studying maintenance of patterns of gene expression during differentiation and development [1]. The process is initiated by a *cis*-acting RNA, the X inactive specific transcript (*Xist*). *Xist* RNA is thought to recruit silencing complexes to the inactive X, which then serve to establish and maintain the inactive state in all subsequent cell divisions [2–4]. Most lineages undergo random X inactivation, there being an equal probability of either the maternally (Xm) or paternally (Xp) inherited X chromosome being inactivated in a given cell [5]. In the extraembryonic trophectoderm and primitive endoderm lineages of mouse embryos, however, there is imprinted X inactivation of Xp [6]. This process is also *Xist* dependent [7]. A recent study has shown that imprinted X inactivation in trophectoderm is not maintained in embryonic ectoderm development (*eed*) mutant mice [8]. Here we show that Eed and a second Polycomb group protein, Enx1, are directly localized to the inactive X chromosome in XX trophoblast stem (TS) cells. The association of Eed/Enx1 complexes is mitotically stable, suggesting a mechanism for the maintenance of imprinted X inactivation in these cells.

Results and Discussion

We established trophoblast stem (TS) cell lines from mouse blastocysts to provide an in vitro model system to analyze imprinted X chromosome inactivation. Crosses were set up between (C57Bl6×CBA)F1 females and PGK strain males. The use of PGK strain allows predefined polymorphisms on the X chromosome to be used to distinguish maternal (Xm) and paternal (Xp) alleles of selected X-linked genes [9, 10]. Blastocysts were isolated at 3.5 days post coitum (dpc), and TS cell lines were derived as described by Tanaka et al. [11]. Initially, we derived three cell lines. PCR analysis using primers for the X- and Y-linked *Ube1X* and *Ube1Y1* loci was carried out to determine the sex chromosome constitution of cell lines, and an *Xist* polymorphism between PGK strain and laboratory strain mice was used to confirm the presence of two X chromosomes in putative XX cell lines (data not shown). Two TS cell lines (A2 and B1) were XX, and a single cell line (B7) was XY.

To determine X inactivation patterns in XX TS cells, we first analyzed expression of the *Xist* gene by RNA FISH. The results demonstrated that *Xist* RNA in B1 XX TS cells localizes to a single nuclear domain (Figure 1A). This pattern is indistinguishable from what is seen in other differentiated XX cell types (for example, see [3]). Allele-specific single-nucleotide primer extension (SNuPE) analysis demonstrated that *Xist* expression in the B1 XX TS cell line is predominantly from the Xp allele, whereas expression of the X-linked *Pgk-1* gene is exclusively from the Xm allele (Figures 1B and 1C). A low level of Xm *Xist* expression was detected in these experiments, although, as stated above, this does not lead to silencing of the Xm *Pgk-1* allele (Figure 1C). A possible explanation is that repression of Xm *Xist* is not rigidly maintained in TS cell cultures. Similar data were obtained for the second XX TS cell line, A2 (data not shown). Taken together, these results demonstrate that XX TS cells have undergone paternally imprinted X inactivation.

The embryonic ectoderm development (Eed) protein is the mouse homolog of the *Drosophila melanogaster* Polycomb group protein extra sex combs [12]. A recent study demonstrated that mice homozygous for mutations in the *eed* gene fail to maintain imprinted X inactivation in the trophectoderm lineage [8]. Formally, this could be due to a direct interaction of Eed protein with the inactive X or an indirect effect on a gene product required for maintenance of X inactivation. To investigate this, we carried out immunofluorescence analysis on TS cells, using a rabbit polyclonal antibody raised against human EED protein. As illustrated in Figure 2A, the analysis revealed a single large focus of Eed protein in the nucleus of XX TS cells but not in XY TS cells (compare Figures 2A and 2B). This finding provides indirect evidence that Eed protein is recruited to the inactive X chromosome in TS cells. It should be noted that there is a more generalized Eed protein localization comprising small foci or diffuse signal, both in XX and XY TS cells (see Figures 2A and 2B). This latter pattern has been observed in lymphocytes and in other cell types (J.B., unpublished data).

The Eed protein (or its direct homolog in other species) has been shown to be present in a complex with a second Polycomb group protein, Enx1 (Enx1 is the mouse homolog of the enhancer of Zeste protein in *D. melanogaster* [13, 14]). We therefore went on to analyze TS cells with an Enx1-specific rabbit polyclonal antibody. Again, we observed a single large focus in XX TS cells (Figure 2C). To test if the Eed and Enx1 signals

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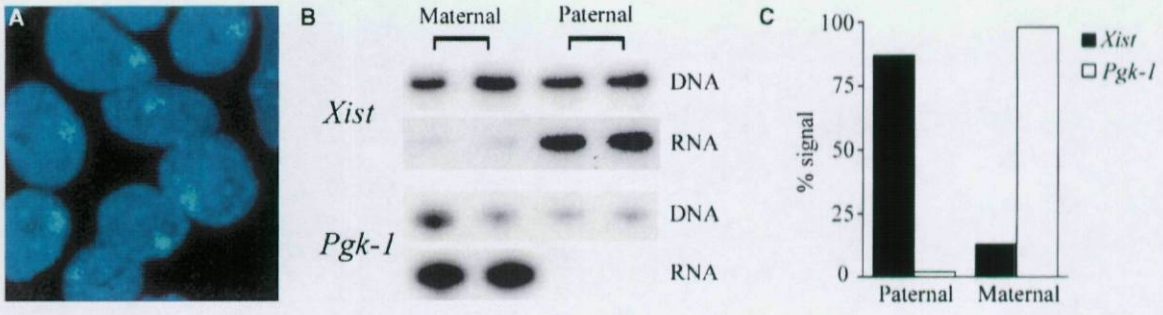


Figure 1. X Inactivation in TS Cells

(A) Detection of *Xist* RNA (green) in B1 XX TS cells by FISH using pEFB probe. DNA was counterstained with DAPI.

(B) Illustration of SNuPE analysis of *Xist* and *Pgk-1* loci in B1 XX TS cells. SNuPE reactions were carried out on PCR products for *Xist* and *Pgk-1* loci using primers specific for cDNA (RNA). Genomic DNA from (PGK×129)F1 female animals (DNA) provided a control. Duplicate loadings of labeled primer from reactions specific for maternal and paternal alleles are shown.

(C) Quantitation of SNuPE data shown in (B), illustrating percentage of signal corresponding to maternal and paternal alleles. The data were normalized relative to control SNuPE reactions from the (PGK×129)F1 female genomic DNA.

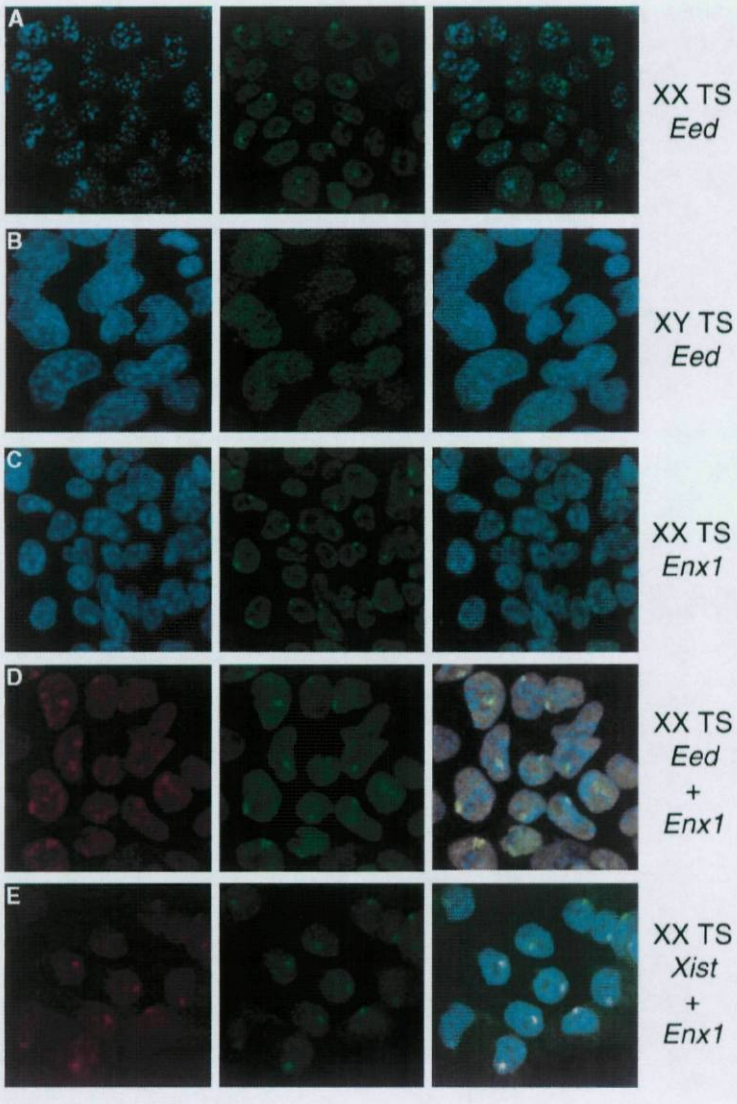


Figure 2. Localization of Polycomb Group Proteins Eed and Enx1 in TS Cells

(A) Indirect immunofluorescence on XX TS cells using antisera to Eed protein. Illustrated are DNA counterstaining with DAPI (left), FITC detection of Eed protein (center), and merged image (right).

(B) Analysis of B7 XY TS cells as in (A) above.

(C) Indirect immunofluorescence on XX TS cells using antisera to Enx1. Panels as in (A) and (B) above.

(D) Double labeling of Eed and Enx1 in XX TS cells. Illustrated are Texas red detection of the Eed mouse monoclonal antibody (left) and FITC detection of antibody to Enx1 (center). The merged image (right) includes DAPI DNA counterstain.

(E) Immuno-FISH labeling of *Xist* RNA and Enx1 protein in XX TS cells. Illustrated are Texas red detection of *Xist* RNA with pEFB probe (left) and FITC detection of antibody to Enx1 protein (center). The merged image (right) also shows DAPI DNA counterstain.

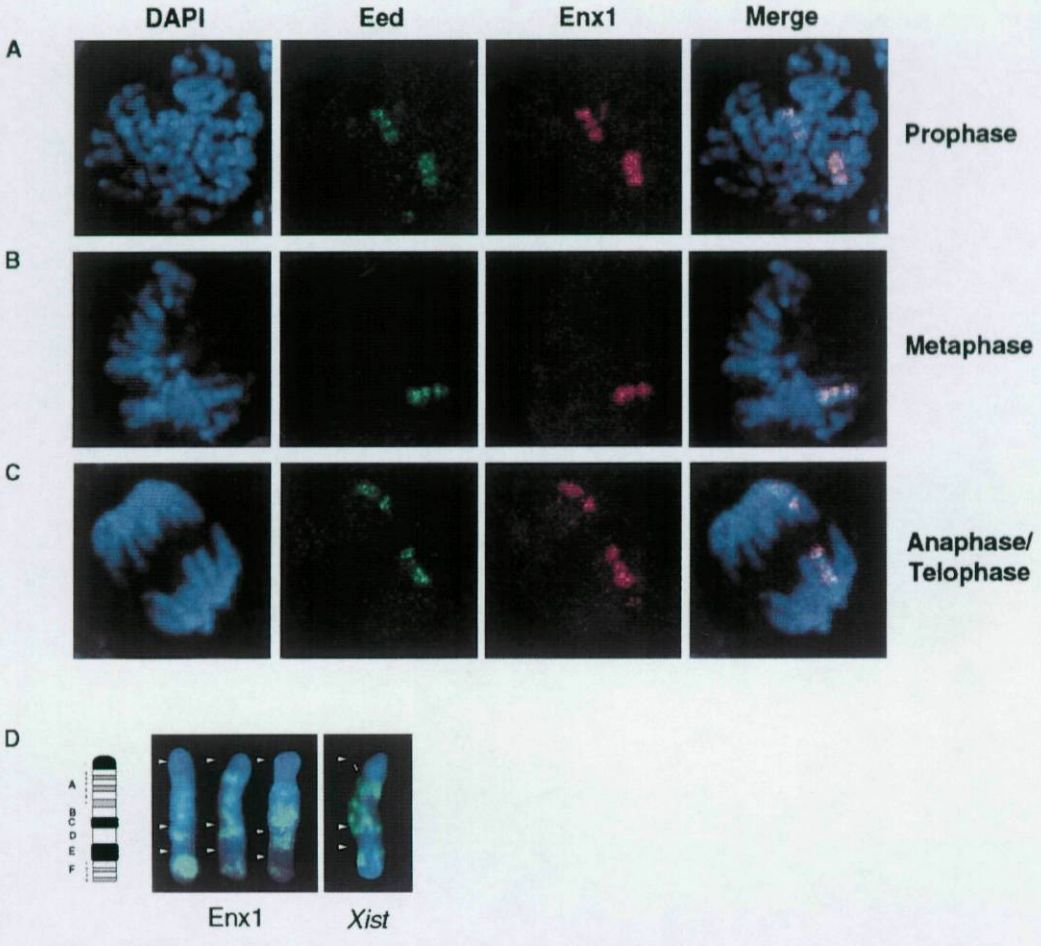


Figure 3. X Chromosomal Localization of Eed/Enx1 Proteins during Mitosis in TS Cells

(A–C) Indirect immunofluorescence of Eed (green) and Enx1 (red) at different stages of mitosis, as indicated. DNA is counterstained with DAPI (blue).

(D) High-resolution images of metaphase X chromosomes showing examples of the inactive X chromosome localization pattern for Eed protein. The ideogram to the left illustrates the Giemsa banding pattern for the mouse X chromosome. Arrowheads indicate exclusion of Eed from the Giemsa dark pericentric heterochromatin band A1 and from regions correlating with the location of the major Giemsa-dark bands C and E. For comparison, an example of *Xist* RNA localization is shown on the right. Eed and *Xist* RNA are detected with FITC (green), and DNA is counterstained with DAPI.

colocalize, we carried out double-labeling experiments with Enx1 polyclonal antiserum and a mouse monoclonal antibody specific for Eed protein. The result, illustrated in Figure 2D, demonstrated clear colocalization of the large nuclear foci for Eed and Enx1 proteins.

To prove that Eed/Enx1 proteins are localized to the inactive X chromosome, we carried out a double-labeling experiment detecting Enx1 protein and *Xist* RNA. The result, illustrated in Figure 2E, demonstrates that the large nuclear focus of Enx1 proteins does indeed correspond to the inactive X chromosome. Thus, Eed/Enx1 complexes are associated with the inactive X chromosome in XX TS cells.

In the course of analyzing the XX TS cells, we observed that the Eed/Enx1 signal on the inactive X chromosome is retained at mitosis. We examined this more carefully, identifying a number of cells in different stages of mitosis and then assessing localization of Eed and Enx1 proteins. Examples are shown in Figure 3. Staining of a distinct chromosome was seen at prophase (Figure 3A) and at metaphase (Figure 3B). The example shown in Figure 3A is of a tetraploid cell in which there are two inactive X chromosomes (increased ploidy occurs relatively frequently in TS cell cultures, as a consequence of endoreduplication which occurs when TS cells differentiate into trophoblast giant cells [11]). The localization of Eed/Enx1 complexes on the inactive X is retained on individual sister chromatids at anaphase and at telophase (Figure 3C). As genetic studies demonstrate a requirement for Eed protein in the maintenance of X inactivation in trophectoderm cells [11], this observation provides a first clear example of retention of Polycomb group proteins at known target loci throughout mitosis.

The examples shown in Figures 3A–3C illustrate that Eed/Enx1 proteins are distributed in a nonlinear banded localization rather than homogeneously over the entire

length of the chromosome. This pattern is highly reproducible and is similar to the banded localization of *Xist* RNA observed previously [15]. To investigate this further, we obtained high-resolution images of Eed localization on metaphase chromosomes (Figure 3D). As previously reported for *Xist* RNA, Eed appears to be excluded from pericentromeric heterochromatin and also from regions that correlate with the location of major Giemsa-dark bands. Based on this, we speculate that Eed/Enx1 complexes could be a direct target for recruitment by *Xist* RNA.

Biochemical analysis has higlighted several interactions between Eed/Enx1 complexes and other proteins. We tested antisera to a number of these proteins: HDACs 1-3 [16, 17]; YY1 [18, 19]; and Bmi1, HPC1, M33, and RING1a [13, 16, 20] (the latter group of proteins belongs to a biochemically distinct Polycomb group complex; recent data, however, have demonstrated a transient interaction between proteins in this complex and the Eed/Enx1 complex early in *D. melanogaster* embryogenesis [19]). All antisera detected specific nuclear signals, but in no case did we observe inactive X chromosome specific foci in XX TS cells (data not shown). While we cannot rule out the possibility that technical factors preclude detection of these epitopes at all sites in our experiments, the results indicate that involvement of Eed/Enx1 complexes in X inactivation in TS cells relies on previously uncharacterized biochemical interactions.

Analysis of *eed* mutant mice indicated that there is no detectable defect in the maintenance of random X inactivation in the embryo proper [8]. Consistent with this, we have not observed Eed/Enx1 localization on the X chromosome in XX somatic cell lines, both primary and transformed (data not shown). Therefore, utilization of Eed/Enx1 in X inactivation may be lineage specific. In addition to trophectoderm cells, imprinted X inactivation also occurs in the extraembryonic primitive endoderm lineage in mouse [6] and in all tissues of marsupial mammals [21]. It will be of interest to determine if Eed/Enx1 complexes are involved in these instances. As imprinted X inactivation is thought to represent the evolutionary progenitor of random X inactivation, a role for Eed/Enx1 Polycomb group proteins would be consistent with their high degree of evolutionary conservation and involvement in heritable gene silencing. An alternative interpretation is that Eed/Enx1 involvement in X inactivation is developmentally regulated. The trophectoderm is the first lineage to differentiate, and Eed/Enx1 association with the inactive X chromosome in TS cells may reflect this. Further analysis of Eed/Enx1 in different lineages during early development will therefore be important.

The Polycomb group proteins were initially identified in genetic screens to identify factors required for heritable silencing of homeobox genes during development. To date, all known members of this group have been shown to be chromatin associated. Heritable silencing must be preserved through both DNA replication during S phase and also throughout mitosis. This could be achieved either directly by continued association of Polycomb group proteins during these processes or indirectly via a histone or DNA modification imposed by enzymatic activity of Polycomb group complexes. Our results demonstrate that Eed/Enx1 complexes remain bound to the inactive X chromosome throughout mitosis in TS cells, indicating, in this situation at least, that heritability may be achieved by a direct mechanism. It is however possible that an indirect effect also plays a role. The Enx1 protein contains a SET domain, a motif that in other proteins has been shown to correspond to the catalytic domain of a histone H3 lysine 9 (H3-K9) methyltransferase [22, 23]. Recent evidence has shown that H3-K9 methylation occurs rapidly at the onset of random X inactivation, although the enzyme responsible for this activity is uncharacterized [24–27]. It is interesting to consider that in trophectoderm cells this function could be carried out by Eed/Enx1 complexes.

Experimental Procedures

Cell Culture

Trophoblast stem (TS) cells were derived from 3.5 dpc blastocysts obtained from crosses between (C57B16×CBA)F1 females and PGK strain males, essentially as described by Tanaka et al. [11]. *Xist* and *Pgk-1* single-nucleotide primer extension (SnuPE) assay was carried out using cDNA from TS cell lines and primers and conditions as described previously [9, 10].

Immunofluorescence

Characterization of Polycomb group protein antibodies has been described previously [13, 16, 28]. Antibodies for HDAC1-3 and YY1 proteins were from Santa Cruz Biotechnology, Inc. Secondary antibodies were obtained from Molecular Probes. All antibody dilutions were carried out in 5% normal goat serum.

TS cells were plated onto glass slides and cultured for 2 days on conditioned medium. The cells were then washed with PBS, fixed in 2% paraformaldehyde for 15 min, quenched with 50 mM NH_4Cl solution for 10 min, and permeabilized with 0.4% Triton X-100 for 5 min (three PBS washes between each step). Cells were then incubated in blocking solution (0.2% fish gelatin [Sigma] in PBS) for 30 min and primary antibody for 1 hr in a humidified chamber. After three consecutive 3 min washes in blocking solution, cells were incubated for a further 30 min with secondary antibody. The cells were washed again with blocking solution (three times), once with PBS only, and then mounted in Vectashield antifade (Vector Laboratories), containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI).

For RNA FISH, the TS cells were prepared for slides as detailed above. RNA FISH was carried out as previously described [29]. For high-resolution analysis of metaphase chromosomes, RNA FISH was performed as described by Duthie et al. [15]. *Xist* RNA was detected with the DNA plasmid probe pEFB containing a 3 kb BamH1 fragment from exon 1 labeled with digoxigenin. For immuno-RNA FISH, RNA FISH was performed first, followed by immunostaining for Enx1. The cells were permeabilized for 3 min in the Triton X-100/cytoskeletal (CSK) buffer and fixed in 4% paraformaldehyde for 10 min (all solutions on ice). Hybridization and washes were done in the usual way except that wash solutions were heated to 42°C. The *Xist* probe detection was performed first. Slides were then washed three times with 4XSSC/0.1% Tween 20 (37°C) and incubated in blocking buffer (4XSSC/0.1% Tween 20/4% BSA) for 30 min at 37°C in a humidified chamber. Enx1 antibody diluted in detection buffer (4XSSC/0.1% Tween 20/1% BSA) was applied and incubated for 1 hr in a humidified chamber at 37°C. The slides were washed three times with 4XSSC/0.1% Tween 20 at 37°C and blocked with 5% NGS in detection buffer for 30 min at 37°C. The slides were then incubated with secondary antibody for a further 30 min. Finally, the slides were washed and mounted in DAPI-Vectashield.

Confocal images were collected using a Leica TCS-Sp1 confocal microscope (63× and 100× oil immersion) and processed with the Leica software and Adobe Photoshop. High-resolution metaphase chromosome localization was detected with a Photometrics CCD camera.

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APPENDIX

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Establishment of Histone H3 Methylation on the Inactive X Chromosome Requires Transient Recruitment of Eed-Enx1 Polycomb Group Complexes

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Summary

Previous studies have implicated the Eed-Enx1 Polycomb group complex in the maintenance of imprinted X inactivation in the trophectoderm lineage in mouse. Here we show that recruitment of Eed-Enx1 to the inactive X chromosome (Xi) also occurs in random X inactivation in the embryo proper. Localization of Eed-Enx1 complexes to Xi occurs very early, at the onset of *Xist* expression, but then disappears as differentiation and development progress. This transient localization correlates with the presence of high levels of the complex in totipotent cells and during early differentiation stages. Functional analysis demonstrates that Eed-Enx1 is required to establish methylation of histone H3 at lysine 9 and/or lysine 27 on Xi and that this, in turn, is required to stabilize the Xi chromatin structure.

Introduction

A single X chromosome is transcriptionally silenced early in development in female mammals (Lyon, 1961). This process, termed X inactivation, equalizes the dosage of X-linked genes in XX females relative to XY males. Initiation and propagation of X inactivation occurs coincident with cellular differentiation (Monk and Harper, 1979) and involves a large nonprotein-coding RNA, the X inactive-specific transcript (*Xist*) (Brown et al., 1991, 1992; Borsani et al., 1991; Brockdorff et al., 1991, 1992). *Xist* RNA spreads over the X chromosome and is thought to induce chromosome inactivation through recruitment of as yet unidentified silencing factors (Clemson et al., 1996; Panning et al., 1997; Sheardown et al., 1997). A recent study demonstrated that the silencing function of *Xist* RNA maps to a short tandemly repeated element at the 5′ end of the transcript (Wutz et al., 2002).

In eutherian mammals X inactivation is initiated at random, with an equal probability for each cell that either the maternally (Xm)- or paternally (Xp)-inherited X is inactivated (Gardner and Lyon, 1971). In marsupials (Sharman, 1971) and also in certain extraembryonic lineages in (some) eutherians (Takagi and Sasaki, 1975), X inactivation is paternally imprinted. Both random and imprinted X inactivation are *Xist* dependent (Penny et al., 1996; Marahrens et al., 1997).

Following establishment of X inactivation, silencing is heritably maintained through all subsequent cell divisions. This is attributable to modifications of the chromatin structure that are hallmarks of developmentally regulated (facultative) and constitutive heterochromatin. Thus, heterochromatin on the inactive X chromosome (Xi) is characterised by hypoacetylation of histones H2A, H2B, H3, and H4 (Jeppesen and Turner, 1993; Belyaev et al., 1996; Boggs et al., 1996), methylation of histone H3 lysine 9 (H3-K9) (Peters et al., 2002; Boggs et al., 2002), loss of methylation at histone H3 lysine 4 (H3-K4) (Boggs et al., 2002), and incorporation of a variant histone H2A molecule (macroH2A) (Costanzi and Pehrson, 1998). Additionally Xi is replicated late in S phase (Priest et al., 1967; Takagi, 1974) and shows a high level of CpG island methylation (Mohandas et al., 1981; Norris et al., 1991).

One approach to identifying the proteins recruited by *Xist* RNA has been to analyze the temporal order of events leading to stable X inactivation after expression of *Xist* in differentiating XX embryonic stem (ES) cells. In this in vitro model system, global H4 hypoacetylation, recruitment of the variant histone, macroH2A, and CpG island methylation are all late events (Keohane et al., 1996; Mermoud et al., 1999), while transition to late replication was found to occur early (Keohane et al., 1996). Recently, deacetylation and methylation of histone H3 lysine 9 (H3-K9) and, also, loss of methylation on H3 lysine 4 (H3-K4) were found to be very early events (Heard et al., 2001; Mermoud et al., 2002). The proteins involved in these steps are therefore candidate targets for *Xist* RNA.

Establishment of X inactivation can only occur during a restricted window of opportunity early in development (Wutz and Jaenisch, 2000). Thus, if *Xist* expression is induced and maintained early during ES cell differentiation, stable chromosome silencing is established. If, on the other hand, induction of expression occurs later or in fully differentiated cells, chromosome silencing does not result (Clemson et al., 1998; Wutz and Jaenisch, 2000). Moreover, during the critical early stages, chromosome silencing is *Xist* dependent and reversible (Wutz and Jaenisch, 2000). In contrast to this, in differentiated cells, maintenance of silencing is *Xist* independent and (generally) irreversible (Brown and Willard, 1994; Csankovszki et al., 1999; Wutz and Jaenisch, 2000). These observations suggest that the silencing factors

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initially recruited by *Xist* RNA may be developmentally regulated. It should be noted that a recent report has demonstrated silencing in response to *Xist* transgenes in the HT-1080 human somatic cell line (Hall et al., 2002).

Similarities between the chromatin structure on Xi and other heritably silenced regions suggest that there may be common factors involved. Proteins involved in heritable silencing have been identified in genetic screens in *Drosophila*. These are the modifiers of position effect variegation (PEV) (reviewed in Weiler and Wakimoto, 1995) and the Polycomb group (PcG) proteins (reviewed in Pirrotta, 1997). The PcG proteins were identified initially as being required for maintenance of repression of homeotic genes. They are thought to have a wider role in heritable gene silencing, with homologs having been found in all multicellular organisms. Biochemical analysis indicates that PcG proteins belong to one of two multimeric complexes, PRC1 (Shao et al., 1999) or ESC-EZ (Sewalt et al., 1998; van Lohuizen et al., 1998; Jones et al., 1998; Tie et al., 1998). These complexes are thought to effect silencing by acting at the level of chromatin structure. Recent data indicates that the PRC1 and ESC-EZ complexes interact transiently during early embryogenesis in *Drosophila* (Poux et al., 2001).

The ESC-EZ complex includes the products of the *extra sex combs* (*esc*) gene (termed *eed* in mouse) and the *enhancer of zeste* [*e(z)*] gene (termed *Enx1* or *Ezh2* in mouse—the mouse ESC-EZ complex is referred to as the Eed-Enx1 complex throughout this study). Eed-Enx1/ESC-EZ complexes have been shown to interact with type 1 histone deacetylases (HDACs) (van der Vlag and Otte, 1999; Tie et al., 2001), and this has been suggested to be the basis for heritable silencing. More recent studies have demonstrated that the SET domain of the EZ protein catalyzes H3 lysine 9/27 methylation by ESC-EZ complexes (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002).

Mutations affecting ESC/Eed and EZ/Enx1 proteins have been shown to act very early in embryogenesis relative to other PcG mutants (Struhl and Brower, 1982; Schumacher et al., 1996; O'Carroll et al., 2001). In addition to its role in heritable silencing, EZ/Enx1 protein is required for maintenance of chromosome integrity during mitosis (Philips and Shearn, 1990) and, possibly related to this, in cell proliferation (Schumacher et al., 1996; O'Carroll et al., 2001; Beuchle et al., 2001).

In a recent study it was demonstrated that mutations in the mouse *eed* gene result in failure to maintain imprinted X inactivation, specifically in extraembryonic trophoblast cells (Wang et al., 2001). No effect was seen on random X inactivation in the embryo proper. Following on from this Mak et al. (2002) showed that, in trophoblast stem (TS) cells, a model system for imprinted X inactivation, Eed and Enx1 proteins are highly concentrated on Xi. No localization was seen in XX somatic cells, consistent with the observations of Wang et al. (2001). Taken together these studies suggested that there is a lineage-specific pathway for establishing X inactivation. Here we report that the recruitment of Eed-Enx1 complexes to Xi is in fact temporally regulated and not lineage specific. Localization of the complexes occurs rapidly at the onset of imprinted and random X inactivation, coincident with the onset of *Xist* RNA accumulation, both in vitro and in vivo. Analysis of *eed* mutant embryos demonstrates that the function of the Eed-Enx1 complex in X inactivation is to establish histone H3 N-terminal methylation at lysines 9/27.

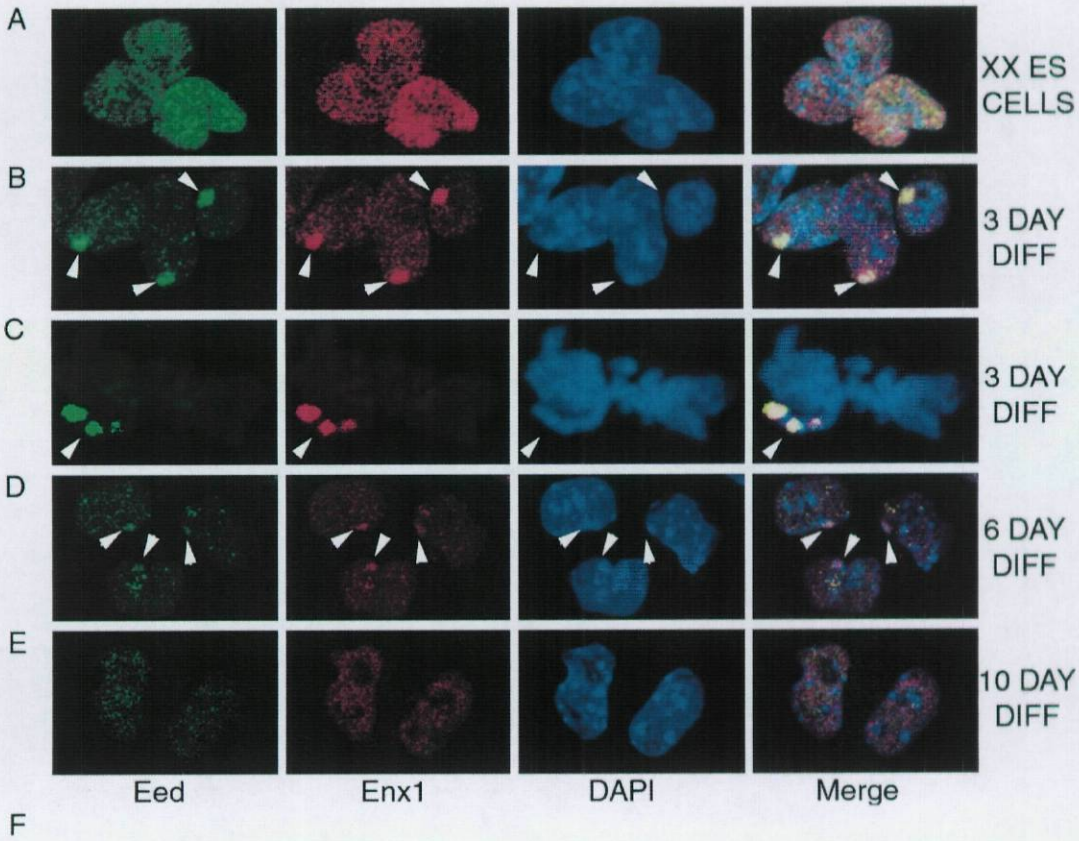
Results

Localization of Eed and Enx1 Proteins to Xi Is a Function of the Differentiation Status of ES and TS Cells

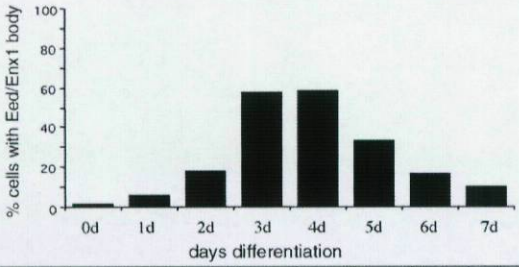
In a recent study we found that Eed-Enx1 PcG complexes localize to Xi in XX trophoblast stem (TS) cells (Mak et al., 2002). Localization was not observed in XX somatic cell lines, although nuclear staining for Eed and Enx1 proteins was detectable. These observations suggested either that Eed-Enx1 complex localization to Xi is lineage specific or, alternatively, that it is temporally regulated, occurring only early in development. To investigate this further we used indirect immunofluorescence to analyze undifferentiated and differentiated PGK12.1 XX ES cells. Previous studies have established that X inactivation in XX ES cells occurs in a progressive stepwise manner after initiation of differentiation in vitro (Keohane et al., 1996; Sheardown et al., 1997; Mermoud et al., 1999).

In undifferentiated ES cells, Eed and Enx1 proteins were seen to localize diffusely throughout the nucleoplasm (Figure 1A). In early stage differentiated cultures (1–4 days), however, a single strong focus of signal was present in addition to diffuse nuclear staining (referred to henceforth as the Eed/Enx1 body). An example of 3 day differentiated cells is illustrated in Figure 1B. On mitotic figures, Eed/Enx1 was seen to localize in a banded pattern on a single chromosome (Figure 1C). That this is the inactive X chromosome was verified by immunoRNA-FISH analysis of *Xist* RNA and Enx1 protein (see below). Interestingly, the Eed/Enx1 body was only detectable transiently. Thus, in 6 day differentiated cultures, staining of the body was weak (Figure 1D), and, after 10 days of differentiation, it was not detectable at all (Figure 1E). Scoring data obtained for a series of time points is shown in Figure 1F. The highest levels of Eed/Enx1 bodies (approximately 60% of cells) were seen at days 3 and 4 of differentiation. Similar results were obtained with a second XX ES cell line, LF2, analyzed at 0, 3, and 7 days differentiation (data not shown). Asynchronicity of differentiation in ES cell cultures accounts for the fact that Eed/Enx1 bodies are not seen in all cells at any given time point.

In addition to Eed/Enx1 bodies being reduced, overall intensity of nuclear staining for the proteins appeared to decrease at later differentiation time points (compare Figures 1A and 1B with Figures 1D and 1E). To examine this in more detail, we carried out Western blots on nuclear extracts prepared from undifferentiated and differentiated PGK12.1 ES cells (Figure 2A). Levels of both Eed and Enx1 proteins were found to be very high in undifferentiated ES cells and also during days 1–3 of differentiation. As differentiation progressed, however, levels fell markedly and, by day 10 of differentiation, were similar to those seen in the lymphocyte control. The reduction in overall protein levels was more pronounced for Eed than for Enx1. These observations suggest that the disappearance of Eed/Enx1 bodies may



F



%Eed/Enx1 bodies	1.7	5.7	18.3	57.3	58.4	33.3	16.8	10
Number scored	179	176	279	122	77	132	125	131

Figure 1. Transient Recruitment of Eed-Enx1 Complexes to the Inactive X Chromosome in Differentiating XX ES Cells

(A–E) Immunofluorescence detection of Eed (green) and Enx1 (red) carried out on undifferentiated PGK12.1 XX ES cells (XX ES cells) and on cells differentiated (diff) for 3, 6, and 10 days, as indicated. DNA is counterstained with DAPI.

(B) Arrowheads indicate Eed/Enx1 bodies.

(C) Arrowhead indicates single chromosome with Eed/Enx1 localization in a cell undergoing mitosis.

(D) Arrowheads indicate weak Eed/Enx1 body staining (see text).

(F) Scoring data showing the percentage of PGK12.1 XX ES cells with Eed/Enx1 bodies during days (d) 0–7 of differentiation.

be attributable to the downregulation of Eed and Enx1 proteins during differentiation.

Levels of HDAC1 and HDAC2, which have been shown to be recruited by the ESC-EZ/Eed-Enx1 complex (van der Vlag and Otte, 1999), remained constant over a similar differentiation time course. They were, however, lower in lymphocytes (Figure 2B). The Bmi-1 and Hpc2 proteins, which are involved in the distinct PRC1 PcG complex (Shao et al., 1999), showed an opposite pattern of expression to Eed and Enx1, with highest levels detectable at late differentiation time points and in lymphocytes (Figure 2B). Immunofluorescence analysis of the HDAC and PRC1 proteins did not reveal specific localization to Xi at any of the differentiation stages analyzed (data not shown).

TS cells are multipotent precursors that can give rise to different cell types of the trophectoderm lineage after withdrawal of FGF4 from the culture medium (Tanaka et

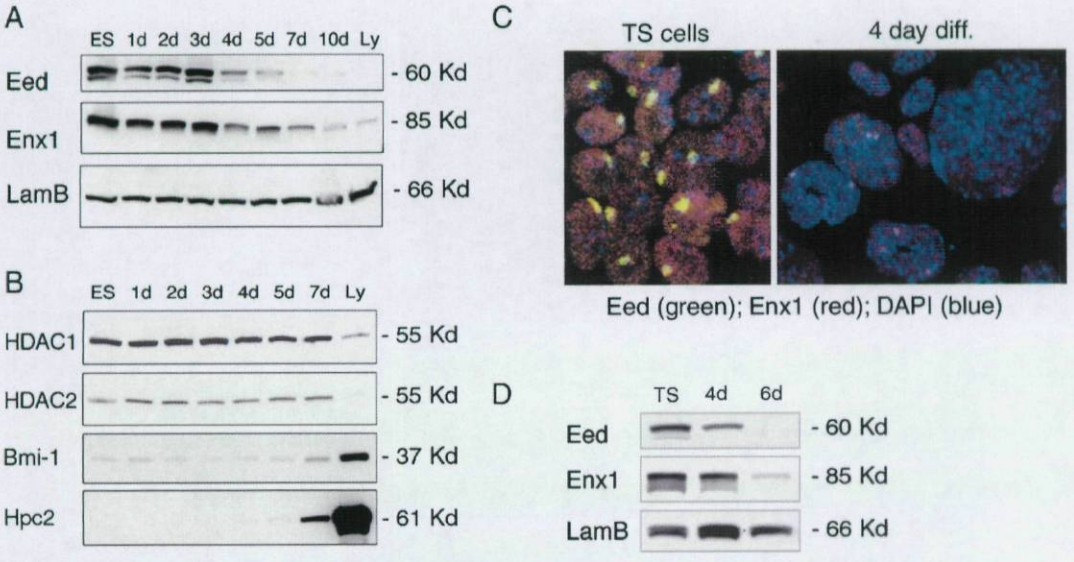


Figure 2. Eed and Enx1 Are Downregulated in Differentiated Cells

(A and B) Western blot analysis of nuclear extracts from PGK12.1 XX ES cells and cells differentiated for 1–10 days (d), as indicated. Lymphocyte (Ly) extracts were used as a somatic cell control. Proteins analyzed were Eed, Enx1, Lamin B (LamB), HDAC1, HDAC2, Bmi-1, and Hpc2. Molecular sizes are indicated. Note doublet for Eed protein is thought to be attributable to alternative RNA transcripts (Denisenko et al., 1998) or posttranslational modification (Ng et al., 2000).

(C) Immunofluorescence analysis of Eed and Enx1 proteins in undifferentiated B1 XX TS cells and in cells differentiated (diff) for 4 days. Note trophectoderm giant cell present in differentiated culture.

(D) Western blot analysis of Eed, Enx1, and LaminB (LamB) proteins in nuclear extracts from TS cells and from cells harvested 4 or 6 days after initiation of differentiation.

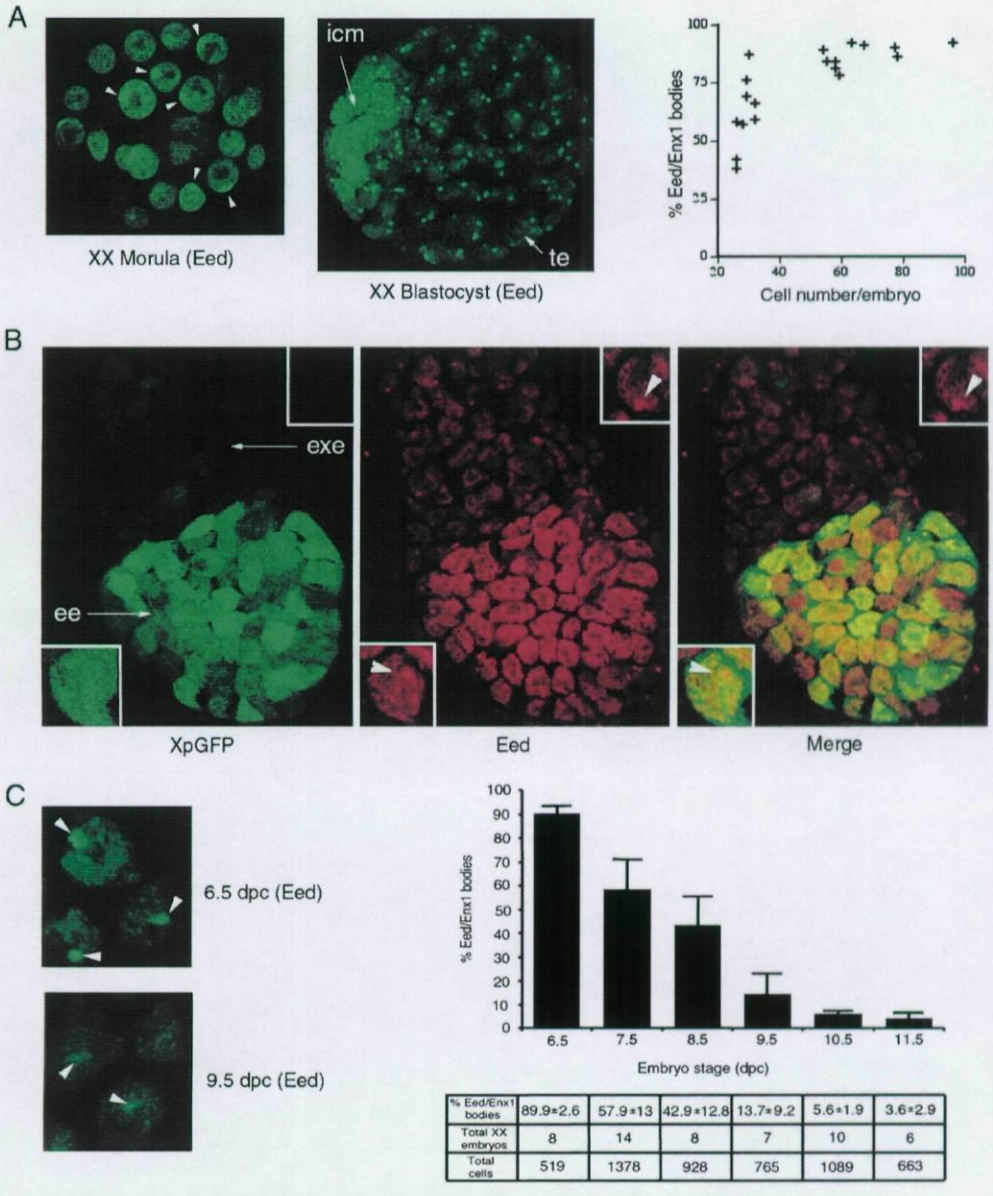
al., 1998). Previously, we analyzed Eed/Enx1 localization only in undifferentiated XX TS cells (Mak et al., 2002). In light of the above results, we decided to analyze differentiated TS cells. Immunofluorescence analysis revealed that the Eed/Enx1 bodies disappear after 4–6 days of differentiation (Figure 2C). Moreover, Western blot analysis revealed that there is a marked reduction in overall levels of Eed and Enx1 proteins (Figure 2D), as occurs in differentiating ES cells.

Taken together these results indicate that, with in vitro cell systems, transient localization of Eed-Enx1 complexes to Xi occurs as a function of the differentiation status of cells. This, in turn, appears to reflect downregulation of Eed/Enx1 proteins during differentiation from totipotent/multipotent precursors.

Transient Eed/Enx1 Localization on the Inactive X Chromosome In Vivo

We next analyzed Eed-Enx1 complex localization on Xi at different stages of mouse embryogenesis in order to confirm that our observations on ES and TS cells reflect events that occur in vivo. To analyze Eed/Enx1 body formation at the onset of imprinted X inactivation, we carried out immunofluorescence on preimplantation embryos at the late morula (16- to 32-cell) stage and the blastocyst stage, the time when the trophectoderm lineage first differentiates. At the morula stage single Eed/Enx1 bodies were clearly detectable in a proportion of cells in approximately 50% of embryos. An example illustrating Eed staining is shown in Figure 3A (left panel). Identical results were obtained for Enx1 (data not shown). Expression of the XGFP transgene, inherited on the Xp (see Experimental Procedures), or *Xist* expression detected in immunoRNA-FISH analysis (see below), confirmed that these were XX embryos. In blastocysts, Eed/Enx1 bodies were seen in most cells. Overall nuclear staining for Eed was much higher in the totipotent inner cell mass (ICM) cells than in differentiating trophectoderm (Figure 3A, center panel). Scoring data for a number of embryos demonstrate that the proportion of cells with an Eed/Enx1 body increases to maximal levels during the morula to blastocyst transition (Figure 3A, right).

We went on to analyze postimplantation embryos at 5.5 days post coitum (dpc), the time when random X inactivation is initiated in cells of the embryonic ectoderm. With the XGFP transgene transmitted on Xp, the embryonic (GFP-positive) and extraembryonic (GFP-negative) regions could be readily identified. In the example shown a mosaic GFP signal could be seen in the embryonic ectoderm, indicating that random X inactivation had initiated (Figure 3B, left panel). Eed/Enx1 bodies were detectable in both embryonic and extraembryonic cells at this stage. However, a clear difference in the overall levels of Eed protein was apparent, with markedly higher signal detected in the undifferentiated embryonic cells than in differentiated extraembryonic cells (Figure 3B, center panel).



% Eed/Enx1 bodies	89.9±2.6	57.9±13	42.9±12.8	13.7±9.2	5.6±1.9	3.6±2.9
Total XX embryos	8	14	8	7	10	6
Total cells	519	1378	928	765	1089	663

Figure 3. Eed/Enx1 Body Formation during Imprinted and Random X Inactivation In Vivo

(A) Examples of immunofluorescence analysis of Eed protein in XX morula (left panel) and blastocyst stage embryos (center panel). Eed/Enx1 bodies in the morula are indicated with arrowheads. The inner cell mass (icm), and trophectoderm regions (te) of the blastocyst are labeled. The scatterplot in the right panel records the percentage of cells with Eed/Enx1 bodies in XX embryos plotted against the total number of cells in individual embryos.

(B) Single optical section illustrating immunofluorescence analysis of Eed localization in a 5.5 dpc XX embryo with an X-linked GFP transgene present on the paternal X chromosome (Xp). Strong green fluorescence marks the embryonic ectoderm (ee) where random X inactivation is occurring (note mosaic pattern of GFP staining). Cells that have undergone imprinted X inactivation, notably extraembryonic ectoderm (exe), have silenced the GFP transgene on Xp. Insets indicate Eed/Enx1 bodies (arrows) in cells of exe (top right) or ee (bottom left) regions.

(C) Analysis of Eed/Enx1 bodies in cells of XX embryos at 6.5–11.5 dpc. Examples on the left show cells of early stages (6.5 dpc) with strong Eed/Enx1 bodies (top panel) and cells at a later stage (9.5 dpc) in which Eed/Enx1 bodies and general Eed staining are much weaker. The graph on the right shows scoring data from a number of experiments, giving the percentage of cells exhibiting Eed/Enx1 bodies at the stages indicated.

Localization of Eed-Enx1 complexes on Xi was subsequently monitored in XX embryos at time points after the onset of random X inactivation (Figure 3C). While most cells exhibited a single Eed/Enx1 body at 6.5 dpc, the frequency of detection began to decline thereafter. Overall levels of Eed and Enx1 proteins were also seen

to decline, as assessed by intensity of staining (see Figure 3C, 9.5 dpc example). By 11.5 dpc Eed/Enx1 bodies were undetectable in the majority of cells. These results demonstrate that transient localization of Eed-Enx1 complexes to Xi is a feature of X inactivation in vivo. As is the case for ES and TS cells, disappearance of Xi localization appears to be due to progressive down-regulation of Eed and Enx1 proteins during differentiation.

Eed/Enx1 Body Formation Occurs at the Onset of X Inactivation

We went on to assess the timing of Eed-Enx1 complex recruitment to Xi relative to other steps in the X inactivation process using the differentiating XX ES cell system. X inactivation is triggered by the accumulation of stable *Xist* RNA, and this is the earliest detectable step, occurring between 2 and 5 days differentiation in XX ES cells (Sheardown et al., 1997). Eed/Enx1 body formation was seen to closely mirror the accumulation of stable *Xist* RNA, being detectable in 80% of cells at 2 days differentiation and in nearly all cells thereafter (Figure 4A).

Low levels of *Xist* transcript and, also, antisense *Tsix* transcript occur on both alleles in undifferentiated cells and transiently on the active X chromosome allele during early differentiation stages (Panning et al., 1997; Sheardown et al., 1997; Lee et al., 1999; Debrand et al., 1999). This is detected as a fine pinpoint signal in RNA-FISH analysis (Figure 4A, left panel). We did not observe a concentration of Eed or Enx1 proteins colocalizing with these signals (Figure 4A). This may be attributable to the low level of *Xist* RNA, or, alternatively, coexpression of *Tsix* antisense RNA may preclude recruitment of the complex.

Recent studies have demonstrated that specific modifications on the N termini of histone H3, namely, deacetylation and methylation of H3-K9 (Heard et al., 2001; Mermoud et al., 2002), and loss of methylation at H3-K4 (Heard et al., 2001) are very early events in X inactivation. Global deacetylation of histone H4, on the other hand, occurs later (Keohane et al., 1996; Heard et al., 2001).

To analyze H3-K9 methylation relative to Eed/Enx1 body formation, we used antisera raised to a branched H3 peptide dimethylated at K9. This antibody detects di- and trimethylH3-K9 (meH3-K9) (T.J. and A.H.F.M.P., unpublished data) and, in previous studies, has been shown to highlight pericentromeric heterochromatin and the inactive X chromosome (Peters et al., 2001; 2002; Mermoud et al., 2002). We analyzed cells from 2, 3, and 6 day differentiated cultures, scoring first for the presence of a single Eed/Enx1 body and then for the presence or absence of underlying H3-K9 methylation (Figure 4B). MeH3-K9 staining of pericentric heterochromatin, as assessed by colocalization with DAPI dense-staining regions of the nucleus, was observed at all stages analyzed (Figure 4B). Strong meH3-K9 signal underlying Eed/Enx1 bodies was seen in 31% of cells at 2 days differentiation. A weaker signal, indicating partial methylation, was seen in a further 35% of cases. At later stages strong meH3-K9 staining was seen in the majority of cases. These results indicate that a progressive increase in detectable H3-K9 methylation on Xi occurs subsequent to recruitment of Eed-Enx1 complexes. Deacetylation of H3-K9 (Figure 4C) and loss of methylation of H3-K4 (Figure 4D) were also seen to occur rapidly at the time of Eed-Enx1 complex recruitment.

Recent studies have demonstrated that purified Eed-Enx1 (ESC-EZ) complexes catalyze methylation of histone H3-K27 and, to a lesser extent, H3-K9 (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). We therefore analyzed H3-K27 methylation using antiserum specific for tri-meH3-K27. In immunofluorescene analysis this antibody highlights the inactive X chromosome, but not pericentromeric heterochromatin (A.H.F.M.P. and T.J., submitted). In differentiating XX ES cells, we observed strong meH3-K27 staining over Eed/Enx1 bodies in nearly all cells at all differentiation stages tested (Figure 4E). This result is consistent with Eed-Enx1 complexes catalyzing H3-K27 methylation on Xi (see below).

As a control for these experiments, we analyzed global deacetylation of H4, which has been reported to occur later than H3 modifications (Keohane et al., 1996; Heard et al., 2001). In agreement with these earlier studies, hypoacetylation of H4 in association with Eed/Enx1 bodies was only detected in a relatively small proportion of cells after 2 and 3 days differentiation, but increased to maximal levels after 6 days differentiation (Figure 4F).

Xist Expression Is Necessary and Sufficient to Recruit Eed-Enx1 Complexes

The above results demonstrate that localization of Eed-Enx1 complexes to Xi is a very early event, both during XX ES cell differentiation and in vivo. Thus, Eed and Enx1 are candidates for proteins recruited by *Xist* RNA. To investigate this idea further, we analyzed Eed-Enx1 complex localization in an XY ES cell line, LH1.2, which carries a multicopy *Xist* transgene integrated into an autsosome (Herzing et al., 1997). The transgene is expressed and accumulates in *cis* in undifferentiated cultures in approximately 30% of cells (see Figure 5A, left panel). Using immunoRNA-FISH analysis we found that Eed/Enx1 bodies are present and colocalize with the ectopic *Xist* RNA (Figure 5A). Thus, expression of *Xist* RNA in undifferentiated XY ES cells is sufficient to recruit Eed-Enx1 complexes. Scoring data showed colocalization in 58% of cells that had some degree of accumulation of *Xist* RNA (n = 119). This is slightly lower than that in differentiating XX ES cells (Figure 4A), probably reflecting the fact that transgene *Xist* RNA levels are sometimes relatively low. Analysis of H3/H4 modifications indicates that chromatin silencing occurs in response to ectopic *Xist* RNA expression in LH1.2 cells (data not shown).

We next examined Eed-Enx1 recruitment in response to ectopic *Xist* expressed in vivo in preimplantation embryos. The Tg15 line carries an *Xist* transgene integrated on the Y chromosome (Matsuura et al., 1996). The transgene is expressed in all cells in XY^{Tg15} preimplantation embryos (M. Ager and S.D.M. Brown, personal communication). To discriminate between XY^{Tg15} and XX blastocysts, Tg15 males were crossed with homozygous XGFP females. Blastocysts were then isolated from crosses between male F1 progeny ($X^{GFP}Y^{Tg15}$) and normal females (XX). XX^{GFP} and XY^{Tg15} embryos could then be distinguished on the basis of GFP expression. ImmunoRNA-FISH analysis was carried out, and Enx1 protein was

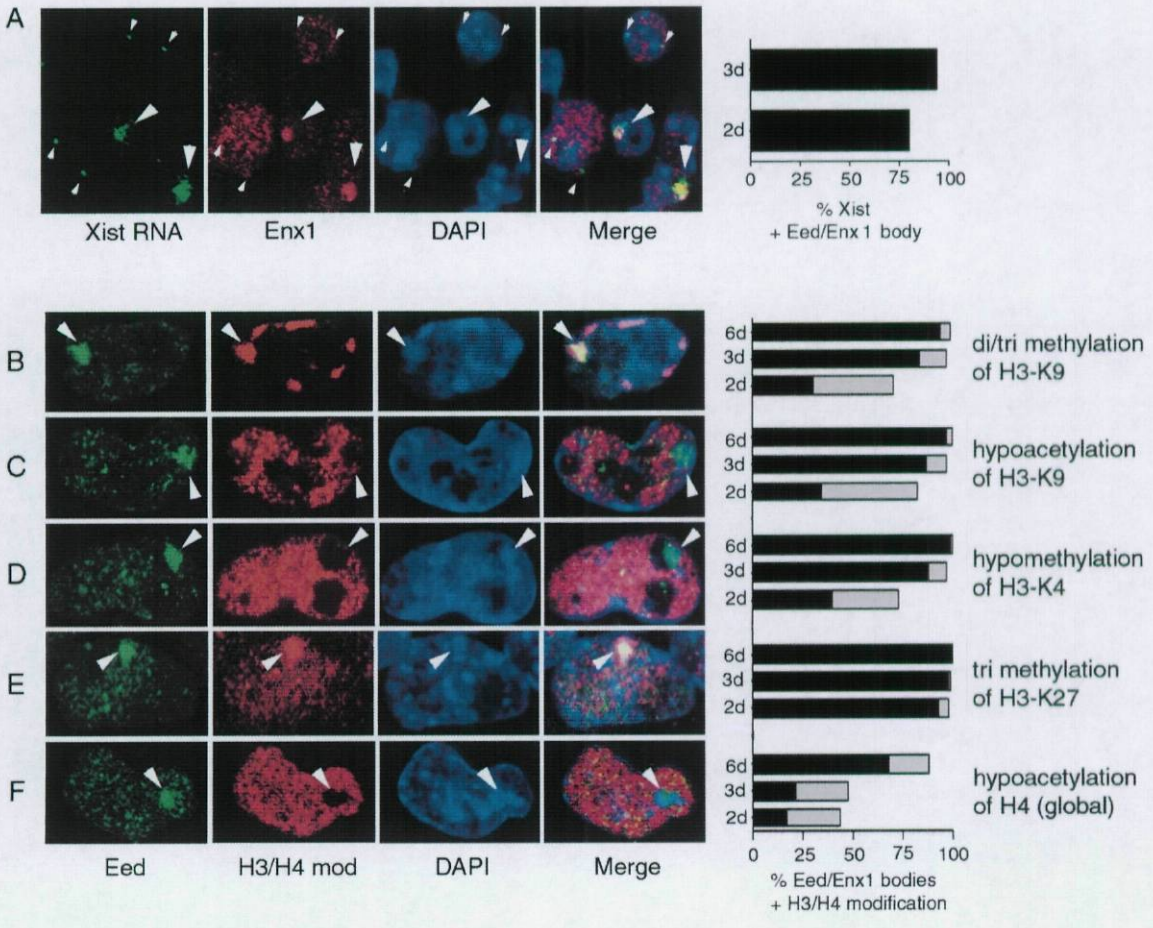


Figure 4. Recruitment of Eed-Enx1 Complexes Is an Early Event in Random X Inactivation

(A) Example showing immunoRNA-FISH detection of *Xist* RNA (green) and Enx1 protein (red) in 2 day differentiated PGK12.1 cells. DNA is counterstained with DAPI. Small arrowheads indicate two pinpoint *Xist/Tsix* signals seen in undifferentiated XX ES cells. Large arrowheads indicate accumulated *Xist* RNA and associated Enx1 staining. The chart on the right records the percentage of cells ($n > 100$) where *Xist* RNA signal has underlying Enx1 staining, after 2 and 3 days (d) differentiation.

(B–F) Examples of early stage differentiated PGK12.1 cells exhibiting histone H3/H4 modifications underlying Eed/Enx1 bodies. Graphs to the right illustrate scoring data ($n > 100$) at 2, 3, and 6 days differentiation. Filled areas represent the proportion of cells where histone modification was clearly detectable. Shaded areas represent cells where an intermediate level of histone modification was detected.

seen to colocalize with transgenic *Xist* RNA in a similar pattern to that seen in normal female blastocysts (compare Figure 5B with 5C).

Finally, we analyzed Eed-Enx1 recruitment in XX blastocysts carrying a mutant *Xist* RNA allele, $Xist^{inv}$. $Xist^{inv}$ is a targeted mutation with a deletion of *Xist* exon IV and an inversion of sequences between 5.9 and 13.2 Kbp of the *Xist* genomic sequence (see Experimental Procedures). The $Xist^{inv}$ allele is able to express a mutant RNA but fails to elicit X inactivation (N.B. and T.B.N., in preparation). XX preimplantation embryos in which $Xist^{inv}$ is inherited on Xp express mutant RNA in all cells. As shown in Figure 5C, little or no Enx1 protein was seen to colocalize with $Xist^{inv}$ RNA. Taken together these results demonstrate that expression of functional *Xist* RNA is both necessary and sufficient for the recruitment of Eed-Enx1 complexes.

Recruitment of Eed-Enx1 Complexes on Xi Is Required to Establish H3-K9/K27 Methylation

Biochemical studies have demonstrated that Eed interacts with type 1 histone deacetylases (HDACs) (van der Vlag and Otte, 1999; Tie et al., 2001). In addition Enx1 protein has a SET domain, the active site in histone methyltransferases (HMTases) (Rea et al., 2000), and recent studies have shown that ESC-EZ (Eed-Enx1) complexes catalyze methylation of H3-K9 and K27, with a strong preference for K27 (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002). Thus, the Eed-Enx1 complex is a good candidate for the enzyme activities catalyzing H3 N-terminal modification at the onset of X inactivation. To investigate this idea we set out to analyze H3 modifications in *eed* mutant embryos.

The eed^{3354SB} allele is a point mutation that disrupts

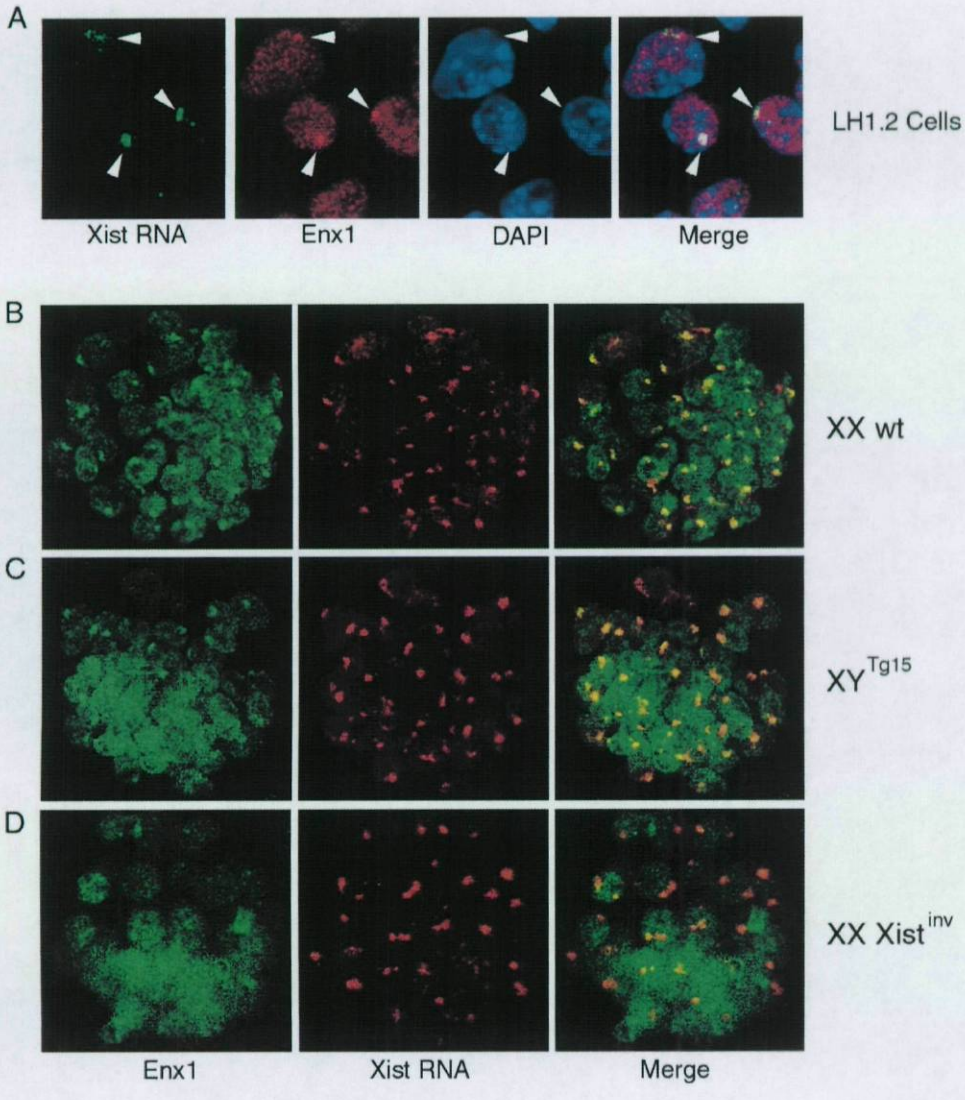


Figure 5. *Xist* Expression Is Necessary and Sufficient for Recruitment of Eed-Enx1 Complexes to Xi

(A) Example of immunoRNA-FISH analysis of undifferentiated LH1.2 XY ES cells showing individual cells expressing transgene *Xist* RNA and colocalizing staining for Enx1 protein.

(B–D) Examples of immunoRNA-FISH analysis of blastocyst stage embryos detecting Enx1 protein (green) and *Xist* RNA (red).

(B and C) Normal XX blastocyst (B) and XY^{Tg15} blastocyst expressing an *Xist* transgene integrated on the Y chromosome (C) show Eed/Enx1 bodies colocalizing with *Xist* RNA.

(D) XX blastocyst bearing the null $Xist^{inv}$ mutation on the paternal X chromosome. Mutant *Xist* RNA fails to elicit X inactivation, and Eed/Enx1 bodies are not detectable.

the structure of a WD40 domain required for the interaction of Eed with Enx1 (Schumacher et al., 1996). It appears to be a null allele, as the phenotype, embryo lethality at about 8.5 dpc, is indistinguishable from that caused by complete deletion of the locus. To study X inactivation in the embryo proper in *eed* mutants, we chose to analyze embryonic ectoderm of 7.5 dpc embryos, i.e., prior to the stage at which homozygous mutants die, but after the onset of random X inactivation.

We first analyzed localization of Eed and Enx1 proteins in $eed^{-/-}$ mutant embryos. Examples are shown in Figure 6A (center panel). Eed protein could not be detected at all, suggesting either that it is destabilized as a consequence of the mutation or, alternatively, that the epitope recognized by the antibody has been destroyed. Enx1 protein shows an abnormal localization, being diffusely distributed both in the nucleus and cytoplasm. An identical pattern was observed in male $eed^{-/-}$ embryos (data not shown). Eed/Enx1 bodies were not detected in mutant XX embryos. Furthermore, staining for Eed/Enx1 bodies was markedly reduced in heterozygous embryos (Figure 6A, compare first three panels).

We went on to examine *Xist* expression and Enx1 protein in mutant XX embryos using immunoRNA-FISH conditions. As shown in Figure 6A (right panels), Enx1 protein does not colocalize with *Xist* RNA domains. In fact, Enx1 staining in $eed^{-/-}$ embryos was nearly completely lost under these conditions. As immunoRNA-FISH involves a cell permeabilization step prior to fixation, the Enx1 protein, which is not bound up in a complex or associated with chromatin, probably diffuses out of the cells.

To determine the consequences of disruption of Eed-Enx1 complexes in $eed^{-/-}$ embryos, we carried out immunoRNA-FISH using either antisera to di/tri-meH3-K9 or tri-meH3-K27. Examples are shown in Figure 6B. In contrast to wild-type controls, the di/tri-meH3-K9 antibody failed to detect signal that colocalized with *Xist* RNA in the majority of cells (Figure 6B, left panels). MeH3-K9 at pericentromeric heterochromatin, however, was unaffected. This latter observation is expected as H3-K9 methylation at these sites is catalyzed by the Suv39h1/2 HMTases (Peters et al., 2001). The tri-meH3-K27 antisera also failed to detect a signal underlying *Xist* RNA (Figure 6B, right panels). General nuclear staining for tri-meH3-K27 was also much reduced, indicating that the Eed-Enx1 complex is the major H3-K27 HMTase present at this developmental stage.

As $eed^{-/-}$ embryos were previously shown to initiate, but not to maintain, imprinted X inactivation in trophectoderm (Wang et al., 2001), we went on to determine whether deacetylation at H3-K9 occurs on Xi following random X inactivation in the mutant embryonic ectoderm. In a large proportion of cells, hypoacetylation of H3-K9 was seen underlying *Xist* RNA signal, similar to wild-type cells (Figure 6C, top and center panels). However, in approximately 20% of cells, H3-K9 hypoacetylation was not detected. Moreover, in these cells, *Xist* RNA was diffusely distributed, consistent with the chromosome being relatively decondensed. Similar results were obtained with antisera to meH3-K4 (data not shown).

We went on to assay the inactivation status of two X-linked genes, *Brx* and *Pgk-1*, using RNA-FISH to detect nascent transcripts (Figure 6D). Cells obtained from 7.5 dpc $eed^{-/-}$ embryos and wild-type littermates were scored for the presence of either one or two nascent RNA foci. In wild-type and heterozygous embryos, we observed a single focus (Xa allele), generally located away from the *Xist* RNA domain, in the majority of cells. In $eed^{-/-}$ embryos, however, 10%–15% of cells were seen to have two signals, one of which localized close to the *Xist* RNA domain (Xi allele). Thus, the Xi allele is expressed in a proportion of cells from mutant embryos. Taken together these results indicate that X inactivation commences in $eed^{-/-}$ embryos but that, in the absence of H3-K9/K27 methylation, hypoacetylation of H3-K9 and loss of H3-K4 methylation are not stably maintained, with consequent reactivation of genes on Xi.

Discussion

In this study we have demonstrated that Eed-Enx1 complexes are transiently recruited to Xi at the onset of X inactivation, both in vitro and in vivo. At later developmental stages localization of Eed-Enx1 to Xi is no longer detectable, coinciding with downregulation of overall levels of these proteins. Recruitment to Xi is a very early event in X inactivation, occurring at the same time as *Xist* RNA accumulation. *Xist* expression is both necessary and sufficient to recruit Eed-Enx1 complexes. Analysis of *eed* mutant embryos demonstrates that Eed-Enx1 complexes are required to establish H3-K9/K27 methylation on the inactive X chromosome and, thereby, to stabilize the Xi chromatin structure.

Function of the Eed/Enx1 Complex in X Inactivation

In $eed^{-/-}$ XX embryos we found that Enx1 protein does not localize to Xi. This result is consistent with in vitro analysis demonstrating that the eed^{3354SB} mutation disrupts a WD40 domain required for the interaction of Eed with Enx1 (Denisenko et al., 1998). Thus, in the absence of functional Eed protein, the Enx1 HMTase cannot be directed toward specific targets. As recent studies have demonstrated that Eed/Enx1 complexes methylate H3-K9 and K27 in vitro, with a strong preference for K27 (Czermin et al., 2002; Muller et al., 2002; Cao et al., 2002; Kuzmichev et al., 2002), failure to localize Enx1 clearly accounts for the absence of H3-K9/K27 methylation on Xi in $eed^{-/-}$ embryos.

It should be noted that, while previous studies have identified H3-K9 methylation as an early mark of silent chromatin on the inactive X chromosome (Peters et al., 2002; Boggs et al., 2002), more recent data indicates that this could be attributable to crossreactivity of di/tri-meH3-K9 antisera toward di/tri-meH3-K27. New antisera highly specific for di/tri-meH3-K9 detect pericentromeric heterochromatin, but not Xi, while the tri-meH3-K27 antibody used in this study detects Xi, but not pericentromeric heterochromatin (A.H.F.M.P. and T.J., submitted). It is also possible that Xi is di/trimethylated both at K9 and K27 and that this configuration is not recognized by the novel di/trimethylH3-K9 antisera.

Our data suggest that H3-K9/K27 methylation serves to stabilize the Xi chromatin structure. Thus, in $eed^{-/-}$ embryos, H3-K9 hypoacetylation and loss of H3-K4 methylation on Xi are not seen in a significant number of cells. Moreover, both the Xa and Xi alleles of two X-linked genes were seen to be expressed in a similar proportion of cells. These observations provide a basis for explaining reactivation of the X-linked GFP transgene in trophectoderm cells of $eed^{-/-}$ embryos reported previously by Wang et al. (2001). However, Wang et al. (2001) did not observe reactivation of the GFP transgene in cells of the embryo proper, leading to the conclusion that Eed is required for the maintenance of imprinted, but not random, X inactivation. This difference can be accounted for by two factors. First, it is probable that reactivation of any given locus on Xi is sporadic and progressive. As embryos exhibit mosaic expression of the XGFP transgene because of random X inactivation, a relatively small increase in the proportion of cells expressing the transgene, as observed for endogenous X-linked genes in this study, would be difficult to quantify. A second factor, suggested by Wang et al. (2001), is that additional levels of epigenetic silencing, for example, DNA methylation (Sado et al., 2000), play a more significant role in maintenance of X inactivation in cells

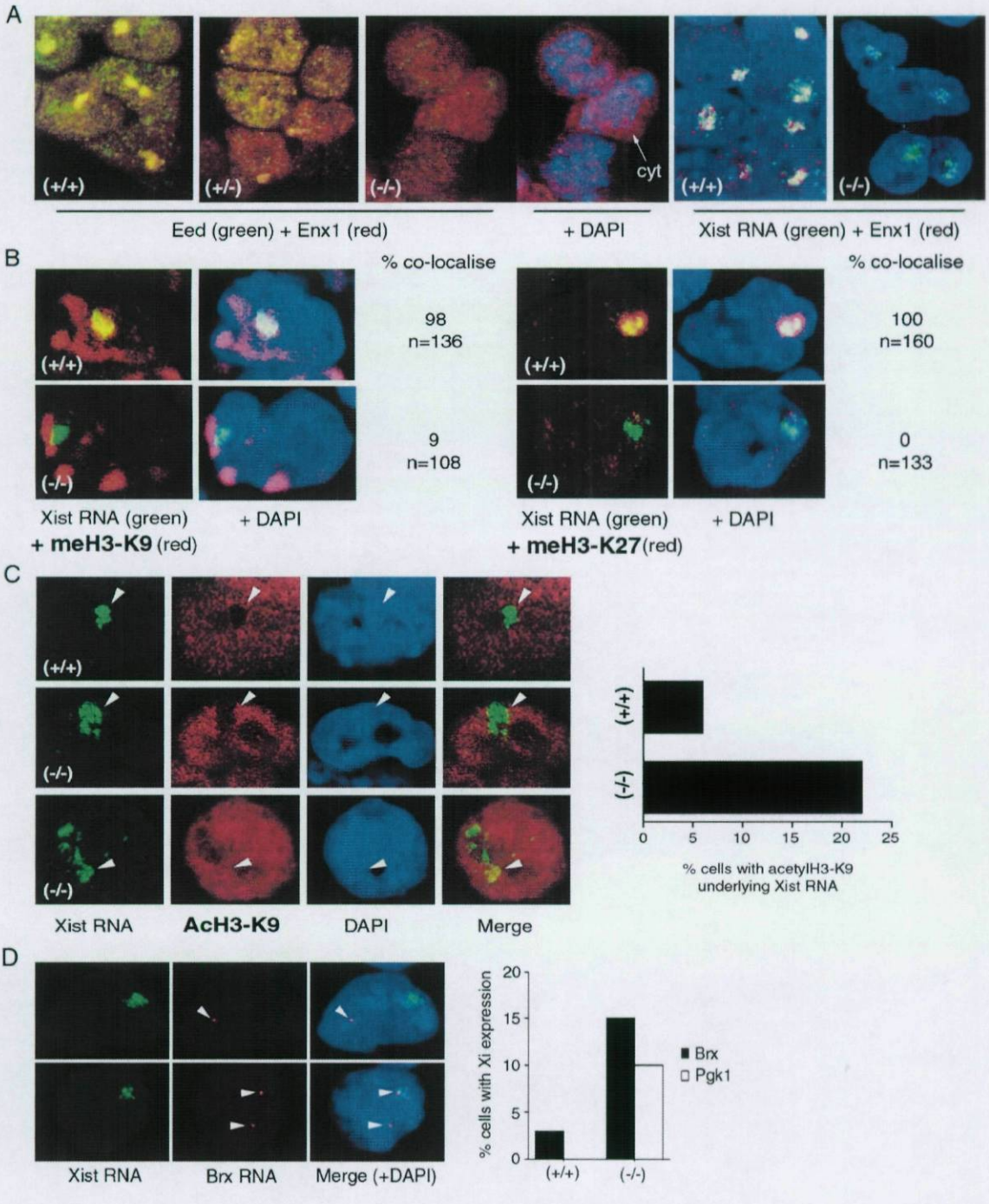


Figure 6. Eed-Enx1 Activity Is Required to Establish H3-K9/K27 Methylation on the Inactive X Chromosome

(A) Localization of Eed (green) and Enx1 (red) in XX *eed*^{3364SB} mutant embryos at 7.5 dpc. Examples of wild-type (+/+), heterozygote (+/−), and homozygous mutants (−/−) are illustrated (panels 1,2, and 3, respectively). Note that Eed/Enx1 bodies stain more weakly in heterozygotes than in wild-type cells. In homozygous mutant cells the Eed signal is nearly undetectable. Enx1 protein is found in the nucleus and cytoplasm (cyt), and there are no Eed/Enx1 bodies detectable. ImmunoRNA-FISH analysis detecting *Xist* RNA (green) and Enx1 (red) further demonstrates this finding (panels 4 and 5).

(B) Examples of immunoRNA-FISH analysis assessing association of di/tri-meH3-K9 (left panels) and tri-meH3-K27 (right panels) with *Xist* RNA domains in homozygous *eed* mutant embryos compared with wild-type littermates. Scoring data recording the percentage of cells with colocalizing signals are shown to the right of the panels.

(C) Examples of immunoRNA-FISH analysis assessing association of *Xist* RNA (green) and acetylH3-K9 (red). In wild-type XX embryos (top panels), the majority of cells show hypoacetylation of H3-K9 underlying *Xist* RNA. This is true in many cells from homozygous mutant embryos

of the embryo proper compared with the trophectoderm, potentially masking the effects of failure to establish H3-K9/K27 methylation.

Methylation of H3-K9 and H3-K27 on Xi are detected throughout development and in adult somatic cells (Heard et al., 2001; Mermoud et al., 2002; A.H.F.M.P. and T.J., submitted). As Eed-Enx1 localization on Xi is only detectable early in development, it is possible that there is a maintenance H3-K9/K27 HMTase that functions at later stages. Alternatively, low levels of Eed-Enx1 complex present in later development could be sufficient to maintain H3-K9/K27 methylation on Xi. A recent study has demonstrated that conditional deletion of Ezh2 (Enx1) in pro-B cells correlates with reduced levels of methylH3-K27 (Su et al., 2002).

Interaction of Eed-Enx1 Complexes and *Xist* RNA

The banded localization of Eed-Enx1 complexes on Xi parallels that observed for *Xist* RNA. We first noted this in TS cells and, on the basis of this observation, suggested that there could be an interaction, either direct or indirect, between *Xist* and the Eed-Enx1 complex (Mak et al., 2002). This view is further supported by observations reported in this study. First, recruitment of Eed-Enx1 complexes occurs extremely rapidly after the onset of stable *Xist* RNA accumulation in differentiating XX ES cells. Second, Eed-Enx1 recruitment occurs in response to expression of ectopic *Xist* RNA transgenes, both in undifferentiated XY ES cells and also in XY^{Tg15} blastocysts. Finally, Eed-Enx1 complexes are not recruited in response to expression of $Xist^{inv}$ mutant RNA, which fails to elicit X inactivation.

While our data support the idea that Eed-Enx1 complexes are recruited by *Xist* RNA, they do not prove that this interaction is direct. In fact, localization of Eed-Enx1 to Xi is first seen to occur at the morula stage, when imprinted X inactivation is initiated, whereas *Xist* RNA expression is detected earlier, in cleavage stage embryos (Nesterova et al., 2001). High levels of Eed-Enx1 complexes are available during the cleavage stages (N.B. and W.M., in preparation), suggesting that a factor(s) required for the interaction of the complex with *Xist* is absent.

Also relevant to the question of whether or not the Eed-Enx1 complex interacts directly with *Xist* RNA is the observation that $eed^{-/-}$ embryos are able to initiate X inactivation. This is, presumably, at least in part, attributable to recruitment of an HDAC complex that can deacetylate H3-K9. It is possible that components of the PcG complex other than Eed and Enx1 are still assembled in $eed^{-/-}$ embryos and that these confer H3-K9 HDAC activity. In the *Drosophila* ESC (Eed) protein, an equivalent mutation disrupts EZ (Enx1) recruitment but does not appear to ablate complex formation (Ng et al., 2000). An alternative scenario is that the complex responsible for H3-K9 deacetylation is a direct target of *Xist* RNA and that the Eed-Enx1 HMTase is then recruited by this complex. In support of this idea, heritable silencing at *Drosophila* homeotic genes is initiated by recruitment of the dMi2 protein, a component of an HDAC and chromatin remodeling complex, by hunchback (Kehle et al., 1998). Moreover, Eed protein can interact with type 1 HDACs, both in mammalian cells (van der Vlag and Otte, 1999) and in *Drosophila* (Tie et al., 2001). Thus, HDAC complexes may recruit Eed-Enx1 to target sites, including Xi, rather than vice versa.

Developmental Regulation of the Eed-Enx1 Complex

It is interesting to note that the time during which high levels of Eed-Enx1 complex are available corresponds closely to the window of opportunity during which cells are responsive to *Xist* RNA (Wutz and Jaenisch, 2000). On the basis of this consideration, we propose the model illustrated in Figure 7. We speculate that *Xist* RNA recruits HDAC and Eed-Enx1 complexes, which lead to establishment of a primary level of chromatin silencing. Only during early differentiation stages would levels of these complexes be sufficient to establish chromosome-wide primary silencing. This would explain why expression of *Xist* in more differentiated cell types cannot induce X inactivation (Wutz and Jaenisch, 2000). We further suggest that maintaining localization of the HDAC/Eed-Enx1 complexes is *Xist* RNA dependent. This would account for reversibility and *Xist* dependence of silencing in undifferentiated ES cells or during early differentiation stages (Wutz and Jaenisch, 2000). Extinguishing *Xist* expression would result in delocalization of HDAC/Eed-Enx1 complexes, loss of H3-K9/K27 methylation, increased H3-K9 acetylation, and, hence, reactivation of Xi.

To account for the fact that X inactivation does subsequently become stabilized and *Xist* independent, we suggest that the chromatin modifications induced by the HDAC and Eed-Enx1 complex provide a template for recruitment of other silencing components. These could be responsible for further histone N-terminal modifications, for example, global H4 deacetylation, and also for DNA methylation at CpG islands and recruitment of macroH2A1.2. It should be noted that proteins of the PRC1 PcG group complex are not localized to Xi at any stage (data not shown) and are therefore unlikely to be involved in maintaining X inactivation in late development.

A key finding from our experiments is that recruitment of Eed-Enx1 to Xi is temporally regulated, rather than lineage specific, and that this, in turn, appears to relate to temporal regulation of overall levels of Eed and, to a lesser extent, Enx1 proteins. A similar expression profile has been reported for ESC (Eed) protein in *Drosophila* embryogenesis (Ng et al., 2000). It is striking that these factors are expressed at highest levels in totipotent or multipotent precursors and during early stages of differentiation. One interpretation of this observation is that

(center panels). However, a significant proportion of homozygous mutant cells exhibit association of acetylH3-K9 signal and *Xist* RNA (lower panels). Quantitative analysis is shown to the right ($n > 100$).

(D) Expression of X-linked genes in $eed^{-/-}$ 7.5 dpc embryos. Examples show cells with Xa *Brx* expression (top panels) and with both Xa and Xi *Brx* expression (lower panels). The graph to the right shows quantitative data illustrating the percentage of cells expressing both Xa and Xi alleles for *Brx* and *Pgk1* loci in wild-type (+/+) or mutant (−/−) embryos.

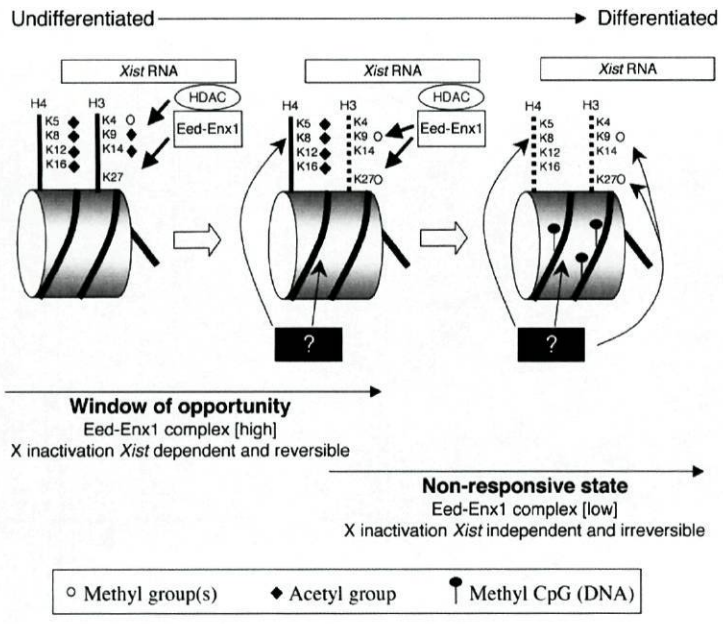


Figure 7. A Model for the Role of Eed-Enx1 Complexes in X Inactivation

The model depicts a histone octamer with H3 and H4 N termini acetylated (filled diamond) or methylated (open circle) at defined lysine (K) residues, as indicated. Recruitment of HDAC and Eed-Enx1 complexes by *Xist* RNA leads to deacetylation of H3-K9, methylation of H3-K9 and H3-K27, and a loss of methylation at H3-K4. The resultant change in H3 conformation (indicated by dashed line) is proposed to represent a primary level of chromatin silencing that is *Xist* dependent. Subsequently, it is proposed that the primary chromatin configuration recruits other modifying complexes, which lead, for example, to deacetylation of H4 and methylation of DNA. The resulting secondary heterochromatin structure (depicted by H4 dashed line) is proposed to be self-maintaining and, therefore, *Xist* RNA independent. See text for further details.

Eed-Enx1 complexes are components of the machinery required to confer genome plasticity. Thus, like X inactivation during the window of opportunity, silent chromatin at other Eed-Enx1 targets may be reversible if the primary signal (for example hunchback at homeotic loci in *Drosophila*) is removed. This would provide cells of the early embryo with the capacity to activate regions of the genome in response to specific differentiation signals, contrasting with the situation in differentiated cells, where heritable silencing is highly stable and is normally irreversible.

Experimental Procedures

Mouse Strains and Cell Lines

ES cell lines PGK12.1, LF2, Efc1, and LH1 were all grown, maintained, and differentiated as described previously (Norris et al., 1994; Sheardown et al., 1997; Herzing et al., 1997). B1 XX TS cells were grown and maintained as described by Mak et al. (2002). TS cell differentiation was carried out as described by Tanaka et al. (1998).

Tg(Cmu-GFP)1Nagy (XGFP) mice were from Jackson Labs. This strain carries a GFP transgene on the X chromosome, and, when inherited on Xp, it allows for XX and XY embryos to be distinguished (Hadjantonakis et al., 1998). The $Xist^{tnv}$ strain was produced by gene targeting (N.B. and T.B.N., in preparation) and maintained in house. The null mutation was induced by inserting a loxP site 5.9 Kb into the gene, in the opposite orientation to a loxP site retained after deletion of *Xist* exon IV. Deletion of exon IV by itself has no effect on X inactivation. eed^{3354SB} mutant mice were obtained from the Oak Ridge National Laboratory. To genotype adult animals we amplified by PCR a 570 bp fragment from genomic DNA using primers eed3 (tgtgcacctcatcagtctctttt) and eed4 (ggtttgcccccagcctttatg) (annealed at 55°C). The eed^{3354SB} and wild-type alleles could then be distinguished after restriction digestion with Alu1.

Embryo Analysis

Normal preimplantation embryos were obtained from crosses between [C57Bl6 × CBA]F1 females and either [C57Bl6 × CBA]F1 or XGFP males. Embryos were harvested either at 0.5, 2.5, or 3.5 dpc. In some cases embryos isolated at 0.5 and 2.5 dpc were cultured in vitro in M16 medium, as described in Hogan et al. (1994), prior to subsequent analysis. Normal postimplantation stage embryos were obtained from crosses between CD1 or PGK strain females and either PGK strain or XGFP males. Embryos were dissected free of decidua, and, where appropriate, the Reicharts membrane was removed. Embryos from 8.5 dpc and later were dissected free of all membranes.

PCR for sexing/genotype analysis was carried out on DNA from extraembryonic ectoderm or embryo fragments. For analysis of the Tg15 line (Matsuura et al., 1996), Tg15 males were first crossed with XGFP females. Embryos were then obtained from male progeny crossed to [C57Bl6 × CBA]F1 females. Thus, $X^{GFP}X$ females and XY^{Tg15} male embryos could be readily distinguished on the basis of GFP fluorescence.

Antibodies

Characterization of antibodies to Eed, Enx1, Bmi-1, and Hpc2 has been described elsewhere (Satijn et al., 1997; Sewalt et al., 1998; van der Vlag and Otte, 1999). Antisera to di/trimethylH3-K9 are described in Peters et al. (2001). The trimethylH3-K27 antibody is described in detail elsewhere (A.H.F.M.P. and T.J., submitted). Antisera to methylH3-K4 were a kind gift of D. Allis and are described in Boggs et al. (2002). Antisera to HDAC2 and Lamin B were from Santa Cruz Biotechnology. Antisera to acetylH3-K9, acetylH4, and HDAC1 were from Upstate Biotechnology. The following dilutions were used for immunofluorescence (IF) and Western blotting (WB): Eed mouse monoclonal, 1:200 (IF) and 1:1,000 (WB); Enx1 rabbit polyclonal, 1:200 (IF) and 1:2,000 (WB); Bmi-1 rabbit polyclonal, 1:10,000 (WB); Hpc2, 1:500 (WB); Lamin B goat polyclonal, 1:2,000 (WB); HDAC1 rabbit polyclonal, 1:2,000 (WB), HDAC2 rabbit polyclonal, 1:1,000 (WB); methylH3-K9 rabbit polyclonal, 1:1,000 (IF); trimethylH3-K27, 1:500 (IF); methylH3-K4 rabbit polyclonal, 1:1,000 (IF); acetylH3-K9 rabbit polyclonal, 1:200 (IF); acetylH4, 1:200 (IF). Secondary antibodies for immunofluorescence were from Molecular Probes, and those for Western blotting were from Amersham Life Science (anti-rabbit or anti-mouse Ig-HRP) or Dako (anti-goat Ig-HRP).

Western Blot Analysis

Whole-cell extracts were prepared from ES cells, TS cells, and lymphocytes as described by Mermoud et al. (1999). Proteins (approximately 20 μg) were electrophoresed by SDS-PAGE and then transferred to PVDF membranes by wet blotting. ECL detection (Amersham) was carried out according to manufacturer's recommendations.

Immunofluorescence and ImmunoRNA-FISH

Undifferentiated and differentiated ES cell samples were prepared for immunofluorescene as described previously (Mermoud et al., 1999). Immunofluorescence was carried out as described by Mak et al. (2002). For preimplantation embryos and for the 5.5 dpc postimplantation stage, immunofluorescence was carried out by transferring embryos in glass capillaries through wells in round bottom 96-well microtitre dishes. Steps were essentially as described by Mak et al. (2002). After incubation with antibodies, embryos were mounted by transferring into 2 μl drops of Vectashield antifade with DAPI (Vector Laboratories) and placed on glass chamber slides. For 6.5 dpc and later postimplantation stages, dissected embryos (or embryo fragments) were washed in PBS, transferred to 5 μl of trypsin solution, and dissociated into small cell clumps with a fine drawn-glass capillary. After the addition of 95 μl of EC10 medium (Mermoud et al., 1999), cells were placed onto Superfrost Plus glass slides (BDH) and allowed to adhere by incubation in a humidified chamber for approximately 3 hr. Immunofluorescence was then carried out as described in Mak et al. (2002).

ImmunoRNA-FISH was carried out as described by Heard et al. (2001). Details of variations used for specific probe/antibody combinations are available on request. Probes and conditions for detection of nascent transcripts for the *Brx* and *Pgk-1* loci have been described previously (Sheardown et al., 1997; Clerc and Avner, 1998;). Preimplantation embryos were cytospun onto glass slides as described by Costanzi et al. (2000).

The GFP signal in XGFP embryos was detected with a Leica M2FLIII dissection microscope. Slides were analyzed on a Leica TCS-Sp1 confocal microscope and processed with the Leica software and Adobe Photoshop.

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