Epigenetic Defects in Stem Cells Deficient in Polycomb Group Function

Nathan D. Montgomery

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology

Chapel Hill
2007

Approved by:

Advisor: Terry Magnuson, Ph.D.

Reader: Scott Bultman, Ph.D.

Reader: Robert Duronio, Ph.D.

Reader: Fernando Pardo Manuel de Villena, Ph.D.

Reader: Brian Strahl, Ph.D.

ABSTRACT

NATHAN MONTGOMERY: Epigenetic Defects in Stem Cells Deficient in Polycomb Group Function
(Under the direction of Terry Magnuson, Ph.D.)

During development, the expression states of many genes must be maintained through cell divisions in order to ensure lineage-, time-, and dose-appropriate patterns of gene expression. This transcriptional memory is independent of permanent DNA sequences changes and instead involves reversible epigenetic mechanisms. Polycomb Group (PcG) proteins represent a conserved family of developmental regulators that mediate heritable transcriptional silencing by covalently modifying histone proteins. Here, we demonstrate that mutations in the PcG gene *Embryonic ectoderm development* (*Eed*) produce a variety of epigenetic defects in mouse embryos and stem cells. EED is a noncatalytic subunit of Polycomb Repressive Complex 2, a 600 kDa complex containing a number of proteins, including the histone H3-lysine 27 (H3K27) methyltransferase EZH2. Consistent with the role of PcG genes in transcriptional memory, *Eed* mutant embryos and trophoblast stem cells have defects in genomic imprinting, a process by which an allele's expression is dependent upon the gender of the parent from which it was inherited. To determine whether these gene expression defects revealed a required role for EED in PRC2 function, we characterized the status of H3K27 methylation in *Eed* mutant stem cells. H3K7 can be mono-, di-, or trimethylated (H3K27me1, H3K27me2, H3K27me3, respectively), but it has been unclear which of these marks are mediated by

PRC2. Here, we demonstrate that EED is required for all three methylation states. Additionally, although EED is present as four distinct isoforms in mammalian cells, these isoforms are not necessary for H3K27 methylation. Instead, EED's core WD-40 motifs and histone binding domain alone are sufficient to mediate histone methylation. Finally, although the histone methylation defects in *Eed* mutant stem cells appear to be global, the imprinted expression defects are restricted to DNA hypomethylated, extraembryonic tissues and to genes that are imprinted normally in *DNA methyltransferase 1* (*Dnmt1*) mutant placentas. Together, these results suggest that histone methylation and DNA methylation may have non-overlapping roles in imprinted gene regulation.

To Stephanie.

ACKNOWLEDGEMENTS

A number of individuals have made critical contributions to my graduate studies and to this dissertation, and I am deeply grateful to each of them. First, I am indebted to my thesis advisor, Dr. Terry Magnuson, who gave me both the freedom to shape my own project and the guidance to ensure that my efforts remained productive. His support and advocacy have given me abundant opportunities. Additionally, the many members of the Magnuson laboratory have been instrumental to my development as a scientist and to my experience as a graduate student. The intellectual climate in the laboratory provided the most rewarding academic experiences of my life. A few members of the laboratory deserve special mention for direct contributions to my dissertation research. In particular, I am deeply indebted to Ms. Della Yee, whose experimental expertise is hiding behind almost every result in this dissertation. The data are not only mine but also hers. Similarly, much of the work included in Chapter 2 was conducted under the supervision of Dr. Jesse Mager. I have included only that work to which I made important intellectual and experimental contributions, but even so, the reader should recognize that Dr. Mager was the driving force behind the embryo experiments; I merely provided the hands to execute that work.

My graduate experience was enhanced dramatically by fellowship support from the Howard Hughes Medical Institute. Their funds not only paid my tuition, fees, and stipend but also provided resources that gave me opportunities otherwise beyond my

means, including attending meetings and training at the Whitehead Institute in Cambridge, MA. My gratitude for their support is matched by my regret that they have since eliminated the predoctoral fellowship program, a great loss to future graduate students.

Finally, my greatest appreciation is reserved for my wife Stephanie, who is an inspiring scientist and my greatest friend.

PREFACE

The trajectory of scientific understanding is often defined by landmark achievements punctuating periods of far more incremental progress. I have been fortunate to be a graduate student in genetics at an extraordinary time in the field's history, in the immediate aftermath of biology's greatest achievement since cracking life's triplet code. The first working drafts of the human genome were published the same month that I interviewed for admission to the Curriculum in Genetics and Molecular Biology at UNC. These genetic blueprints to life have revolutionized modern biology, and I have been fortunate to have had a front row view of the advances that have followed their publication.

Ironically, during the same era when the amount of curated genomic sequence in public databases was increasing logarithmically, a parallel movement had begun to unravel the molecular mysteries underlying non-DNA based mechanisms of heredity, a field known as epigenetics. Eighteen months before I started my graduate training, Dr. Brian Strahl, who was then a postdoctoral researcher at the University of Virginia but who would later become a member of my thesis committee, published a landmark paper with Dr. David Allis, in which they proposed that covalent modifications on the histone proteins that package our DNA function as a heritable, regulatory code, instructing cells how to respond to the associated genes. Seven months later and less than a year before I arrived at UNC, the molecular players responsible for this code began to be identified

when Dr. Thomas Jenuwein and his colleagues in Vienna reported the identification of the first enzyme, SUV39H1, capable of adding methyl groups to histone tails.

Hence, I started my graduate studies at an exciting time, and already, I had a considerable interest in histone biology. As an undergraduate, I had studied the yeast Chromatin Assembly Factor-I (CAF-I), which is responsible for loading histones onto DNA to produce nucleosomes. Excited by Jenuwein's results as well as a flurry of papers that followed, it was a relatively easy decision to focus my graduate studies on epigenetics. It was clearly a field poised to make important progress on fundamental biological questions.

Whereas my decision to study epigenetics was largely a conscious calculation about the future, my decision to focus specifically on the mouse Polycomb Group gene *Eed* was probably more a consequence of its past. Perhaps no regulator of epigenetic phenomena in the mouse has a richer history than *Eed*. The gene was first uncovered by Drs. Bill and Lee Russell, who identified deletions encompassing *Eed* as part of specific locus testing at Oak Ridge National Laboratory. Later, Dr. Eugene Rinchik generated point mutations that failed to complement those deletions, and in 1996, a postdoctoral researcher in the Magnuson laboratory, Dr. Armin Schumacher, positionally cloned the gene. In between, work by Dr. Salome Waelsch and by Dr. Cindy Faust, another postdoctoral researcher in the Magnuson laboratory, began to reveal the fascinating biology uncovered by these mutations. In short, *Eed* is a rare mouse gene in that it was identified by classical genetic approaches more commonly employed in Drosophila laboratories. That history appealed greatly to me, and I was more than a little enamored by the opportunity to be linked, albeit indirectly by a thesis project, to great geneticists.

Given that background, it is more than a little ironic then that the work that follows is more molecular biology than genetics. Throughout my graduate career, I have tried to go where the science has taken me, even when it has taken me places I did not initially expect to go. After demonstrating with Jesse Mager that *Eed* is required for the imprinted expression of a number of genes in the mouse (Chapter 2), I decided that fundamental, mechanistic questions remained unanswered. Those questions required the tools of molecular biology rather than the tools of genetics. The pages that follow are my attempts to answer those questions.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

LIST OF ABBREVIATIONS

Ascl2	*Achaete-scute complex homolog-like 2*
Cdkn1c	*Cyclin-dependent kinase inhibitor 1c*
cDNA	complementary DNA
CT	Threshold cycle
DMR	Differentially methylated region
Dnmt1	*DNA methyltransferase 1*
E7.5	Embryonic day 7.5 (post fertilization)
Eed	*Embryonic ectoderm development* gene
EED	Embryonic ectoderm development protein
EED-1	EED isoform 1
EED-2	EED isoform 2
EED-3	EED isoform 3
EED-4	EED isoform 4
Eed^{hypo}	*Eed* $^{l7Rn5\text{-}1989SB}$ allele
Eed^{hypo}	Eed $^{l7Rn5\text{-}1989SB}$ protein
Eed^{null}	*Eed* $^{l7Rn5\text{-}3354SB}$ allele
Eed^{null}	Eed $^{l7Rn5\text{-}3354SB}$ protein
$Eed^{-/-}$	Homozygous for the *Eed* $^{l7Rn5\text{-}3354SB}$ allele
ENU	N-ethyl-N-nitrosurea
ES cell	Embryonic stem cell
esc	*extra sex combs* gene

ESC	extra sex combs protein
escl	*extra sex combs like* gene
E(z)	*Enhancer of zeste* gene
E(Z)	Enhancer of zeste protein
Ezh2	*Enhancer of Zeste Homolog 2* gene
EZH2	Enhancer of Zeste Homolog 2 protein
Gapdh	*Glyceraldehyde –3-phosphate dehydrogenase*
H3K9	Histone H3 Lysine 9
H3K27	Histone H3 Lysine 27
HMTase	Histone Methyltransferase
HP1-∝	Heterochromatin Protein 1-alpha
ICR	Imprinting Control Region
Indel	Insertion-deletion polymorphism
kb	Kilobase pairs (nucleotides)
kDa	Kilodalton
Kcnq1ot1	*Kcnq1 overlapping transcript 1*
Kcnq1	*Potassium voltage-gated channel, subfamily Q, member 1*
KvDMR	Differentially methylated region in *Kcnq1*
LOI	Loss of imprinting
MEF	Murine embryonic fibroblast
mes-6	*Maternal effect sterile 6* gene
MES-6	Maternal effect sterile 6 protein
PcG	Polycomb Group

PCR	Polymerase Chain Reaction
PRC1	Polycomb Repressive Complex 1
PRC2	Polycomb Repressive Complex 2
SET domain	Su(var)3-9, E(Z), Trithorax domain.
Suz12	*Supressor of Zeste 12* gene
SUZ12	Supressor of Zeste 12 protein
TS cell	Trophoblast stem cell
Tssc3	*Tumor-suppressing subchromosomal fragment 3*
Tssc4	*Tumor-suppressing subchromosomal fragment 4*
Tssc5	*Tumor-suppressing subchromosomal fragment 5*
Wap-T_{121}	Large T antigen fragment expressed from the *Whey acidic protein* promoter
WD-40	Tryptophan-Aspartic Acid repeat motif
Xi	Inactive X chromosome

CHAPTER 1

BACKGROUND AND INTRODUCTION

1.1 EPIGENETIC INHERITANCE AND THE HISTONE CODE

During development, gene expression states established in progenitor cells are often maintained through cellular divisions in descendant cell populations[1]. This "transcriptional memory" insures lineage-, time-, and dose-appropriate patterns of gene expression. In recent years, considerable effort has been focused towards elucidating the molecular mechanisms underlying transcriptional memory. Of particular importance, the ability to derive viable clones from terminally differentiated somatic cells and the ability of some differentiated cell types to transdifferentiate demonstrate that transcriptional memory is independent of permanent DNA sequence changes and instead involves reversible, non-genetic phenomena[2-4]. Collectively, these DNA sequence independent mechanisms of cellular heredity are known as epigenetics.

Units of epigenetic inheritance are expected to share at least two critical features with DNA, the unit of genetic inheritance. First, they must harbor information, and second, they must be able to be propagated. Distinguishing them from DNA, units of epigenetic inheritance must also be reversible, as epigenetic states are often reset during germline or preimplantation development and after somatic cell nuclear transfer[5]. In recent decades, the molecular mechanisms underlying epigenetic inheritance have begun to be revealed. From this work, it is now clear that two prominent mechanisms of epigenetic regulation are DNA methylation and covalent histone modifications[6]. These mechanisms appear to fulfill the criteria expected of units of epigenetic inheritance.

In many species, the cytosines of CpG dinucleotides are often symmetrically methylated. Methylated cytosines control gene expression states, are propagated by maintenance DNA methyltransferases, and can be reversed by both passive and active

means[6]. Consequently, DNA methylation fulfills the criteria expected for a unit of epigenetic inheritance. However, although DNA methylation is clearly an important epigenetic mark in both plants and mammals, it is unlikely to be the sole unit of epigenetic inheritance. In other species, including the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, little if any DNA methylation is present[7, 8]. Moreover, even in mammals, DNA methylation is not universally required for epigenetic phenomena[9-11]. As a result, alternative mechanisms must also be crucial for epigenetic inheritance.

Histones are small, basic, evolutionarily conserved proteins that associate with eukaryotic DNA to produce DNA-protein complexes called nucleosomes[12]. Each nucleosome consists of an octamer of histone proteins (two each of histones H2A, H2B, H3, and H4) around which approximately 147 bp of DNA is wrapped[13]. Histones have long been appreciated to perform an important function in packaging massive eukaryotic genomes into relatively small eukaryotic nuclei, but more recently, it has become clear that histones also perform important regulatory roles[12].

Diverse types of chemical modifications are found on histone amino-terminal tails, including acetylation, methylation, phosphorylation, poly-ADP ribosylation, sumoylation, and ubiquitination[14]. Recently, combinations of such modifications have been proposed to function as a "histone code" that controls epigenetic states[14, 15]. In this model, histone modifying enzymes, such as histone acetyltransferases and histone methyltransferases (HMTases), write a code that instructs cellular machinery how to respond to the associated DNA. Subsequently, this code is recognized by other proteins, which bind histone tails harboring a specific modification or combination of

modifications, much in the same way that sequence-specific transcription factors bind DNA sequences. These recognition proteins then execute the code originally written by the histone modifying enzymes.

Early support for the histone code came from work with Su(Var) 3-9 Homolog 1 (SUV39H1), Heterochromatin Protein 1 (HP1) and their homologs in yeast and Drosophila. Jenuwein and colleagues demonstrated that SUV39H1 is a HMTase with enzymatic activity directed towards histone H3 at lysine 9 (H3K9)[16]. This catalytic activity resides in an evolutionary conserved domain called the SET domain, which is found in many lysine methyltransferases[17]. Consistent with the histone code hypothesis, after SUV39H1 methylates H3K9, HP1 binds to the modified histone via its chromodomain and promotes higher order chromatin structures that inhibit transcription[18]. In the last decade, the number of enzymes demonstrated to covalently modify histones and the number of proteins shown to bind specifically to modified histones have grown dramatically[19].

In addition to harboring regulatory information, covalent histone modifications appear capable of fulfilling the remaining requirements for a heritable unit of epigenetic inheritance, at least in some cases. First, at least some histone modifications may be propogated through mitotic divisions. Histones H3 and H4 exist as a heterotetramer in intact nucleosmes, and historically, that tetramer was thought to remain intact during DNA replication[20]. This suggests that, unlike DNA replication, histone deposition may not be a semiconservative process, meaning that one sister chromatid may inherit the parental H3/H4 tetramer with appropriate modifications while the other sister chromatid inherits a nascent tetramer lacking those modifications[21]. By itself, this mode of

histone replication appears to be incompatible with histone modifications functioning as a unit of epigenetic inheritance. However, at least in the case of H3K9 methylation, the mark is still propogated. HP1, via its chromoshadow domain, can recruit SUV39H1 to methylate adjacent nucleosomes, potentially propagating H3K9 methylation to nascent histones after replication, provided that multiple, adjacent nucleosomes harbored the modification prior to replication and provided that modified parental tetramers are transmitted to each sister chromatid (Figure 1.1)[22]. Such a mechanism would allow histone modifications to function as primary epigenetic marks, even if H3/H4 tetramers are not inherited semiconservatively. Interestingly, however, more recent data is challenging traditional views of H3/H4 deposition. These studies demonstrate that histone H3 and H4 associated with chromatin assembly complexes exist as dimers, rather than as tetramers, arguing that H3/H4 tetramers may actually separate during DNA replication[23]. If true, histone deposition may be truly semiconservative, and all histone modifications may be faithfully propogated through cell divisions (Figure 1.2). In either case, the competing models both provide compelling mechanisms by which histone modifications may be self-propagating, as required for any primary epigenetic mark. Finally, fulfilling the last requirement for any unit of epigenetic inheritance, covalent histone modifications are typically reversible. For instance, histone deacetylases and histone demethylases remove acetyl groups and methyl groups, respectively, from histones, and in some cases, patterns of histone modifications are erased completely by histone replacement[24-27].

The complexity of the histone code is a consequence not only of the large number of modified residues and the diverse types of chemical modifications found on histones,

but also it is a product of the number of moieties added to a particular residue. For instance, lysines can be mono-, di-, or trimethylated, and potentially, these three methyl states could mediate three distinct biological outcomes[28]. Consistent with this expectation, the chromodomain of HP1 binds trimethylated H3K9 with a binding affinity seven- and ten-fold greater than its affinity for dimethylated and monomethylated H3K9, respectively[29]. Additional support for functional distinctions between lysine methyl states comes from localization data. HP1α colocalizes only with the trimethylated form of H3K9 at pericentric heterochromatin *in vivo*[18, 28]. Additionally, histone H4 trimethylated at lysine 20 is a marker of the pericentric heterochromatin, whereas the monomethylated form of that residue is instead a marker of the inactive X chromosome[30, 31]. Even without knowing the precise functions of all of these marks, these results demonstrate that the number of methyl groups added to a particular nucleosome is a regulated process.

1.2. POLYCOMB GROUP BACKGROUND

One prediction of the histone code hypothesis is that proteins that mediate and recognize histone modifications will have important roles in development. Polycomb Group (PcG) proteins represent one conserved family of developmental regulators that mediate heritable transcriptional silencing by modifying histones[32, 33]. Bona fide PcG genes produce a specific class of developmental defects when mutated in Drosophila[7, 8]. The fly thorax normally consists of three segments, known as T1, T2, and T3. In male flies, the legs of the first thoracic segment, T1, are distinguishable by the presence of characteristic mating structures called sex combs. Mutations in PcG genes transform

the identity of one or more thoracic segments, producing flies with T1-T1-T1 or T1-T1-T3 patterning defects instead of the typical T1-T2-T3 thorax. Accordingly, male PcG mutants have inappropriate sex combs on the legs of their second and even third thoracic segments, which explains the nomenclature of PcG genes, including *Polycomb*, *extra sex combs*, and *Sex combs on the midleg*.

The patterning defects in PcG mutant flies are a consequence of misexpression of homeotic genes in the *Antennapedia* and *bithorax* complexes[7, 8, 34]. During early Drosophila development, homeotic gene expression patterns are established by gap and pair-rule segmentation proteins, which are DNA sequence specific transcription factors. However, expression of gap and pair-rule proteins ceases by mid-embryogenesis. Subsequently, the patterns established by those initiating molecules are maintained by the combined action of PcG proteins, which are required to keep repressed homeotic genes silent in descendant cells, and trithorax group proteins, which are required to keep expressed homeotic genes active in descendant cells. The molecular mechanisms linking gap- and pair rule-mediated initiation to PcG- and trithorax group-mediated maintenance remain elusive.

Homologs of fly PcG genes have been identified in many other species, including plants, nematodes, and mammals, and in each case, the homologous genes appear to play crucial roles in mediating heritable transcriptional silencing[7, 33, 35]. In the mouse, *Mus musculus*, mutations in PcG genes produce patterning defects conceptually similar to the homeotic transformations observed in Drosophila. As in fly, these phenotypes involve misexpression of homeotic genes, often resulting in transformation of various vertebrae to the identity of their anterior or posterior neighbors[36].

1.3. BIOCHEMICAL CHARACTERIZATION OF PcG COMPLEXES

Biochemical characterization of PcG complexes has provided considerable insight into the molecular mechanisms underlying PcG-mediated silencing. In both flies and mammals, PcG proteins exist in two biochemically separable complexes, generally known as Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). Because a number of fly PRC1 genes are duplicated in the mammalian lineage, in the mouse, PRC1 refers to a heterogeneous collection of 2 MDa complexes, which have not been fully characterized[32]. Critical subunits of PRC1 include chromodomain containing proteins (Cbx2, Cbx4, Cbx6, Cbx7, or Cbx8 in mouse and Pc in fly) and ubiquitin E3 ligases (Ring1a or Ring1b in mouse and Sex combs extra in fly)[37]. The latter allows PRC1 to monoubiquitinate histone H2A at lysine 119[38]. While this mark appears essential for PcG-mediated silencing, it is not clear how the presence of monoubiquitin on histone H2A interferes with transcription.

PRC2 is an approximately 600 kDa complex defined in the mouse by the presence of the SET-domain containing histone methyltransferase EZH2 (fly E(Z)), the WD-repeat protein EED (fly ESC), the Zn-finger protein SUZ12 (fly Su(Z)12), and additional proteins, including histone deacetylases, which appear to be at least transiently associated with the complex[39-41]. EZH2 and its homologs in fly and nematode have all been shown to methylate histone H3 at lysine 27 (H3K27)[40-44]. However, the functions of the other, noncatalytic subunits are less clear. *In vitro* EZH2 lacks HMTase activity in the absence of EED and SUZ12, indicating that these subunits have some undefined role in EZH2-mediated histone methylation[45]. Additionally, the N-terminus of both EED

and ESC appear to bind histones, and fly Su(Z)12 may be necessary for PRC2 association with chromatin[46, 47].

In mammals, the composition of PRC2 is complicated by the presence of four distinct isoforms of the noncatalytic subunit EED[48, 49]. These isoforms are thought to be produced by utilizing four, in-frame translational start sites in a common *Eed* mRNA. Although most eukaryotic proteins initiate translation at canonical methionine-encoding AUG codons, an increasing number of proteins are known to initiate translation at non-AUG codons. Often, these noncanonical initiation sites produce upstream isoforms of proteins also translated from downstream AUG codons, and generally, the noncanonical start codons differ from the canonical AUG sequence at only one nucleotide position[50, 51]. The putative EED isoform start sites are proposed to conform with both of these general trends; EED-1 and EED-2 are thought to be translated from non-canonical, upstream GUG codons at nucleotide positions 169-171 and 274-276 in the *Eed* mRNA, and EED-3 and EED-4 are believed to be translated from downstream, AUG codons at positions 454-456 and 496-498, respectively (Figure1.3)[48, 52]. However, these sites were proposed on the basis of limited, *in vitro* studies utilizing a rabbit reticulocyte, cell-free translation system[52]. At the outset of this work, EED translational start sites had not been characterized in living cells.

The functions of the four EED isoforms remain unclear, although isoform usage appears to be developmentally regulated. In particular, EED-2 has been reported to be expressed only in undifferentiated stem cells and in tumor tissue, suggesting a potential role for this isoform in pluripotency[49]. Mechanistically, initial *in vitro* studies suggested that EED isoforms may control the substrate specificity of EZH2. Specifically,

EED-1 and EED-2 were suggested to direct EZH2 activity towards histone H1 at lysine 26, while EED-3 and EED-4 were reported to direct EZH2 activity towards the conventional H3K27 substrate[48]. However, subsequent *in vitro* studies failed to confirm these results, and to date, the only target of EZH2 or its homologs that has been confirmed *in vivo* is H3K27[53].

H3K27 can be mono-, di-, or trimethylated (H3K27me1, H3K27me2, and H3K27me3, respectively)[28]. While the functions of the former two marks remain unclear, H3K27me3 alone appears to function as a binding platform for PRC1 chromodomain containing proteins, in much the same way that trimethylated H3K9 recruits HP1 binding[29, 54]. Support for this conclusion comes from both *in vitro* and *in vivo* work. *In vitro*, the binding affinity of fly Pc for H3K27me3 is four- to five-fold greater than its affinity for H3K27me1 or H3K27me2[29]. *In vivo*, Pc and H3K27me3 staining largely colocalize on fly polytene chromosomes, but Pc and H3K27me2 do not[55]. Additionally, Pc can be competed away from chromatin by trimethylated H3K27 peptides but not by dimethylated H3K27 peptides[55]. Hence, considerable evidence implicates H3K27me3 in recruiting PRC1. However, the relationship between PRC1 and H3K27me3 appears to be more complicated than the relationship between HP1 and H3K9me3. Even where PRC1 and H3K27me3 colocalize cytologically, that colocalization is limited to only a subset of H3K27 trimethylated nucleosomes[56]. Finally, at the outset of this work, it was unclear whether PRC2 alone was capable of mediating mediating H3K27 methylation, or whether other complexes could mediate or were even required for one or more of the H3K27 methylation states.

Despite extensive characterization of PcG complexes, it remains unclear how PcG proteins and PcG-mediated histone modifications inhibit transcription. It is generally assumed that PcG proteins condense local chromatin environments to block access to the transcriptional machinery, and in fact, preincubating nucleosomal arrays with PRC1 blocks SWI/SNF-mediated chromatin remodeling and transcription *in vitro*, suggesting that the downstream-acting PcG complex mediates a chromatin state that is refractory to transcription[37, 57]. More recently, reconstituted PRC1 complexes were shown to physically compact a chromatinized template[58]. However, because PRC1 proteins were present at extremely high concentrations in all of those experiments, it remains unclear whether PcG proteins are able to condense chromatin under physiological conditions, and no existing data in living cells confirms this conclusion[32]. In fact, chromatin immunoprecipitation experiments demonstrate that RNA polymerase II localizes to PcG-silenced promoters, implying that PcG-mediated repression is not simply a consequence of promoter inaccessibility[59]. As an alternative to local chromatin condensation, PcG proteins could interfere with cellular machinery required for transcription. Fly PRC1 copurifies with TBP-associated factors (TAFs), suggesting that PcG proteins might inhibit transcription by directly associating with components of the generation transcription machinery[60]. However, similar interactions have not been observed in mammalian cells[32]. Finally, PcG-mediated histone modifications could directly impact transcription. Although PRC2-mediated H3K27me3 may function primarily to recruit PRC1, the function of PRC1-mediated H2A ubiquitination is unclear. It is possible that this downstream mark interferes directly with transcription by some unknown mechanism.

Whatever mechanism explains PcG-mediated silencing, it is unlikely to involve a spreading mechanism comparable to position effect variegation in the fly or SIR-mediated silencing in yeast. In flies, PcG proteins are recruited by sequence-specific DNA binding proteins to Polycomb Response Elements (PREs)[61]. Similar elements have not been identified in mammals, where little is known about targeted recruitment of PcG proteins. Although a single insulator placed between a PRE and a promoter blocks PcG-mediated silencing, this block is bypassed when an even number of insulators separates the PRE and the promoter, implying that PcG proteins do not track along the DNA from their recruitment site to their target[62].

1.4. CHARACTERIZATION OF *EED* FUNCTION *IN VIVO*

The *in vivo* functions of mammalian PRC2 have been revealed largely by work with mice and mouse embryos harboring mutations in *Eed*, *Ezh2*, and *Suz12*. Of these, an allelic series of mutations in *Eed* has been particularly revealing. Deletions encompassing *Eed* were first generated by William and Lee Russell at Oak Ridge National Laboratory, as part of specific locus tests designed to characterize the dangers of ionizing radiation in mammals[63, 64]. *Eed* is tightly linked to *Tyrosinase (Tyr)*, a mouse coat color gene used as a visible marker in the Russells' specific locus test, and lesions removing *Tyr* frequently deleted *Eed* as well. Mapping deletion breakpoints around *Tyr* identified six regions required for mouse viability, including one region that caused lethality at embryonic day 8.5 (E8.5) when deleted[63, 65, 66]. Subsequently, using the chemical mutagen N-ethyl-N-nitrosurea (ENU), point mutations were generated that failed to complement this deletion phenotype[67, 68]. Mice harboring these point

mutations were then used to positionally clone the gene responsible for the E8.5 lethality in the deletion mutants. That gene, now known as *Embryonic ectoderm development* (*Eed*), is a five-WD repeat protein homologous to the fly PcG gene *esc*[69].

The phenotypic consequences of two ENU-generated *Eed* point mutations have been characterized extensively. $Eed^{l7Rn5\text{-}3354SB}$ is a leucine-to-proline substitution in the third of EED's five confirmed WD-40 motifs (Figure 1.3)[69]. $Eed^{l7Rn5\text{-}3354SB}$ homozygous embryos appear to recapitulate the phenotype caused by *Eed* deletion mutations, suggesting that the $Eed^{l7Rn5\text{-}3354SB}$ is a null allele (as a result, $Eed^{l7Rn5\text{-}3354SB}$ will subsequently be referred to as Eed^{null} or Eed^{-})[70]. Supporting the possibility that EED is absolutely required for PRC2 function, $Eed^{null/null}$ embryos arrest at perigastrulation stages comparable to the stages at which *Ezh2* and *Suz12* mutant embryos also arrest[71, 72].

Consistent with *Eed*'s classification as a mouse PcG gene, animals homozygous for a second, hypomorphic *Eed* allele, $Eed^{l7Rn5\text{-}1989SB}$ (herein referred to as Eed^{hypo}) exhibit segmental patterning defects reminiscent of the homeotic transformations observed in *esc* mutant embryos[69, 73]. In this mutation, a nonpolar isoleucine is converted to a polar asparagine in the same WD-40 motif in which the substitution caused by the Eed^{null} allele resides (Figure 1.3)[69]. On outbred backgrounds, $Eed^{hypo/hypo}$ animals are generally viable but runted.[68, 69] As with fly PcG mutant patterning defects, the homeotic transformations associated with the hypomorphic allele are a consequence of shifted homeotic gene expression boundaries[73, 74]. $Eed^{hypo/null}$ compound heterozygotes exhibit an intermediate phenotype, with homeotic expression defects identical to $Eed^{hypo/hypo}$ animals and with midgestation lethality, due to the absence of secondary trophoblast giant cells.

In addition to regulating anterior-posterior patterning, mouse PcG genes also appear to play important roles in epigenetic regulation of additional targets, most notably the inactive X chromosome (Xi). In mammals, XX female mammals achieve dosage compensation by inactivating one of their two X chromosomes. In mouse extraembryonic tissues as well as in preimplantation mouse embryos, the paternal X chromosome is preferentially, if not universally, silenced[75]. As a consequence, X-chromosome inactivation in mouse extraembryonic tissues is "imprinted", meaning that one of the two X-chromosomes must inherit an epigenetic mark dictating either that the paternal X-chromosome be silenced or that that maternal X-chromosome remain active. Mutations in the mouse PcG gene *Eed* lead to aberrant reactivation of the normally silent paternal X chromosome in a subset of extraembryonic cells, indicating that PcG proteins are required for the maintenance of imprinted X-chromosome inactivation[74, 76]. Additionally, PcG proteins colocalize with the Xi, as do H3K27me3 and ubiquitinated histone H2A, histone modifications associated with PcG activity[77-79].

In mammals, a number of autosomal genes are also imprinted, and the demonstration that *Eed* is required for imprinted X-chromosome inactivation led to an examination of *Eed*'s role in the regulation of autosomal imprinted genes. That work is presented in Chapter 2. Chapters 3 and 4 detail mechanistic studies addressing the molecular details of EED function. In chapter 3, work is presented characterizing EED's role in H3K27 methylation. In chapter 4, that work is extended by mapping regions of the EED protein required to mediate H3K27 methylation.

1.5. REFERENCES

1. Brock, H.W., and Fisher, C.L. (2005). Maintenance of gene expression patterns. Dev Dyn *232*, 633-655.

2. Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature *385*, 810-813.

3. Eggan, K., Baldwin, K., Tackett, M., Osborne, J., Gogos, J., Chess, A., Axel, R., and Jaenisch, R. (2004). Mice cloned from olfactory sensory neurons. Nature *428*, 44-49.

4. Tosh, D., and Slack, J.M. (2002). How cells change their phenotype. Nat Rev Mol Cell Biol *3*, 187-194.

5. Dean, W., Santos, F., and Reik, W. (2003). Epigenetic reprogramming in early mammalian development and following somatic nuclear transfer. Semin Cell Dev Biol *14*, 93-100.

6. Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet *3*, 662-673.

7. Bultman, S., Montgomery, N., and Magnuson, T. (2004). Chromatin-Modifying Factors and Transcriptional Regulation During Development. In Handbook of Stem Cells, Volume 1, 1st Edition, R. Lanza, ed. (San Diego, CA: Elsevier), pp. 63-91.

8. Montgomery, N.D., Magnuson, T., and Bultman, S. (2006). Epigenetic Mechanisms of Cellular Memory During Development. In Essentials of Stem Cell Biology, 1st Edition, R. Lanza, ed. (San Diego: Elsevier), pp. 69-80.

9. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004). Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet *36*, 1291-1295.

10. Tanaka, M., Puchyr, M., Gertsenstein, M., Harpal, K., Jaenisch, R., Rossant, J., and Nagy, A. (1999). Parental origin-specific expression of Mash2 is established at the time of implantation with its imprinting mechanism highly resistant to genome-wide demethylation. Mech Dev *87*, 129-142.

11. Sado, T., Fenner, M.H., Tan, S.S., Tam, P., Shioda, T., and Li, E. (2000). X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. Dev Biol *225*, 294-303.

12. Kornberg, R.D., and Lorch, Y. (1999). Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. Cell *98*, 285-294.

13. Luger, K., and Richmond, T.J. (1998). The histone tails of the nucleosome. Curr Opin Genet Dev *8*, 140-146.

14. Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

15. Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

16. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature *406*, 593-599.

17. Dillon, S.C., Zhang, X., Trievel, R.C., and Cheng, X. (2005). The SET-domain protein superfamily: protein lysine methyltransferases. Genome Biol *6*, 227.

18. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature *410*, 116-120.

19. Margueron, R., Trojer, P., and Reinberg, D. (2005). The key to development: interpreting the histone code? Curr Opin Genet Dev *15*, 163-176.

20. Leffak, I.M., Grainger, R., and Weintraub, H. (1977). Conservative assembly and segregation of nucleosomal histones. Cell *12*, 837-845.

21. Henikoff, S., Furuyama, T., and Ahmad, K. (2004). Histone variants, nucleosome assembly and epigenetic inheritance. Trends Genet *20*, 320-326.

22. Richards, E.J., and Elgin, S.C. (2002). Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. Cell *108*, 489-500.

23. Tagami, H., Ray-Gallet, D., Almouzni, G., and Nakatani, Y. (2004). Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. Cell *116*, 51-61.

24. Holbert, M.A., and Marmorstein, R. (2005). Structure and activity of enzymes that remove histone modifications. Curr Opin Struct Biol *15*, 673-680.

25. Takeuchi, T., Watanabe, Y., Takano-Shimizu, T., and Kondo, S. (2006). Roles of jumonji and jumonji family genes in chromatin regulation and development. Dev Dyn *235*, 2449-2459.

26. Ahmad, K., and Henikoff, S. (2002). The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly. Mol Cell *9*, 1191-1200.

27. Klose, R.J., Kallin, E.M., and Zhang, Y. (2006). JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet *7*, 715-727.

28. Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H., and Jenuwein, T. (2003). Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol Cell *12*, 1577-1589.

29. Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev *17*, 1870-1881.

30. Schotta, G., Lachner, M., Sarma, K., Ebert, A., Sengupta, R., Reuter, G., Reinberg, D., and Jenuwein, T. (2004). A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev *18*, 1251-1262.

31. Kohlmaier, A., Savarese, F., Lachner, M., Martens, J., Jenuwein, T., and Wutz, A. (2004). A chromosomal memory triggered by Xist regulates histone methylation in X inactivation. PLoS Biol *2*, E171.

32. Schwartz, Y.B., and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet *8*, 9-22.

33. Cao, R., and Zhang, Y. (2004). The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev *14*, 155-164.

34. Gebuhr, T.C., Bultman, S.J., and Magnuson, T. (2000). Pc-G/trx-G and the SWI/SNF connection: developmental gene regulation through chromatin remodeling. Genesis *26*, 189-197.

35. Calonje, M., and Sung, Z.R. (2006). Complexity beneath the silence. Curr Opin Plant Biol *9*, 530-537.

36. Gould, A. (1997). Functions of mammalian Polycomb group and trithorax group related genes. Curr Opin Genet Dev *7*, 488-494.

37. Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C.T., Bender, W., and Kingston, R.E. (1999). Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell *98*, 37-46.

38. Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873-878.

39. van der Vlag, J., and Otte, A.P. (1999). Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet *23*, 474-478.

40. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev *16*, 2893-2905.

41. Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197-208.

42. Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185-196.

43. Bender, L.B., Cao, R., Zhang, Y., and Strome, S. (2004). The MES-2/MES-3/MES-6 complex and regulation of histone H3 methylation in C. elegans. Curr Biol *14*, 1639-1643.

44. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039-1043.

45. Cao, R., and Zhang, Y. (2004). SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell *15*, 57-67.

46. Tie, F., Stratton, C.A., Kurzhals, R., and Harte, P.J. (2007). The N-Terminus of Drosophila ESC Binds Directly to Histone H3 and is Required for E(Z)-Dependent Trimethylation of H3 Lysine 27. Mol Cell Biol.

47. Nekrasov, M., Wild, B., and Muller, J. (2005). Nucleosome binding and histone methyltransferase activity of Drosophila PRC2. EMBO Rep *6*, 348-353.

48. Kuzmichev, A., Jenuwein, T., Tempst, P., and Reinberg, D. (2004). Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell *14*, 183-193.

49. Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T.S., Scher, M., Kirmizis, A., Ouyang, X., Brockdorff, N., Abate-Shen, C., Farnham, P., and Reinberg, D. (2005). Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A *102*, 1859-1864.

50. Touriol, C., Bornes, S., Bonnal, S., Audigier, S., Prats, H., Prats, A.C., and Vagner, S. (2003). Generation of protein isoform diversity by alternative initiation of translation at non-AUG codons. Biol Cell *95*, 169-178.

51. Tikole, S., and Sankararamakrishnan, R. (2006). A survey of mRNA sequences with a non-AUG start codon in RefSeq database. J Biomol Struct Dyn *24*, 33-42.

52. Denisenko, O.N., and Bomsztyk, K. (1997). The product of the murine homolog of the Drosophila extra sex combs gene displays transcriptional repressor activity. Mol Cell Biol *17*, 4707-4717.

53. Martin, C., Cao, R., and Zhang, Y. (2006). Substrate preferences of the EZH2 histone methyltransferase complex. J Biol Chem *281*, 8365-8370.

54. Min, J., Zhang, Y., and Xu, R.M. (2003). Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. Genes Dev *17*, 1823-1828.

55. Ringrose, L., Ehret, H., and Paro, R. (2004). Distinct contributions of histone H3 lysine 9 and 27 methylation to locus-specific stability of polycomb complexes. Mol Cell *16*, 641-653.

56. Schwartz, Y.B., Kahn, T.G., Nix, D.A., Li, X.Y., Bourgon, R., Biggin, M., and Pirrotta, V. (2006). Genome-wide analysis of Polycomb targets in Drosophila melanogaster. Nat Genet *38*, 700-705.

57. King, I.F., Francis, N.J., and Kingston, R.E. (2002). Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. Mol Cell Biol *22*, 7919-7928.

58. Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science *306*, 1574-1577.

59. Dellino, G.I., Schwartz, Y.B., Farkas, G., McCabe, D., Elgin, S.C., and Pirrotta, V. (2004). Polycomb silencing blocks transcription initiation. Mol Cell *13*, 887-893.

60. Saurin, A.J., Shao, Z., Erdjument-Bromage, H., Tempst, P., and Kingston, R.E. (2001). A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. Nature *412*, 655-660.

61. Muller, J., and Kassis, J.A. (2006). Polycomb response elements and targeting of Polycomb group proteins in Drosophila. Curr Opin Genet Dev *16*, 476-484.

62. Comet, I., Savitskaya, E., Schuettengruber, B., Negre, N., Lavrov, S., Parshikov, A., Juge, F., Gracheva, E., Georgiev, P., and Cavalli, G. (2006). PRE-mediated bypass of two Su(Hw) insulators targets PcG proteins to a downstream promoter. Dev Cell *11*, 117-124.

63. Gluecksohn-Waelsch, S. (1979). Genetic control of morphogenetic and biochemical differentiation: lethal albino deletions in the mouse. Cell *16*, 225-237.

64. Russell, L.B., Russell, W.L., and Kelly, E.M. (1979). Analysis of the albino-locus region of the mouse. I. Origin and viability. Genetics *91*, 127-139.

65. Niswander, L., Yee, D., Rinchik, E.M., Russell, L.B., and Magnuson, T. (1988). The albino deletion complex and early postimplantation survival in the mouse. Development *102*, 45-53.

66. Rinchik, E.M., Carpenter, D.A., and Selby, P.B. (1990). A strategy for fine-structure functional analysis of a 6- to 11-centimorgan region of mouse chromosome 7 by high-efficiency mutagenesis. Proc Natl Acad Sci U S A *87*, 896-900.

67. Rinchik, E.M., and Carpenter, D.A. (1993). N-ethyl-N-nitrosourea-induced prenatally lethal mutations define at least two complementation groups within the embryonic ectoderm development (eed) locus in mouse chromosome 7. Mamm Genome *4*, 349-353.

68. Wang, J., Tie, F., Jane, E., Schumacher, A., Harte, P.J., and Magnuson, T. (2000). Mouse homolog of the Drosophila Pc-G gene esc exerts a dominant negative effect in Drosophila. Genesis *26*, 67-76.

69. Schumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *384*, 648.

70. Holdener, B.C., Rinchik, E.M., and Magnuson, T. (1995). Phenotypic and physical analysis of a chemically induced mutation disrupting anterior axial development in the mouse. Mamm Genome *6*, 474-475.

71. O'Carroll, D., Erhardt, S., Pagani, M., Barton, S.C., Surani, M.A., and Jenuwein, T. (2001). The polycomb-group gene Ezh2 is required for early mouse development. Mol Cell Biol *21*, 4330-4336.

72. Pasini, D., Bracken, A.P., Jensen, M.R., Denchi, E.L., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J *23*, 4061-4071.

73. Wang, J., Mager, J., Schnedier, E., and Magnuson, T. (2002). The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. Mamm Genome *13*, 493-503.

74. Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J.C., Nagy, A., and Magnuson, T. (2001). Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat Genet *28*, 371-375.

75. Sado, T., and Ferguson-Smith, A.C. (2005). Imprinted X inactivation and reprogramming in the preimplantation mouse embryo. Hum Mol Genet *14 Spec No 1*, R59-64.

76. Kalantry, S., and Magnuson, T. (2006). The Polycomb group protein EED is dispensable for the initiation of random X-chromosome inactivation. PLoS Genet *2*, e66.

77. Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

78. Mak, W., Baxter, J., Silva, J., Newall, A.E., Otte, A.P., and Brockdorff, N. (2002). Mitotically stable association of polycomb group proteins eed and enx1 with the inactive x chromosome in trophoblast stem cells. Curr Biol *12*, 1016-1020.

79. Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jenuwein, T., Otte, A.P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481-495.

FIGURE 1.1. Model for the propagation of histone marks from intact parental H3H4 tetramers

(Top Panel) A series of nucleosomes with methylated H3K9 (red triangle), a mark bound specifically by HP1. For simplicity, only histones H3 and H4 are shown.
(Middle Panel) After replication, modified, parental tetramers are inherited intact. Modified, parental tetramers and unmodified, nascent tetramers are distributed to both sister chromatids. HP1's chromoshadow domain binds SUV39H1, recruiting that H3K9 HMTase to methylate the unmodified, nascent tetramers. For simplicity, spreading of H3K9 methylation is shown unidirectionally.
(Bottom Panel) After SUV39H1 propagates the mark, both sister chromatids harbor the parental chromatin profile, allowing the epigenetic state to be inherited by both daughter cells after cell division.

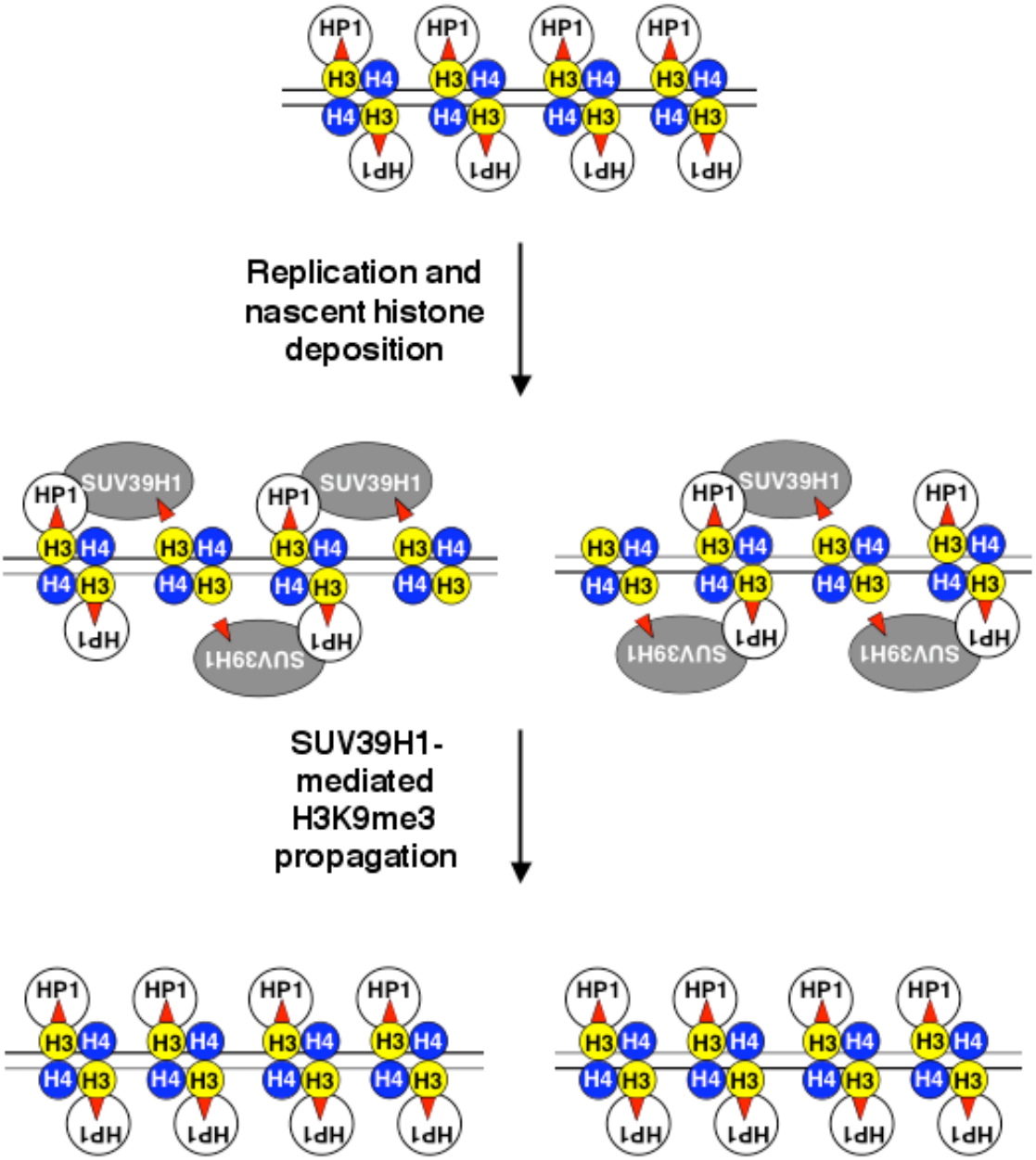

HP1
H3
H4
Replication and nascent histone deposition
SUV39H1
SUV39H1-mediated H3K9me3 propagation

FIGURE 1.2. Model for the propagation of histone marks from disassembled H3H4 tetramers

(Top Panel) A series of nucleosomes with methylated H3K9 (red triangle), a mark bound specifically by HP1. For simplicity, only histones H3 and H4 are shown.

(Second Panel) During replication, parental H3/H4 tetramers are disassembled to form H3/H4 dimers, which mix with nascent, unmodified H3/H4 dimers to generate tetramers containing both parental, modified histones and nascent, unmodified histones.

(Third panel) Hybrid nucleosomes retain the parental modification on one H3 in each tetramer. This mark is propagated to the nascent histones in the same or neighboring nucleosomes by SUV39H1 which is recruited by its association with HP1's chromodomain. For simplicity, spreading of H3K9 methylation is shown unidirectionally.

(Bottom Panel) After SUV39H1 propagates the mark, both sister chromatids harbor the parental chromatin profile, allowing the epigenetic state to be inherited by both daughter cells after cell division.

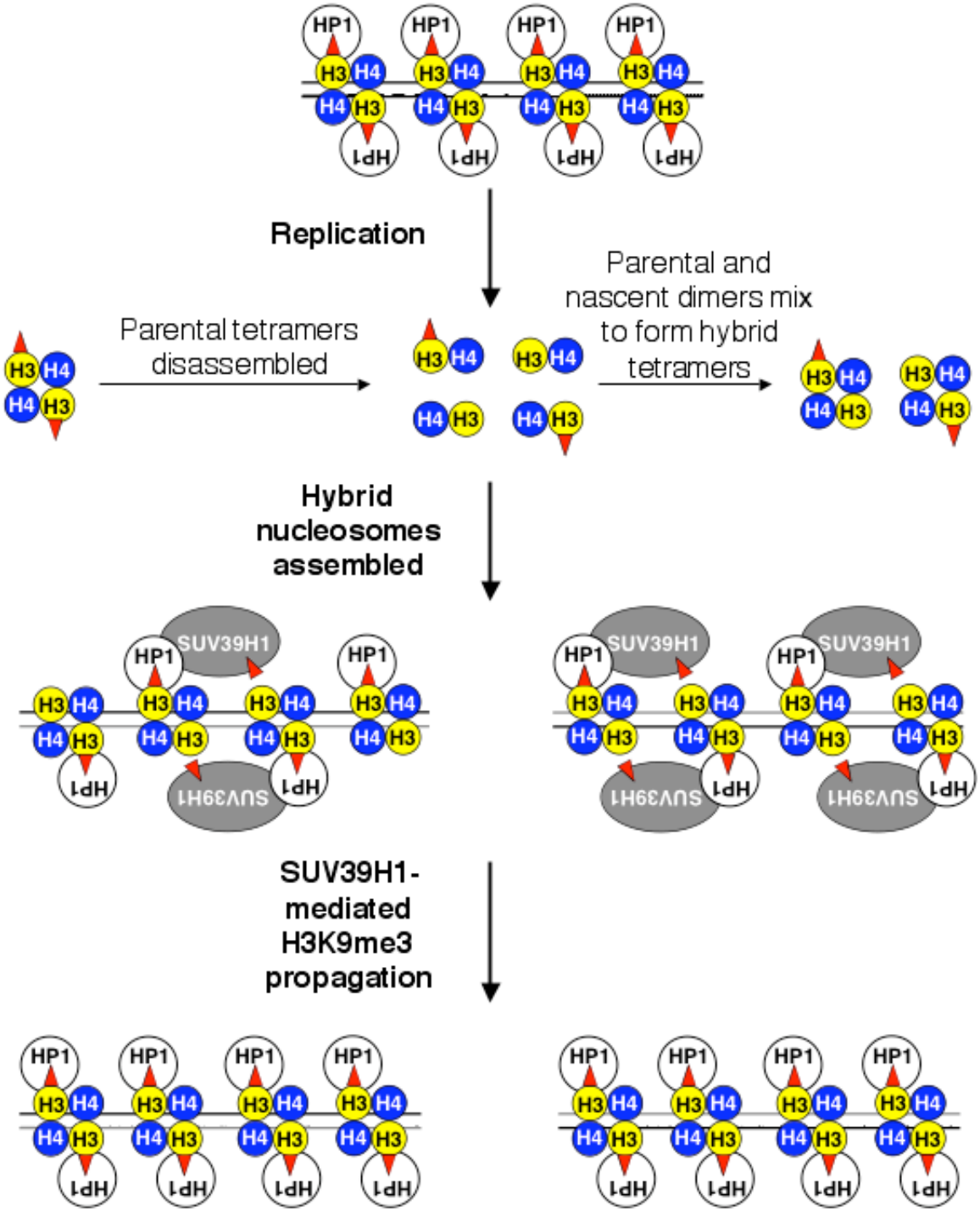
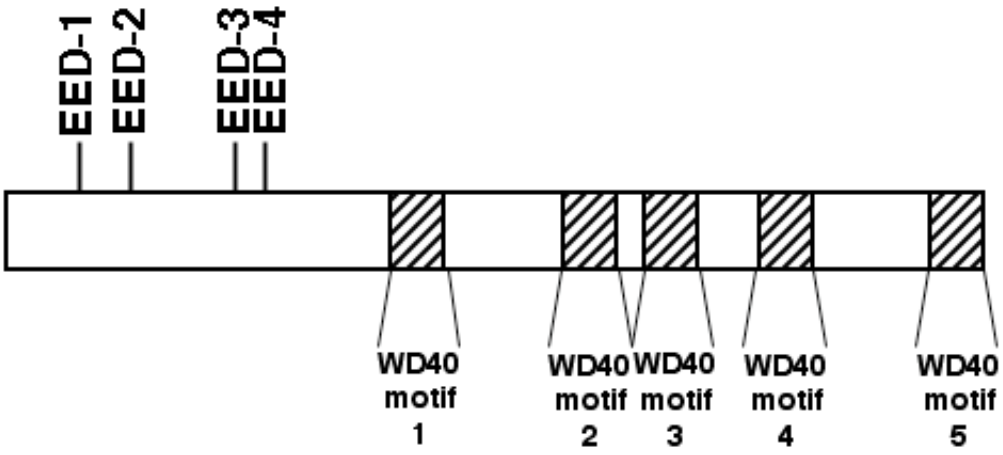

HP1
H3
H4
Replication
Parental tetramers disassembled
Parental and nascent dimers mix to form hybrid tetramers
Hybrid nucleosomes assembled
SUV39H1
SUV3 9H1
SUV39H1-mediated H3K9me3 propagation

FIGURE 1.3. Putative EED translation start sites and WD-40 motifs

EED isoform translation start sites proposed on the basis of *in vitro* translation studies are indicated as EED-1, -2, -3, and -4, respectively [52]. These numbers refer to EED start sites proposed by Denisenko and Bomsztyk to reside at mRNA positions GUG 169-171, GUG 274-276, AUG 454-456, and AUG 496-498. Diagonally-hatched boxes refer to EED-40 motifs at sequences 721-808 (WD-40 motif 1), 1012-1105 (WD-40 motif 2), 1150-1240 (WD-40 motif 3), 1330-1444 (WD-40 motif 4), and 1672-1762 (WD-40 motif 5). (Figure not drawn to scale)


EED-1
EED-2
EED-3
EED-4
WD40 motif 1
WD40 motif 2
WD40 motif 3
WD40 motif 4
WD40 motif 5

CHAPTER 2

IMPRINTING DEFECTS IN *EED* MUTANT EMBRYOS AND CELLS

A portion of the work in this chapter was published in *Nature Genetics* 33(4): 502-507.

2.1. INTRODUCTION

In mammals, maternally-inherited and paternally-inherited genomes are functionally nonequivalent due to the presence of at least 80 imprinted autosomal genes and the imprinted X-chromosome [1]. Unlike most genes, imprinted genes are monoallelically expressed in a parent-of-origin dependent manner, and as a consequence, both parental genomes are required for normal mammalian development and physiology [2-4].

Most imprinted genes reside in clusters that are co-regulated by imprinting control regions (ICR) [5]. These ICRs harbor germline imprints controlling the expression of nearby imprinted genes and are often identifiable by distinct germline DNA methylation patterns in eggs and in sperm. Imprinting defects in various DNA methyltransferase mutant mice demonstrate that these and other, post-zygotic differentially methylated regions (DMRs) are important for imprinted gene expression [6-10]. However, the mechanisms controlling imprinted gene expression are poorly understood and may differ between clusters.

One of the most striking examples of imprinting in mammals is imprinted X-chromosome inactivation. Female mammals achieve dosage compensation by inactivating one of their two X-chromosomes [11]. In marsupials and monotremes and also in mouse preimplantation embryos and extraembryonic tissues, X-chromosome inactivation is imprinted, and the paternally inherited X-chromosome is preferentially inactivated [12]. Studies on the inactive X-chromosome (Xi) have been particularly

revealing in the effort to elucidate molecular mechanisms underlying transcriptional silencing in general and imprinting in particular, because the size of the Xi allows proteins enriched on that chromosome to be identified cytologically. These studies suggest that transcriptional silencers identified in other systems are frequently enriched on the inactive X-chromosome. For instance, PcG proteins and PcG-mediated histone modifications are both found on the Xi, and the PcG gene *Eed* is required to maintain imprinted X-chromosome inactivation in a subset of mouse extraembryonic cells and in differentiating trophoblast stem cells, confirming that PcG-mediated silencing is functionally necessary for imprinted X-chromosome inactivation in those cells [13-17].

Less is known about the molecular mechanisms controlling regulation of autosomal imprinted genes. The imprinted genes on distal mouse chromosome 7 represent one of the best studied examples of autosomal imprinted genes in the mouse. At least fifteen imprinted genes are found in a 1.2 Mb region of this chromosome. These 15 genes are part of two separable imprinting clusters, which employ distinct regulatory mechanisms and are defined by distinct germline ICRs [5]. Imprinting of the more proximal *H19/Igf2* cluster appears to be regulated by DNA-methylation sensitive and allele-specific binding of the insulator CTCF, which blocks of the association of several genes with an upstream enhancer on the maternal but not on the paternal chromosome [18, 19].

Imprinting at the more distal *KvDMR* cluster appears to be more complicated. The *KvDMR* cluster contains ten maternally expressed genes and one paternally expressed gene spread over 800 kb (Figure 2.1). The most proximal of these genes, *Ascl2*, sits less than 300 kb from *Ins2*, the most distal gene in the *H19/Igf2* cluster, but

regulation of the two clusters is completely independent, with deletion of the germline ICRs in either cluster having no impact on imprinted expression in the other cluster [20, 21]. The regulation of the *KvDMR* cluster appears to share a number of conceptual and mechanistic similarities with imprinted X-chromosome inactivation. First, although DNA methylation plays a prominent role in imprinting at other clusters and in the random X-chromosome inactivation that occurs in mouse embryonic tissues, neither imprinted X-chromosome inactivation nor imprinted expression of a number of genes in the *KvDMR* cluster are disrupted in *DNA methyltransferase 1* (*Dnmt1*) mutant extraembryonic tissues [22-24]. Additionally, both processes are dependent on *cis*-acting, non-coding RNAs. *Xi specific transcript* (*Xist*) is required in *cis* to silence genes on the Xi, and *Kcnq1ot1* expressed from the paternal allele is similarly required to silence the neighboring, paternal alleles of the protein-coding genes in the *KvDMR* cluster [21, 25, 26]. Given the requirement for *Eed* in imprinted X-chromosome inactivation and given the similarities between the Xi and the *KvDMR* cluster, we hypothesized that PRC2 might also be required for imprinting at this autosomal locus.

2.2. METHODS AND MATERIALS

Cell lines and cultures

CD1.JF1 (F1) hybrid trophoblast stem (TS) cells lines 5-4 (*Eed*$^{+/null}$) and 3-5 (*Eed*$^{null/null}$) were grown on irradiated fibroblast feeders as previously described [27, 28]. To differentiate, these cells were removed from Fgf4 and Activin. Before harvesting for expression analyses, cells were passaged at least twice in the absence of feeders to avoid contamination.

Expression analyses

RNA was isolated from wild-type or *Eed*$^{null/null}$ E7.5 embryos, wild-type or *Eed*$^{hypo/null}$ E9.5 embryos, and wild-type or *Eed*$^{null/null}$ TS cells using TRIzol® Reagent (Invitrogen). RNA was extracted from the TRIzol® lysate in phenol-chloroform and precipitated with an equal volume of isopropanol. Precipitated nucleic acids were washed in 70% ethanol and resuspended in sterile water. In order to eliminate contaminating genomic DNA, RNA preparations were incubated for one hour at 37º C with DNase (Ambion).

cDNA was prepared using SuperScript™ II Reverse Transcriptase (Invitrogen) and then subjected to RT-PCR. Whenever possible, intron-spanning amplicons were utilized to avoid DNA contamination. After RT-PCR, single nucleotide polymorphisms were discriminated by sequencing or restriction digest as indicated below. Insertion/deletion polymorphisms were discriminated by single strand conformation polymorphism (SSCP) analysis or by non-denaturing high performance liquid chromatography (HPLC). For SSCP analysis, RT-PCR reactions were spiked with ^{32}P-dCTP and denatured by boiling in formamide. Then, reactions were loaded onto acrylamide gels (0.5X MDE, 0.6X TBE,

0.04% TEMED, 0.04% APS) and run overnight. For HPLC analysis of indels, PCR reactions are run over an affinity column and then eluted with increasing concentrations of acetonitrile.

Primers and assays utilized for these experiments are show below.

Ascl2
Fwd: TTTCCAGTTGGTTAGGGGGC
Rev: GGGACAGAGGTCATCTTTATTGTGC
Eed$^{null/null}$ Embryo Assay: Direct sequencing
CD1: A at position 86 beginning with the 5' end of the reverse primer
JF1: G at position 86 beginning with the 5' end of the reverse primer
Eed$^{hypo/null}$ Embryo and *Eed*$^{null/null}$ TS Cell Assay: Direct sequencing
CD1: T at position 290 beginning with the 5' end of the forward primer
JF1: C at position 290 beginning with the 5' end of the forward primer

Cdkn1c
Fwd: CAGAACCGCTGGGACTTCAAC
Rev: TGGGCTGCTCTACGCAACC
Assay: *Tsp*509I digest
CD1: 680, 323 bp products
JF1: 1003 bp product

Cd81 (Tapa1)
Fwd: GATCCCTGGAGTGACCAGAG
Rev: CCCATGTGTGATGTCAGCTC
Assay: Nondenaturing High Performance Liquid Chromatography
CD1: 183 bp product
JF1: 188 bp product

Kcnq1 (Kvlqt1)
Fwd: GATCACCACCCTGTACATTGG
Rev: CCAGGACTCATCCCATTATCC
Embryo Assay: Direction sequencing digest
CD1: T at position 325 beginning with the 5' end of the forward primer
JF1: G at position 325 beginning with the 5' end of the forward primer
TS Cell Assay: *Alu*I digest
CD1: 215, 164, 108, 29 bp products
JF1: 379, 108, 29 bp products

Kcnq1ot1 (Lit1)

Fwd: GCTCCATCTTCGTTTTGCCG
Rev: ACTCCACTCACTACCTTGGTGCTG
Assay: *Hpy*CH4IV digest
CD1: 228, 118 bp products
JF1: 346 bp product

Msuit
Fwd: AGCTGCTGAGAGGACTGACTGAAC
Rev: GGAGAAAGCAAGTGATGCAAGC
Assay: Direct sequencing
CD1: G at position 104 beginning with the 5' end of the forward primer
JF1: C at position 104 beginning with the 5' end of the forward primer

Tssc3 (Ipl/Phlda2)
Fwd: CTGGAGAAGCGAAGCGACAG
Rev: CAACTGGTCCCGTGCGTTTC
Embryo Assay: Direct sequencing
CD1: T at position 352 beginning with the 5' end of the reverse primer
JF1: G at position 352 beginning with the 5' end of the reverse primer
TS Cell Assay: Direct sequencing
CD1: A at position 33 beginning with the 5' end of the forward primer
JF1: C at position 33 beginning with the 5' end of the forward primer

Tssc4
Fwd: ATGGCAGCAAGAAGCGGAG
Rev: CCTAAACACTGGGGCACAAAGG
Assay: *Alu*I digest digest
CD1: 239 bp product
JF1: 172, 66 bp products

Tssc5 (Slc22a1l/Impt1)
Fwd: TCACGCATACCCTCTGCCC
Rev: CCAGTCCCACAACAGCAAAGAC
Assay: *Nde*I digest
CD1: 559 bp product
JF1: 416 + 143 bp product

For all analyses of imprinted expression in embryos, CD1 animals were used as dams, and JF1 animals were used as sires. F1 TS cells were derived from a cross between JF1 dams and CD1 sires.

Fluorescence *in situ* hybridization (FISH)

BAC preparation

Bacterial Artificial Chromosomes (BACs) containing genomic DNA corresponding to either the biallelically expressed gene *Dlx1* (Incyte Genomics A0199N02) or the imprinted genes *Ascl2, Cd81/Tapa1*, and *Tssc4* (BACPAC, clone RP24-376H20) were utilized as templates in nick translation reactions in order to generate DNA FISH probes. BACs were prepared using Qiagen maxiprep kits, following the manufacturer's protocol for plasmid DNA preparation. However, after the addition of Neutralization Buffer P3, lysates were centrifuged twice for fifteen minute at 9000 x *g*. DNA was precipitated from the supernatant with isopropanol, washed in 70% ethanol, and then resuspended in Qiagen Elution Buffer. BAC identity and preparation purity were assessed by restriction digest and comparison to BAC fingerprints predicted by Internet Contig Explorer (iCE).

Probe labeling

dCTP-Cy3 labeled FISH probes were generated using BAC templates and an Amersham nick translation kit, according to the manufacturers instructions and as previously described [29]. Subsequently, smaller probe fragments were generated by incubating labeled DNA with dilute DNase for 4 hours at 15° C. The digestion was terminated by treatment with 0.2 M EDTA, and double stranded probes were denatured by incubating for 3 minutes at 95° C. Probe fragments were separated from unincorporated dCTP-Cy3 on a G50 sephadex column (Roche). In order to reduce nonspecific hybridization, unlabeled Cot-1 DNA and salmon sperm DNA were added to block repetitive sequences in the BAC probes. Next, the labeled and blocked probes were precipitated with one-tenth volume of sodium acetate and three volumes of ethanol.

Finally, precipitated DNA was washed in 70% ethanol and resuspended in hydrization buffer (50% formamide, 10% dextran sulfate, 1X SSC).

Probe hybrdization

Wild-type and *Eed*$^{null/null}$ TS cells were grown to mid-confluency and pulse labeled for one hour in BrdU (20 μM) to label cells in S phase. Then, the cells were harvested, swollen in hypotonic solution (75 mM Kcl), fixed (3:1 methanol:acetic acid), and dropped onto poly-lysine coated slides (Poly-prep™, Sigma). DNA from dropped cells was denatured (70% deionized formamide, 2X SSC for 2 minutes at 70° C) and then dehydrated by a series of ethanol washes. Slides were incubated overnight in a humid chamber at 37° C with FISH probes diluted in hybridization buffer, and then washed, first in 50% formamide/2X SSC and then in a 2X, 1X, 4X series of SSC washes without formamide.

Immunofluorescence

After FISH probe hybridization, nuclei actively replicating DNA were identified by immunofluorescence. Briefly, slides were blocked by incubation for 30 minutes with anti-BrdU blocking buffer (4X SSC, 4mg/mL bovine serum albumin (BSA), 0.1% Tween). After blocking, slides were incubated for 90 minutes with FITC-conjugated anti-BrdU antibodies (Becton Dickinson) diluted 1:200 in blocking buffer. Blocking and antibody incubations were always performed at 37° C in a humid chamber. After the antibody incubation, slides were washed in 4X SSC, first with and then without 0.1% Tween. Finally, slides were mounted with Vectashield™ containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories). Stained slides were visualized by fluorescence microscopy, and then black and white images were captured

with a Spot CCD digital camera before being pseudo-colored and merged with Spot software V3.5.9 (Diagnostic Instruments Inc.). FISH hybridization patterns in BrdU-positive nuclei were scored as single-single (SS, neither allele replicated), single-double (SD, one allele replicated), or double-double (DD, both alleles replicated).

2.3. RESULTS

Imprinted expression defects in *Eed*$^{null/null}$ embryos

In order to assess EED's role in imprinted expression of genes in the *KvDMR* cluster, assays were necessary that could discriminate maternal and paternal allele gene products. CD1 and JF1 mouse strains are sufficiently diverged from one another such that polymorphisms are frequently present in the expressed regions of genes, usually in 5' and 3' untranslated regions. Assays were developed that would discriminate alleles on the basis of those polymorphisms, and RNA was analyzed from CD1.JF1 (F1) wild-type and *Eed*$^{null/null}$ E7.5 embryos.

Ascl2, which is also called *Mash2*, is the most centromeric gene in the *KvDMR* cluster (Figure 2.1). A T/C CD1/JF1 polymorphism was identified in the 3'UTR of *Ascl2*, and this SNP was used to assess the status of imprinted expression. Direct sequencing of *Ascl2* RT-PCR products revealed that the normally silent, paternally-inherited allele becomes expressed in *Eed*$^{null/null}$ E7.5 embryos (Figure 2.2A).

Genes within an imprinted cluster are believed to be regulated by common mechanisms. As a result, the loss of *Ascl2* imprinting observed in *Eed*$^{null/null}$ embryos suggested that *Eed* may be required for imprinting of the entire *KvDMR* cluster. *Cd81*, also known as *Tapa1*, is the first gene distal to *Ascl2* in the *KvDMR* cluster (Figure 2.1). A 5 bp insertion present in the 3' UTR of the JF1 allele of *Cd81* allows maternal and paternal allele gene products to be discriminated on a single strand conformation polymorphism gel. At E7.5, biallelic *Cd81* expression was observed in both wild-type and *Eed*$^{null/null}$ whole conceptuses, indicating that *Cd81* is not imprinted in some tissues at

these stages (Figure 2.2B). However, only maternal *Cd81* was detected in wild-type E7.5 extraembryonic tissues, demonstrating that *Cd81* is imprinted in extraembryonic tissues at this stage. Similarly, in *Eed*$^{null/null}$ E7.5 extraembryonic tissues, expression was only detected from the maternally-inherited allele, suggesting that *Eed* is not required for *Cd81* imprinting.

The *KvDMR* cluster is a large imprinted cluster with a centrally located imprinting control region (Figure 2.1). Both *Ascl2* and *Cd81* are located on the proximal end of the cluster. In order to assess the status of imprinted expression of genes closer to the ICR and genes at the distal end of the cluster, the status of *Kcnq1*, *Msuit*, and *Tssc3* imprinted expression were assessed in wild-type and in *Eed*$^{null/null}$ E7.5 embryos. Direct sequencing of RT-PCR products from all three genes revealed monoallelic expression of both wild-type and in *Eed*$^{null/null}$ embryos (Figure 2.2C-E). Together, these results indicate that *Eed* is required for the normal imprinting of *Ascl2* but not of several other genes in the *KvDMR* cluster.

Imprinted expression analysis in *Eed*$^{hypo/null}$ embryos

The *Eed*^{null} allele is an ENU-generated leucine-to-proline substitution in the third of EED's five WD-40 motifs [30]. A second, ENU-generated allele, *Eed*^{hypo}, causes a less severe phenotype, with homozygous animals exhibiting homeotic skeletal transformations but surviving to adulthood on outbred backgrounds [30]. Like *Eed*$^{null/null}$ embryos, *Eed*$^{hypo/null}$ embryos have defects in imprinted X-chromosome inactivation [13]. To assess whether *Eed*$^{hypo/null}$ embryos share the *Ascl2* imprinting defects observed in null embryos, *Ascl2* RT-PCR products from wild-type and *Eed*$^{hypo/null}$ embryos were directly

sequenced. However, monoallelic expression was observed in all embryos, suggesting that *Eed*^{hypo} retains sufficient activity to silence the paternal allele of *Ascl2* (Figure 2.3).

Imprinted expression defects in *Eed*$^{null/null}$ TS cells

Even though imprinted genes within the same cluster are typically thought to be coregulated, in E7.5 conceptuses, *Eed* was required for imprinted expression of *Ascl2* but not of the neighboring *Cd81*, which is only 84 kb from *Ascl2*. Recent work has demonstrated that *Eed* is only functionally required for X-chromosome inactivation in a small subset of extraembryonic cells [14]. These defects are recapitulated *in vitro* in trophoblast stem (TS) cells, a cell line representing primitive, extraembryonic tissues. If *Eed*'s role in imprinting were similarly tissue-specific, our ability to detect imprinting defects could be obscured by normal imprinted expression in the vast majority of embryonic cells. As a result, I re-analyzed imprinted expression of genes in the *KvDMR* cluster in wild-type and *Eed*$^{null/null}$ trophoblast stem (TS) cells.

Consistent with our observations in *Eed*$^{null/null}$ conceptuses, *Ascl2* was biallelically expressed in *Eed*$^{null/null}$ TS cells (Figure 2.4A). To assess the expression of *Cd81*, I employed a non-denaturing HPLC assay, in which the mobility of RT-PCR products through the affinity column is determined by size. As a result, the larger, maternal JF1 allele of *Cd81* elutes later than the smaller CD1 allele. Although *Cd81* was expressed primarily from the maternal allele in wild-type TS cells, approximately equal levels of maternal and paternal allele gene product were observed in *Eed*$^{null/null}$ TS cells, demonstrating that *Eed*$^{null/null}$ TS cells have broader imprinting defects than were observed in *Eed*$^{null/null}$ conceptuses (Figure 2.4B).

The gene immediately distal to *Cd81* is *Tssc4* (Figure 2.1). Imprinting analyses of *Tssc4* in wild-type and in *Eed*$^{null/null}$ conceptuses failed to produce definitive results (data not shown). In wild-type TS cells, *Tssc4* is expressed predominantly from the maternal allele, with much lower levels of paternal allele gene product (Figure 2.4C). Conversely, in *Eed*$^{null/null}$ TS cells, the levels of maternal and paternal allele gene product were approximately equal, suggesting that *Eed* is also required for *Tssc4* imprinting in these cells (Figure 2.4C).

PcG proteins have been implicated in stem-cell maintenance, with some results suggesting that PcG-deficient stem cells may inappropriately differentiate [31-33]. If *Eed* mutant TS cells prematurely differentiate and if imprinting were to be relaxed during differentiation, the imprinting defects observed in *Eed*$^{null/null}$ TS cells could be due simply to differences in the differentiation state of wild-type and mutant cells, as opposed to *Eed* having a direct role in imprinted expression. To assess this possibility, we differentiated wild-type and *Eed*$^{null/null}$ TS cells by removing Fgf4 and activin from the growth media. These growth factors are required to prevent TS cells from differentiating and endoreduplicating both *in vivo* and *in vitro* [28]. *Tssc4* imprinting remained intact in differentiated wild-type TS cells, demonstrating that the observed loss of imprinting in *Eed*$^{null/null}$ TS cells is not simply a consequence of premature differentiation (Figure 2.4C).

Ascl2, *Cd81*, and *Tssc4* all reside on the proximal end of the *KvDMR* imprinting control region. To determine whether *Eed* is also required for imprinted expression of genes near and distal to the ICR, I assessed expression of *Kcnq1*, *Cdkn1c*, *Tssc5,* and *Tssc3*, which represent four of the six known imprinted genes expressed from promoters

on the distal side of the ICR. Unlike our observations on the proximal end of the cluster, *Eed* was dispensible for imprinted expression of all four of these genes (Figure 2.5A-D).

The *KvDMR* imprinting control region overlaps with the promoter of the non-coding RNA *Kcnq1ot1*, which is the only gene in the cluster expressed exclusively from the paternally-inherited allele. Paternally-inherited deletions eliminating the *Kcnq1ot1* promoter and mutations prematurely terminating *Kcnq1ot1* transcription both disrupt silencing of the paternal alleles of the maternally-expressed genes in the *KvDMR* cluster [21, 25]. These results indicate that the *Kcnq1ot1* RNA itself or transcriptional elongation of that RNA are required for *KvDMR* cluster imprinting. To determine whether defects in *Kcnq1ot1* expression or imprinting were responsible for the imprinting defects in *Eed* mutant TS cells, I assessed *Kcnq1ot1* imprinting in $Eed^{null/null}$ TS cells. *Kcnq1ot1* was expressed exclusively from the paternally-inherited, CD1 allele in both wild-type and $Eed^{null/null}$ TS cells, and imprinted expression was maintained even upon differentiation of those cells (Figure 2.5E).

Maintained replication asynchrony in $Eed^{null/null}$ TS cells

In addition to allele-specific gene expression, imprinted genes are characterized by additional asymmetries, including asymmetric DNA replication timing [34]. Generally, active alleles of imprinted genes replicate early in S-phase, whereas inactive alleles replicate late during S-phase. Given that an approximately 100 kb region of the *KvDMR* cluster from *Mash2* to *Tssc4* appears to lose its transcriptional imprinting in $Eed^{null/null}$ TS cells (Figure 2.3A-C), we analyzed the replication behavior of this segment to determine whether Eed is also required for asynchronous replication of the subdomain.

These experiments employed a DNA FISH-based assay that allows direct counting of resolved sister chromatids in actively replicating cells (Figure 2.6A). In cells that have replicated neither allele, the FISH probe detects two single dots, corresponding to each of the two unreplicated, homologous chromosomes. Conversely, once both alleles have replicated, the FISH probe detects two double dots, corresponding to the replicated sister chromatids on both homologous chromosomes. Finally, if only one of the two alleles has replicated, the FISH probe detects one single dot and one double dot, corresponding to one unreplicated homolog and the two sister chromatids of the replicated homolog, respectively. This final class, termed "single-double", is the informative class in replication asynchrony assays.

Roughly consistent with previous findings, 38% of wild-type S-phase nuclei had a single-double hybridization pattern with the *Mash2-Cd81-Tssc4* probe, and 22% of nuclei had a single-double hybridization pattern with a probe for *Dlx1*, a biallelically expressed and synchronously replicating, control gene [34]. These values establish the behavior of asynchronously replicating and synchronous replicating genes in my hands. Surprisingly, the percentage of cells exhibiting single-double hybridization patterns were approximately the same in $Eed^{null/null}$ TS cells (22 and 41%, respectively, Figure 2.6B). These results suggest that *Mash2-Cd81-Tssc4* asynchronous replication is not dependent on *Eed*.

2.4. DISCUSSION

Imprinting is one of the archetypal examples of epigenetic gene regulation in mammals. The role of DNA methylation in imprinted gene expression has been characterized extensively [6-10]. However, the roles of other epigenetic marks, such as covalent histone modifications, have received less attention. Here, we have demonstrated that the PcG gene *Eed* is required for imprinted expression of several genes in the *KvDMR* cluster in both mouse conceptuses and TS cells.

While this work was underway, two groups reported that EED, EZH2, and H3K27me3 are all enriched on the silent, paternal alleles of several maternally-expressed genes in the *KvDMR* cluster in midgestation placental tissues [22, 35]. Together with the imprinting defects we observed in *Eed* mutant conceptuses and TS cells, these results suggest that EED is directly required for imprinting in the *KvDMR* cluster. However, whereas PRC2 subunits and H3K27me3 were enriched throughout the *KvDMR* in those studies, the imprinting defects in *Eed* mutant TS cells reported here were restricted to the three most proximal genes, *Ascl2, Cd81*, and *Tssc4*. These results suggest that *Eed*-independent regulatory mechanisms must be critical for the imprinting of the genes distal to *Tssc4*. The most compelling candidate to fulfill this function is DNA methylation. *Dnmt1* mutant placentas have imprinting defects nearly reciprocal to those observed in *Eed* mutant trophoblast stem cells (Figure 2.7) [22]. Together, these results suggest that the *KvDMR* imprinting cluster can be subdivided into two subdomains. In the proximal subdomain, PRC2-mediated histone modifications may be the essential epigenetic mark in extraembryonic tissues, whereas *Dnmt1*-mediated DNA methylation is likely to fulfill this function for more distal genes in the cluster.

The broader imprinting defects in *Eed*$^{null/null}$ TS cells relative to *Eed*$^{null/null}$ conceptuses suggests that *Eed*'s role in imprinting, like its role in X-chromosome inactivation, may be tissue specific. One possible explanation for such tissue specificity may be that covalent histone modifications are reinforced by DNA methylation to a far greater extent in embryonic tissues than in extraembryonic tissues. Mouse extraembryonic tissues have much lower levels of DNA methylation than embryonic tissues. This distinction is a consequence of DNA methylation dynamics during development [36]. After fertilization, the genome is passively demethylated until embryonic and extraembryonic lineages are committed at the blastocyst stage. After implantation, the embryonic lineages are rapidly remethylated, but the extraembryonic lineages remain hypomethylated. As a consequence, covalent histone modifications, such as PRC2-mediated H3K27me3, may be especially critical in these DNA-hypomethylated extraembryonic tissues, where the absence of those marks cannot be overcome by DNA methylation.

Although *Ascl2*, *Cd81*, and *Tssc4* were all biallelically expressed in *Eed* mutant TS cells, the maternal and paternal alleles of these genes continued to replicate asynchronously. Accordingly, imprinted gene expression and replication asynchrony are uncoupled in *Eed*$^{null/null}$ TS cells, suggesting that the transcriptional machinery and the replication machinery may recognize distinct epigenetic marks at imprinted loci. However, this interpretation is complicated by the normal imprinted expression observed in genes distal to *Tssc4*. Mammalian replicons are poorly defined but are frequently as large as several hundred kilobases [37]. Consequently, it is possible that the origin of replication for the *Eed*-regulated genes *Ascl2*, *Cd81*, and *Tssc4* actually lies in the *Eed*-

insensitive distal end of the *KvDMR* cluster. The epigenetic marks controlling expression of those genes remain intact in *Eed*$^{null/null}$ TS cells and could be responsible for replication timing at the proximal end of the cluster. However, asynchronous replication timing and imprinted gene expression have been truly uncoupled for entire imprinted clusters in *Dnmt1*$^{-/-}$ ES cells, suggesting that replication timing and transcriptional imprinting actually are governed by distinct epigenetic marks [38].

The work included in this chapter suggests that PRC2-mediated H3K27me3 is required to silence the paternal alleles of genes at the proximal end of the *KvDMR* cluster. However, EZH2, not EED, is not directly responsible for PRC2-mediated H3K27me3, and in this chapter, we have not demonstrated that EED is required for the catalytic activity of the complex. That possibility is explored in Chapter 3.

2.5. REFERENCES

1. Ferguson-Smith, A.C., and Surani, M.A. (2001). Imprinting and the epigenetic asymmetry between parental genomes. Science *293*, 1086-1089.

2. Surani, M.A., and Barton, S.C. (1983). Development of gynogenetic eggs in the mouse: implications for parthenogenetic embryos. Science *222*, 1034-1036.

3. McGrath, J., and Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell *37*, 179-183.

4. Cattanach, B.M., and Kirk, M. (1985). Differential activity of maternally and paternally derived chromosome regions in mice. Nature *315*, 496-498.

5. Reik, W., and Walter, J. (2001). Genomic imprinting: parental influence on the genome. Nat Rev Genet *2*, 21-32.

6. Li, E., Beard, C., and Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. Nature *366*, 362-365.

7. Howell, C.Y., Bestor, T.H., Ding, F., Latham, K.E., Mertineit, C., Trasler, J.M., and Chaillet, J.R. (2001). Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. Cell *104*, 829-838.

8. Hata, K., Okano, M., Lei, H., and Li, E. (2002). Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development *129*, 1983-1993.

9. Bourc'his, D., Xu, G.L., Lin, C.S., Bollman, B., and Bestor, T.H. (2001). Dnmt3L and the establishment of maternal genomic imprints. Science *294*, 2536-2539.

10. Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., and Sasaki, H. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature *429*, 900-903.

11. Lyon, M.F. (1961). Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature *190*, 372-373.

12. Sado, T., and Ferguson-Smith, A.C. (2005). Imprinted X inactivation and reprogramming in the preimplantation mouse embryo. Hum Mol Genet *14 Spec No 1*, R59-64.

13. Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J.C., Nagy, A., and Magnuson, T. (2001). Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat Genet *28*, 371-375.

14. Kalantry, S., Mills, K.C., Yee, D., Otte, A.P., Panning, B., and Magnuson, T. (2006). The Polycomb group protein Eed protects the inactive X-chromosome from differentiation-induced reactivation. Nat Cell Biol *8*, 195-202.

15. Mak, W., Baxter, J., Silva, J., Newall, A.E., Otte, A.P., and Brockdorff, N. (2002). Mitotically stable association of polycomb group proteins eed and enx1 with the inactive x chromosome in trophoblast stem cells. Curr Biol *12*, 1016-1020.

16. Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jenuwein, T., Otte, A.P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481-495.

17. Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

18. Bell, A.C., and Felsenfeld, G. (2000). Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature *405*, 482-485.

19. Hark, A.T., Schoenherr, C.J., Katz, D.J., Ingram, R.S., Levorse, J.M., and Tilghman, S.M. (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature *405*, 486-489.

20. Caspary, T., Cleary, M.A., Baker, C.C., Guan, X.J., and Tilghman, S.M. (1998). Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. Mol Cell Biol *18*, 3466-3474.

21. Fitzpatrick, G.V., Soloway, P.D., and Higgins, M.J. (2002). Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. Nat Genet *32*, 426-431.

22. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004). Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet *36*, 1291-1295.

23. Sado, T., Fenner, M.H., Tan, S.S., Tam, P., Shioda, T., and Li, E. (2000). X inactivation in the mouse embryo deficient for Dnmt1: distinct effect of hypomethylation on imprinted and random X inactivation. Dev Biol *225*, 294-303.

24. Tanaka, M., Puchyr, M., Gertsenstein, M., Harpal, K., Jaenisch, R., Rossant, J., and Nagy, A. (1999). Parental origin-specific expression of Mash2 is established

at the time of implantation with its imprinting mechanism highly resistant to genome-wide demethylation. Mech Dev *87*, 129-142.

25. Mancini-Dinardo, D., Steele, S.J., Levorse, J.M., Ingram, R.S., and Tilghman, S.M. (2006). Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes Dev *20*, 1268-1282.

26. Plath, K., Mlynarczyk-Evans, S., Nusinow, D.A., and Panning, B. (2002). Xist RNA and the mechanism of X chromosome inactivation. Annu Rev Genet *36*, 233-278.

27. Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942-947.

28. Tanaka, S., Kunath, T., Hadjantonakis, A.K., Nagy, A., and Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. Science *282*, 2072-2075.

29. Singh, N., Ebrahimi, F.A., Gimelbrant, A.A., Ensminger, A.W., Tackett, M.R., Qi, P., Gribnau, J., and Chess, A. (2003). Coordination of the random asynchronous replication of autosomal loci. Nat Genet *33*, 339-341.

30. Schumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *384*, 648.

31. Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., Bell, G.W., Otte, A.P., Vidal, M., Gifford, D.K., Young, R.A., and Jaenisch, R. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349-353.

32. Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K., Koseki, H., Fuchikami, T., Abe, K., Murray, H.L., Zucker, J.P., Yuan, B., Bell, G.W., Herbolsheimer, E., Hannett, N.M., Sun, K., Odom, D.T., Otte, A.P., Volkert, T.L., Bartel, D.P., Melton, D.A., Gifford, D.K., Jaenisch, R., and Young, R.A. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. Cell *125*, 301-313.

33. Sparmann, A., and van Lohuizen, M. (2006). Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer *6*, 846-856.

34. Kitsberg, D., Selig, S., Brandeis, M., Simon, I., Keshet, I., Driscoll, D.J., Nicholls, R.D., and Cedar, H. (1993). Allele-specific replication timing of imprinted gene regions. Nature *364*, 459-463.

35. Umlauf, D., Goto, Y., Cao, R., Cerqueira, F., Wagschal, A., Zhang, Y., and Feil, R. (2004). Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. Nat Genet *36*, 1296-1300.

36. Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet *3*, 662-673.

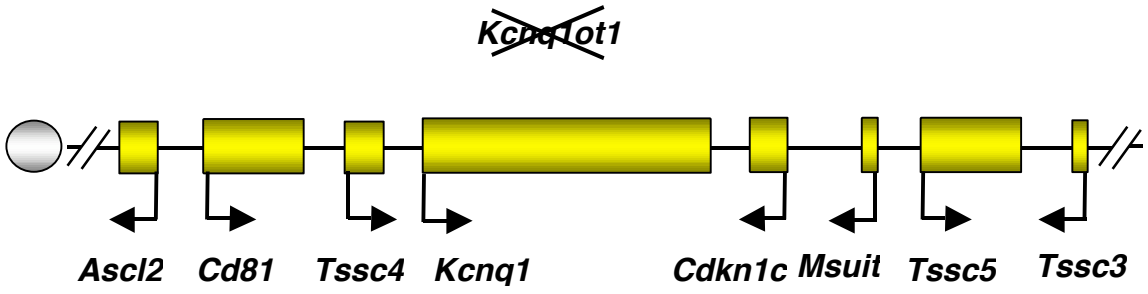
37. Takebayashi, S.I., Manders, E.M., Kimura, H., Taguchi, H., and Okumura, K. (2001). Mapping sites where replication initiates in mammalian cells using DNA fibers. Exp Cell Res *271*, 263-268.

38. Gribnau, J., Hochedlinger, K., Hata, K., Li, E., and Jaenisch, R. (2003). Asynchronous replication timing of imprinted loci is independent of DNA methylation, but consistent with differential subnuclear localization. Genes Dev *17*, 759-773.

FIGURE 2.1. Schematic illustration of the *KvDMR* imprinting cluster

Depicted is the *KvDMR* imprinting cluster on mouse chromosome 7. The two most proximal genes, *Obph1* and *Nap1l4*, were not analyzed and are not included on the diagram. On the maternally-inherited chromosome (maternal- above), the noncoding RNA *Kcnq1ot1* is silenced, but all other genes in the cluster are expressed. On the paternally-inherited chromosome (paternal- below), *Kncq1ot1* is expressed, and all other genes in the cluster are silenced.

Maternal



Paternal

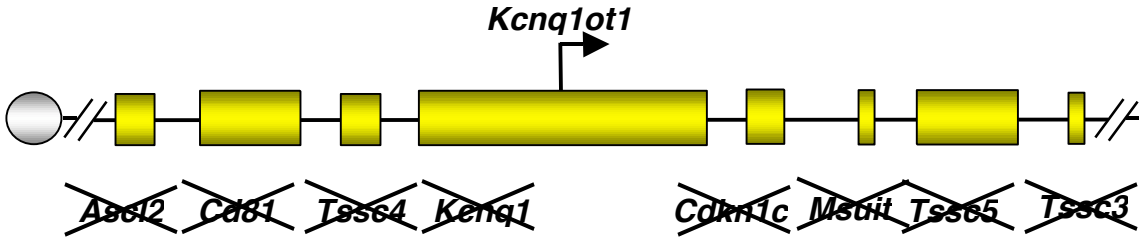


FIGURE 2.2. Imprinted expression analysis in *Eed*$^{null/null}$ E7.5 conceptuses and extraembryonic tissues

Imprinted expression analysis in wild-type (wt or +/+) and *Eed*$^{null/null}$ (mut or null/null) CD1.JF1 (F1) E7.5 embryos **(A)** Direct sequencing of *Ascl2* RT-PCR products. **(B)** Single Strand Conformation Polymorphism gel analysis of *Cd81* imprinting. A 5 bp insertion in the JF1 paternal allele (p) makes that band run more slowly than the smaller CD1 maternal allele (m). Unless otherwise indicated, samples were prepared from whole conceptuses. Samples prepared from dissected extraembryonic tissues are indicated by "Ex. Em." **(C)** Direct sequencing of *Kcnq1* RT-PCR products. **(D)** Direct sequencing of *Msuit* RT-PCR products. **(E)** Direct sequencing of *Tssc3* RT-PCR products.

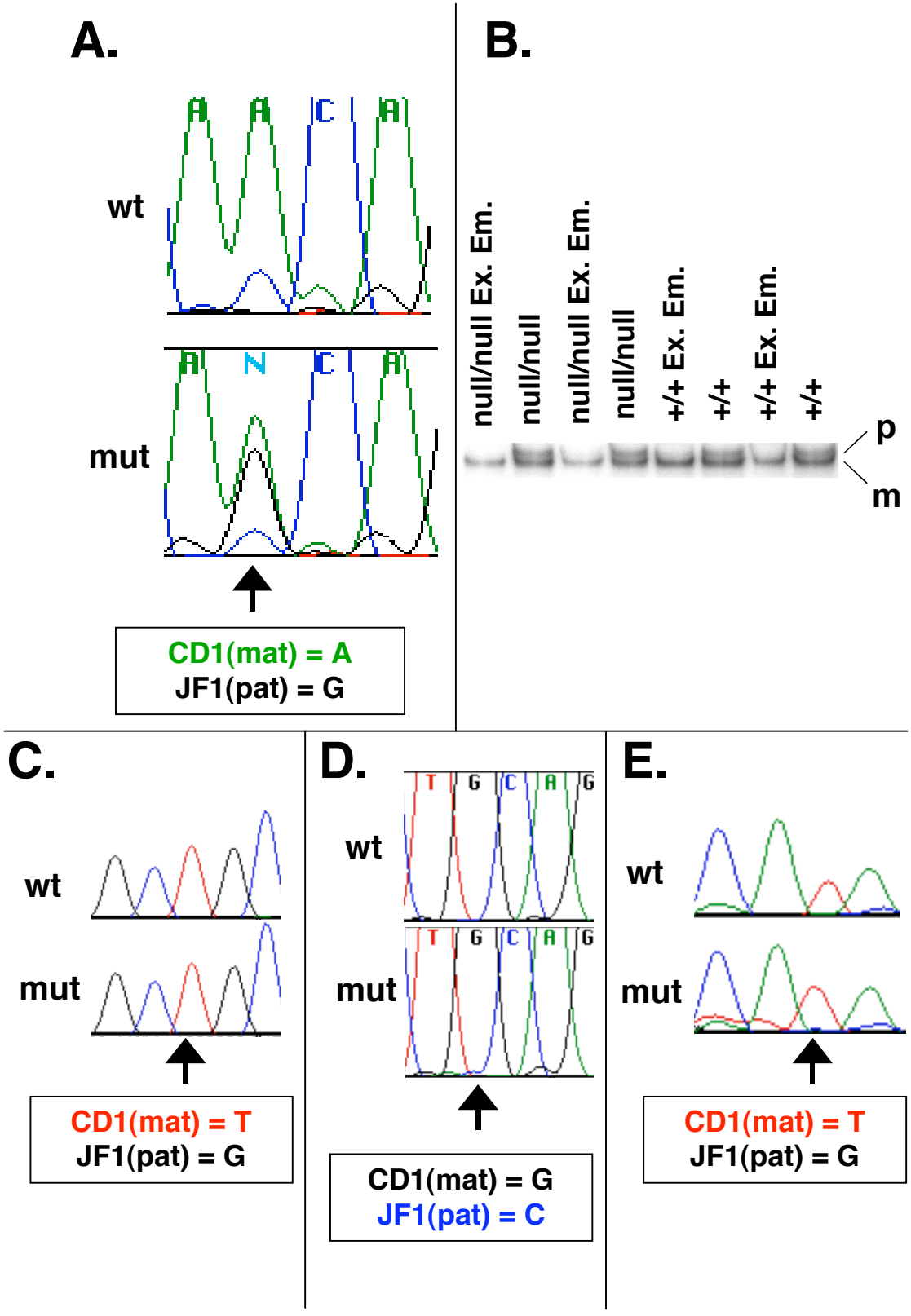

A.
wt
mut
CD1(mat) = A
JF1(pat) = G
B.
null/null Ex. Em.
null/null
null/null Ex. Em.
null/null
+/+ Ex. Em.
+/+
+/+ Ex. Em.
+/+
p
m
C.
wt
mut
CD1(mat) = T
JF1(pat) = G
D.
wt
mut
CD1(mat) = G
JF1(pat) = C
E.
wt
mut
CD1(mat) = T
JF1(pat) = G

FIGURE 2.3. Imprinted expression analysis in *Eed*$^{hypo/null}$ conceptuses

Ascl2 imprinted expression analysis in wild-type and *Eed*$^{hypo/null}$ CD1.JF1 (F1) E9.5 embryos by direct sequencing. The amplicon is the same as that used in Figure 2.2A. However, the forward primer was used for sequencing here, and the reverse primer was used there.

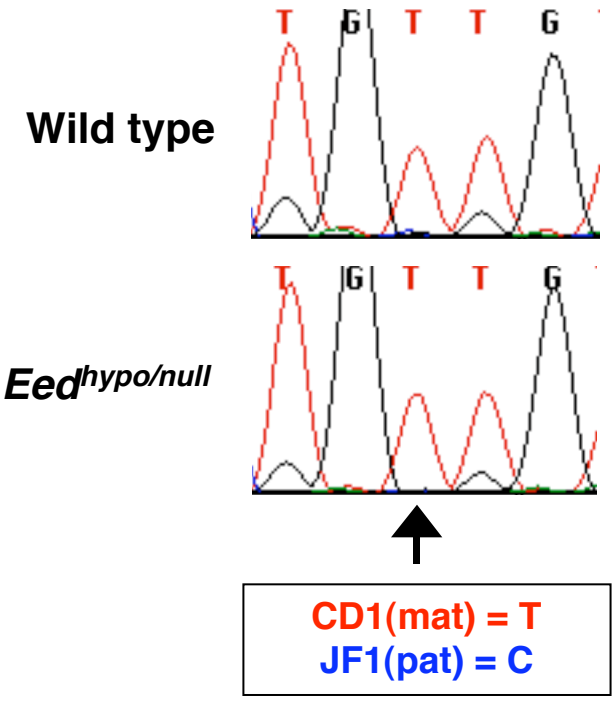
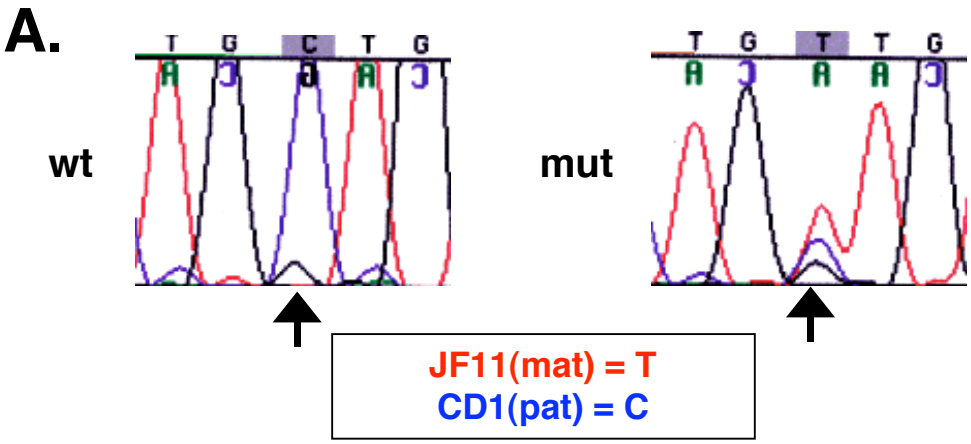
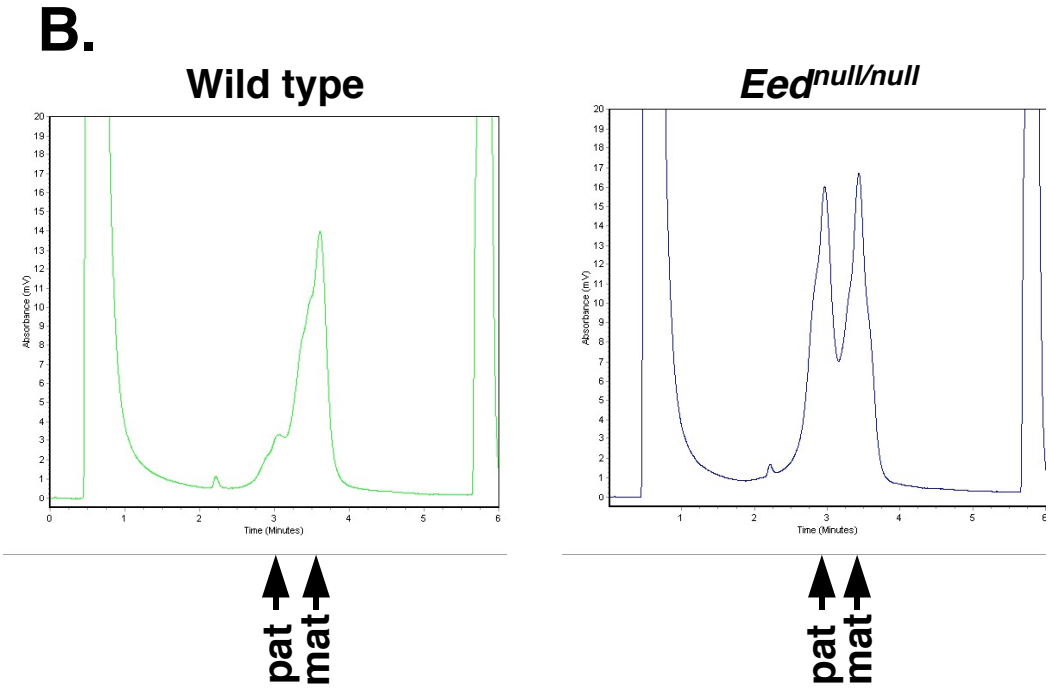

Wild type
T G T T G
Eed hypo/null
T G T T G
CD1(mat) = T
JF1(pat) = C

FIGURE 2.4. Loss of imprinting in *Eed*$^{null/null}$ trophoblast stem (TS) cells

Imprinted expression analysis in wild-type (wt) and *Eed*$^{null/null}$ (mut) JF1.CD1 (F1) trophoblast stem cells. **(A)** Direct sequencing of *Ascl2* RT-PCR products. **(B)** *Cd81* imprinted expression assessed by non-denaturing HPLC of RT-PCR products. The smaller, CD1 paternal allele (pat) elutes from the column after approximately 3 minutes, and the larger, JF1 maternal allele (mat) elutes at approximately 3.75 minutes. **(C)** *Tssc4* imprinted expression utilizing a JF1-CD1 *Alu*I RFLP. Maternally-encoded, JF1 RT-PCR products are cut, but paternally-encoded, CD1 RT-PCR products are not.


A.
wt
mut
T G C T G
T G T T G
JF11(mat) = T
CD1(pat) = C


B.
Wild type
Eed null/null
Absorbance (mV)
Time (Minutes)
pat
mat
pat
mat

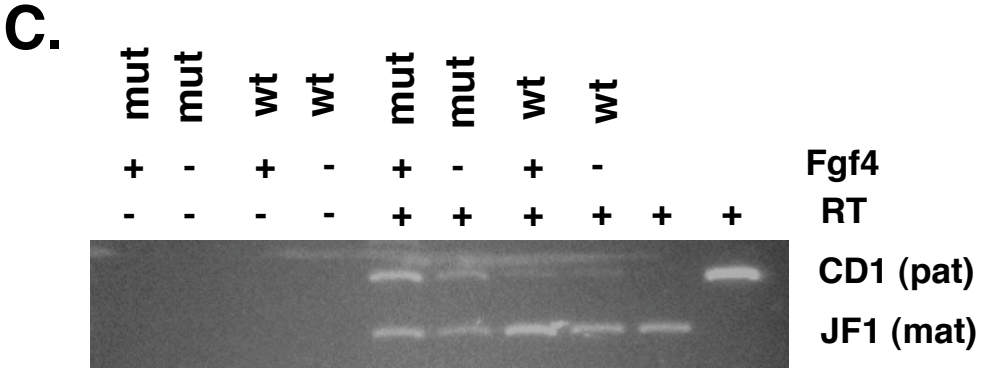

C.
mut mut wt wt mut mut wt wt
+ - + - + - + -
- - - - + + + + + +
Fgf4
RT
CD1 (pat)
JF1 (mat)

FIGURE 2.5. Maintained imprinting in *Eed*$^{null/null}$ trophoblast stem (TS) cells

Imprinted expression analysis in wild-type (wt) and *Eed*$^{null/null}$ (mut) JF1.CD1 (F1) trophoblast stem cells. **(A)** *Kcnq1* imprinted expression utilizing an *Alu*I RFLP. Paternally-encoded, CD1 RT-PCR products are cut, but maternally-encoded, JF1 RT-PCR products are not. **(B)** *Cdkn1c* imprinted expression analysis using a *Tsp*509I RFLP. Paternally-encoded, CD1 RT-PCR products are cut, but maternally-encoded, JF1 RT-PCR products are not. **(C)** *Tssc5* imprinted expression analysis using a *NdeI* RFLP. Maternally-encoded, JF1 RT-PCR products are cut, but paternally-encoded, CD1 RT-PCR products are not. **(D)** Direct sequencing of *Tssc3* RT-PCR products. **(E)** *Kcnq1ot1* imprinted expression analysis utilizing a *Hpy*CH4IV RFLP. The paternally-encoded, CD1 RT-PCR products are cut, but the maternally-encoded, JF1 RT-PCR products are not.

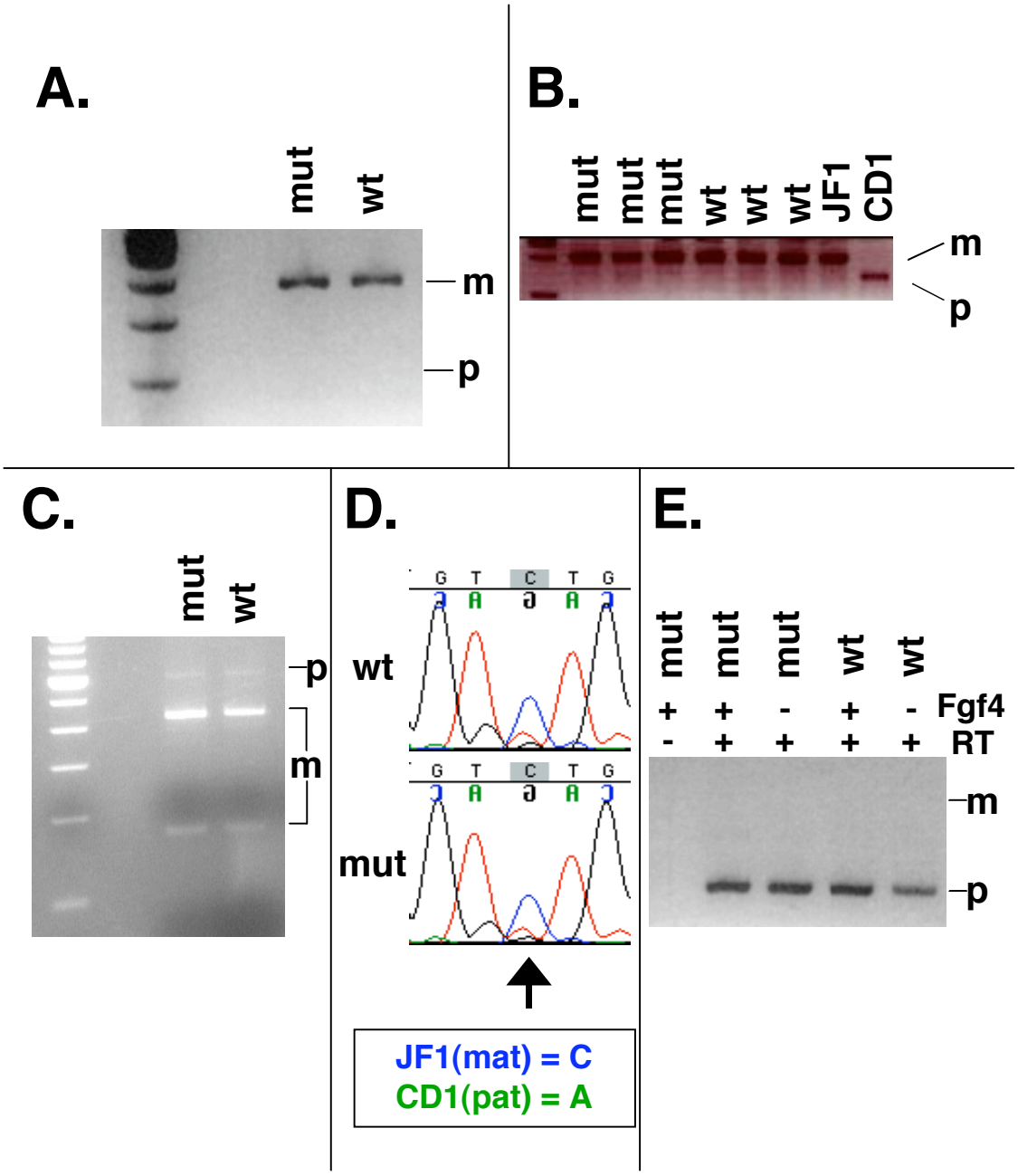
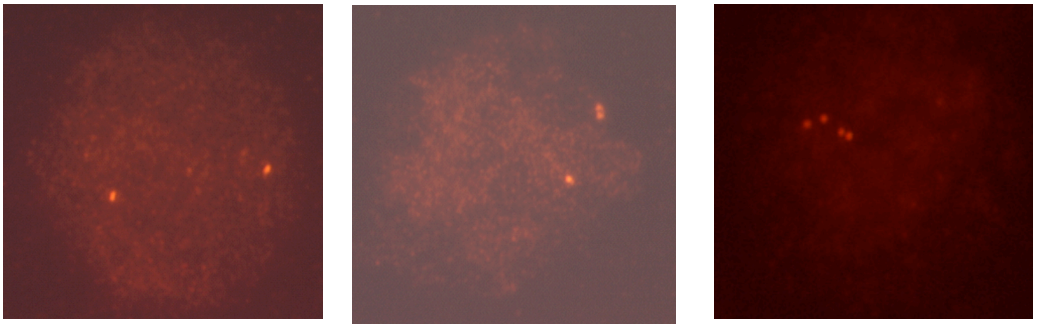

A.
mut
wt
m
p
B.
mut
mut
mut
wt
wt
wt
JF1
CD1
m
p
C.
mut
wt
p
m
D.
G T C T G
wt
mut
JF1(mat) = C
CD1(pat) = A
E.
mut
mut
mut
wt
wt
+ + - + - Fgf4
- + + + + RT
m
p

FIGURE 2.6. Maintained replication asynchrony in the *KvDMR* imprinted cluster in *Eed*$^{null/null}$ TS cells

Replication asynchrony assessed in wild-type and in *Eed*$^{null/null}$ TS cells. **(A)** Fluorescence microscopy images of S-phase nuclei hybridized with DNA FISH probes. When neither allele has replicated, two single dots are observed (Single-Single). When one allele has not replicated but one allele has replicated, one single dot and one double dot are observed (Single-Double). After both alleles have replicated, two double dots are observed (Double-Double). The Single-Double class is a readout of replication asynchrony. **(B)** Results of replication asynchrony assays in wild-type and in *Eed*$^{null/null}$ TS cells. 22% of both wild-type and *Eed*$^{null/null}$ S-phase nuclei exhibit Single-Double staining patterns with a probe to *Dlx1*, a known synchronously replicating control gene. Conversely, 38% of wild-type and 41% of *Eed*$^{null/null}$ S-phase nuclei exhibit Single-Double staining patterns with a probe corresponding to the *Ascl2-Cd81-Tssc4* region of the *KvDMR* cluster.

A.



Single-Single | Single-Double | Double-Double

B.

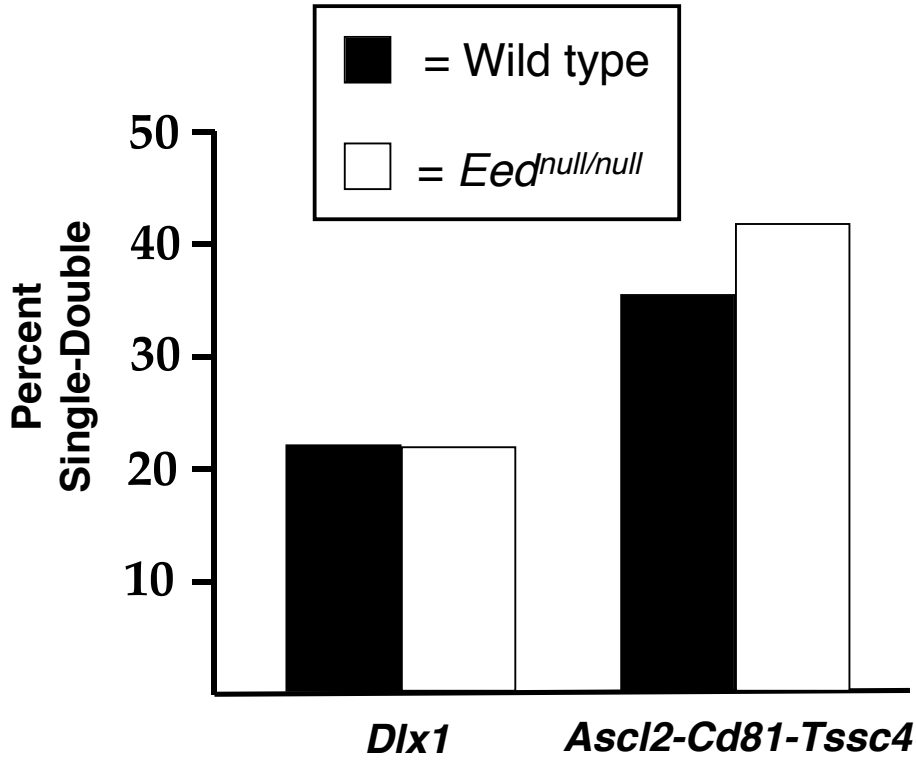


FIGURE 2.7. Summary of imprinting defects in *Eed*$^{null/null}$ TS cells and in *Dnmt1*$^{null/null}$ placentas

Imprinting defects previously reported in *DNA methyltransferase 1* (*Dnmt1*) mutant placentas and reported here in *Eed*$^{null/null}$ TS cells [22]. *Dnmt1* and *Eed* are required to regulate nearly reciprocal groups of genes, although the centrally located *Kcnq1* is imprinted normally in both cases.

	Dnmt1$^{-/-}$ Placentas	*Eed*$^{null/null}$ TS Cells
Ascl2	no	LOI
Cd81	no	LOI
Tssc4	no	LOI
Kcnq1	no	no
Kcnq1ot1	LOI	no
Cdkn1c	LOI	no
Tssc5	LOI	no
Tssc3	LOI	no

CHAPTER 3

THE MURINE POLYCOMB GROUP PROTEIN EED IS REQUIRED FOR GLOBAL HISTONE H3 LYSINE-27 METHYLATION

The Murine Polycomb Group Protein Eed is Required for Global Histone H3 Lysine-27 Methylation

Nathan D. Montgomery[1,2,3], Della Yee[1,3], Andrew Chen[1,3], Sundeep Kalantry[1,3], Stormy J. Chamberlain[1,3], Arie P. Otte[4], & Terry Magnuson[1,2,3]

[1]Department of Genetics, [2]Curriculum in Genetics and Molecular Biology, and [3]Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, CB#7264 Chapel Hill, NC 27599, USA

[4]Swammerdam Institute for Life Sciences, University of Amsterdam, 1098 SM Amsterdam, The Netherlands

This work was published in *Current Biology* 15(10): 942-7.

3.1. SUMMARY

PcG proteins mediate heritable transcriptional silencing by generating and recognizing covalent histone modifications. One conserved PcG complex, PRC2, is composed of several proteins including the histone methyltransferase (HMTase) Ezh2, the WD-repeat protein Eed, and the Zn-finger protein Suz12. Ezh2 methylates histone H3 on lysine 27 (H3K27) [1-4], which serves as an epigenetic mark mediating silencing. H3K27 can be mono-, di-, or trimethylated (H3K27me1, H3K27me2, and H3K27me3, respectively) [5]. Hence, either PRC2 must be regulated so as to add one methyl group to certain nucleosomes but two or three to others, or distinct complexes must be responsible for H3K27me1, H3K27me2, and H3K27me3. Consistent with the latter possibility, H3K27me2 and H3K27me3, but not H3K27me1, are absent in *Suz12*$^{-/-}$ embryos, which lack both Suz12 and Ezh2 protein [6]. Mammalian proteins required for H3K27me1 have not been identified. Here, we demonstrate that, unlike Suz12 and Ezh2, Eed is required not only for H3K27me2 and H3K27me3 but also global H3K27me1. These results provide a functionally-important distinction between PRC2 complex components and implicate Eed in PRC2-independent histone methylation.

3.2. RESULTS

Reduced Histone H3 K27 Methylation in *Eed*$^{null/null}$ cells

To assess Eed's role in H3K27 methylation, H3K27me1, H3K27me2, and H3K27me3 were analyzed by immunofluorescence in wild-type and *Eed*$^{l7Rn5\text{-}3354SB}$ homozygous (herein referred to as *Eed*$^{null/null}$) embryonic stem (ES) cells and trophoblast stem (TS) cells. Consistent with published reports from *Eed*$^{null/null}$ embryos [7], H3K27me3 was undetectable in *Eed*$^{null/null}$ ES cells and TS cells (Figures 3.1C,F). Additionally, H3K27me1 and H3K27me2 were also undetectable in *Eed*$^{null/null}$ ES cells and TS cells (Figure 3.1A,B,D,E). However, no difference in trimethylation of histone H3 lysine 9 staining was observed between wild-type and *Eed*$^{null/null}$ TS and ES cells (data not shown), suggesting that the defect is specific to H3K27 methylation.

To confirm the immunofluorescence data, histones were isolated from wild-type and *Eed*$^{null/null}$ ES cells by acid extraction, and H3K27 methylation was analyzed by Western blotting. H3K27me1, H3K27me2, and H3K27me3 were all dramatically reduced in *Eed*$^{null/null}$ ES cells (Supplementary Figure 3.1). These results implicate Eed not only in di- and trimethylation of H3K27, as had previously been suggested for other PRC2 subunits, but also in monomethylation of H3K27.

Reduced Ezh2 Subunit Protein Levels in *Eed*$^{null/null}$ ES cells

To gauge the molecular basis for the loss of H3K27 methylation in *Eed*$^{null/null}$ cells, Eed and Ezh2 subunit protein levels were compared in wild-type and *Eed*$^{null/null}$ ES cells by Western blotting. Consistent with the genetic classification of *Eed*^{null} as a null allele,

Eed protein is nearly undetectable in *Eed*$^{null/null}$ ES cells (Figure 3.2A) and in *Eed*$^{null/null}$ TS cells (data not shown).

Suz12$^{-/-}$ embryos lack not only Suz12 but also Ezh2, suggesting that the stability of Ezh2 may be dependent upon its incorporation into a functional PRC2 complex [6]. Similarly, Ezh2 protein levels were dramatically reduced in *Eed*$^{null/null}$ ES cells (Figure 3.2A) and in *Eed*$^{null/null}$ TS cells (data not shown).

Despite the dramatic reduction in Eed and Ezh2 protein levels in *Eed*$^{null/null}$ ES cells, the mRNA levels of both gene products were unchanged (Figure 3.2B-D). Together, these results suggest that Ezh2 is unstable outside of intact PRC2 complexes.

Eed Rescues H3K27 Methylation Defects in *Eed*$^{null/null}$ ES Cells

To confirm Eed's role in mono-, di- and trimethylation of H3K27 and to assess whether Eed is involved not only in the maintenance of these marks but also in their *de novo* establishment, *Eed*$^{null/null}$ ES cells were transiently transfected with plasmids expressing *Eed* or *Eed*^{null} full length cDNAs containing all four putative Eed translation start sites [8]. Expression of *Eed* but not *Eed*^{null} rescued the H3K27 methylation defects in a subset of *Eed*$^{null/null}$ ES cells (Figure 3.3. and Supplementary Figures 3.2 and 3.3). We hypothesized that the subpopulation of rescued cells represent the successfully transfected cells in each population. Consistent with this interpretation, the H3K27me1 defect was uniformly rescued in *Eed*$^{null/null}$ ES cells stably expressing wild-type Eed (Figure 3.3).

Surprisingly, no H3K27me2 or H3K27me3 rescue was observed in these stable lines (Supplementary Figures 3.2 and 3.3). However, similar to the parental line,

transient transfection of these lines with the Eed-expression plasmid successfully rescued the H3K27me2 and H3K27me3 defects in a fraction of cells, confirming that the stable lines remain competent for di- and trimethylation (Supplementary Figures 3.2 and 3.3). We hypothesized that the discrepancy in H3K27 methylation in transient versus stable lines might reflect differences in Eed expression levels. Supporting this hypothesis, much higher levels of Eed were observed in transient lines than in stable lines, even though only a fraction of the cells in the transiently transfection receive the Eed-expression plasmid (Figure 3.4A). Additionally, consistent with previous data demonstrating that Ezh2 is dispensable for H3K27me1 but required for H3K27me2 and H3K27me3 [6], Ezh2 protein levels were rescued in transient but not stable lines (Figure 3.4A).

Eed, Eed^{hypo}, and Eed^{null} protein levels correlate with phenotypic severity

Whereas *Eed$^{null/null}$* embryos die during embryogenesis at gastrulation stages, animals homozygous for a hypomorphic allele, *Eed$^{l7Rn5\text{-}19895B}$* (herein referred to as *Eed$^{hypo/hypo}$*), are viable and fertile but are runted and exhibit skeletal transformations [9]. Despite this dramatic phenotypic difference, no biochemical features have been identified that distinguish Eed^{hypo} and Eed^{null} proteins [10-12]. To determine whether complexes containing Eed^{hypo} mediate H3K27 methylation, *Eed$^{null/null}$* ES cells were transiently transfected with plasmids expressing Eed^{hypo}. Consistent with the mild phenotype of *Eed$^{hypo/hypo}$* animals, Eed^{hypo}, like Eed but unlike Eed^{null}, was able to rescue the H3K27 methylation defects in a percentage of *Eed$^{null/null}$* ES cells (Figure 3.3, Supplementary

Figures 3.2 and 3.3). Additionally, *Eed*$^{hypo/hypo}$ fibroblasts retain H3K27me1, H3K27me2, and H3K27me3 (data not shown).

Eed^{hypo}'s ability to mediate H3K27 methylation suggests that, unlike Eed^{null}, Eed^{hypo} must be stable. In order to assess the relative stability of Eed^{hypo} and Eed^{null}, *Eed*$^{null/null}$ ES cells were transiently transfected with plasmids expressing Eed, Eed^{hypo}, or Eed^{null}. Consistent with the hypomorphic phenotype conferred by the mutation, Western blotting of whole cell lysates from transfected cells indicated that the Eed^{hypo} protein is present at a level intermediate to Eed and Eed^{null} (Figure 3.4B). Although reduced relative to wildtype, the level of Eed^{hypo} on a per cell basis is apparently sufficient for assembly of functional PRC2 complexes. However, the qualitative nature of the immunofluorescence assay precluded determination of whether Eed^{hypo} mediates qualitatively less H3K27 methylation than Eed.

3.3. DISCUSSION

The requirement of Eed for global H3K27me1, H3K27me2, and H3K27me3 suggests either that PRC2 is necessary for all H3K27 methylation or that distinct Eed-containing complexes mediate mono-, di- and trimethylation. In support of the former concept, the Drosophila *Ezh2* homologue *E(z)* was recently shown to be required for H3K27me1, H3K27me2, and H3K27me3 [13], suggesting that all H3K27 methylation is mediated by PRC2 in that organism. However, in mammals, the mechanism may be more complex. *Suz12*$^{-/-}$ embryos, which lack Suz12 and Ezh2 but retain Eed, maintain H3K27me1 [6]. These results indicate that PRC2 may mediate only H3K27me2 and H3K27me3 in mammalian cells. This model is similar to H3K9 methylation, where distinct enzymes mediate mono/dimethylation and trimethylation [5].

Importantly, the persistence of Eed in *Suz12*$^{-/-}$ embryos, which also retain H3K27me1, and the absence of H3K27me1 in *Eed*$^{null/null}$ cells are consistent with the possibility that Eed associates with a PRC2-independent H3K27 monomethylase. This interpretation is supported by the observation that H3K27me1 is rescued in *Eed*$^{null/null}$ ES lines stably expressing low levels of Eed independent of any rescue of Ezh2 protein levels. It is unclear why the low level of Eed in the stable rescue lines failed to stabilize a corresponding level of Ezh2. However, one interpretation of these results is that, when levels of Eed are limiting, Eed is preferentially assembled into the proposed monomethylase complex.

The extensive covalent modifications on histone amino-terminal tails have been proposed to serve as a histone code that controls chromatin conformations and transcriptional states [14, 15]. The complexity of this code is a product not only of the

large number of modified residues and the diverse types of chemical modifications (acetylation, methylation, phosphorylation, etc.) but also the different number of chemical moieties added to a particular residue. H3K27me3 recruits a second PcG complex, PRC1, which condenses local chromatin [1, 4, 16, 17]. However, the functions of H3K27me1 and H3K27me2 are undefined. The involvement of Eed in all three forms of methylation suggests that the marks may be functionally related, perhaps reflecting that H3K27me1 is a primed state that facilitates H3K27me2 and H3K27me3. In this model, Eed could bridge PRC2 and the putative monomethylase complex. Alternatively, H3K27me1 may itself be a functional mark, and preliminary analyses suggest that the presence of H3K27me1 alone in our stable lines is sufficient to rescue a subset of morphological defects in mutant cells (N.D.M., D.Y., and T.M., unpublished observations).

While Ezh2 clearly provides catalytic activity to PRC2, the functional roles of the noncatalytic subunits remain largely undefined. Eed^{null} protein is unable to bind Ezh2 [11, 12]. Here, we demonstrate that Ezh2 protein levels are dramatically reduced in $Eed^{null/null}$ cells. Together these results suggest that Ezh2 is unstable outside of functional PRC2 complexes. The *S. cerevisiae* WD-repeat protein Swd2p is similarly required for the stability of the HMTase Set1p, perhaps reflecting a more general requirement for WD-repeat proteins in the stable assembly of HMTase complexes [18].

3.4. MATERIALS AND METHODS

Immunofluorescence

Eed$^{null/null}$ ES cell line 21 and wild-type (*Eed*$^{+/+}$) ES cell line 25.5 [19] or *Eed*$^{null/null}$ TS cell line 3-5 and wild-type (*Eed*$^{+/null}$) TS cell line 5-4 were grown on irradiated fibroblast feeders, plated onto coverslips without feeders, and grown to subconfluency. For immunofluorescence of transfected cells, ES cells were passaged two times without fibroblast feeders, plated onto coverslips and subsequently transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA).

Coverslips and attached cells were treated with CSK buffer (100 mM NaCl, 300mM sucrose, 3mM $MgCl_2$, 10mM PIPES pH=6.8) containing 0.5% Triton-X, fixed in 4% paraformaldehyde/1X PBS, and stored in 1X PBS, 0.2% Tween-20. Subsequently, cells were washed in 1X PBS and incubated in a humid chamber with blocking buffer (1X PBS, 5% goat serum, 0.2% Tween-20, 0.2% Fish skin gelatin). Blocked samples were incubated in a humid chamber with primary antibodies (anti-H3K27me1 (Upstate, Charlottesville, VA), anti-H3K27me2 [5], and anti-H3K27me3 [5]) diluted 1:250 in blocking buffer. Then, the cells were washed in 1XPBS/0.2% Tween-20, blocked again in blocking buffer, and incubated in a humid chamber with secondary antibody (Goat anti-Rabbit Alexa 594, Molecular Probes, Eugene, OR). Finally, samples were washed in 1XPBS/0.2% Tween-20, and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Stained slides were visualized by fluorescence microscopy.

Plasmid Construction and Generation of Stable Lines

Full length *Eed, Eed*^{hypo}, and *Eed*^{null} cDNAs were cloned into the mammalian expression vector pTarget™ (Promega, Madison, WI) by conventional molecular biology techniques. For generation of stable lines, the full length *Eed* cDNA was subcloned into pcDNA3.1/Hygro (Invitrogen, Carlsbad, CA) to generate pNDM45.

PNDM45 was linearized by *Bgl*II restriction digest and electroporated into *Eed*$^{null/null}$ ES cell line 21. Clones stably expressing Eed were picked after eight days of hygromycin selection. Incorporation of the transgene was confirmed by PCR.

Coomassie Staining and Western blotting

For histone isolation, 1-2 x 10^7 ES cells were harvested and lysed with 0.002% NP-40. Nuclei were isolated by gentle centrifugation and then lysed in 0.4 N sulfuric acid. Nuclear proteins were precipitated with 20% trichloroacetic acid, washed in acetone, and resuspended in sterile water. For all other protein isolation, ES or TS cells were grown to near confluency in 35mm dishes. Whole cell lysates were generated by lysing in urea lysis buffer (7.75M Urea, 0.01M Tris pH=8, 0.1M NaH_2PO_4).

For Coomassie staining, histone preparations from wild-type and *Eed*^{null} ES cells were electrophoresed on a 15% SDS-PAGE gel. Gels were then washed in water, stained 1 hour in Simply Blue® SafeStain (Invitrogen, Carlsbad, CA), and then destained overnight in distilled water.

For Western blotting, protein preparations were separated on 10% (for nonhistone proteins) or 15% (for histone proteins) SDS-PAGE gels in Tris-Glycine. Subsequently,

proteins were transferred to Immun-Blot® PVDF membranes (BIO-RAD, Hercules, CA) in Tris-Glycine-Methanol Transfer Buffer. Membranes were blocked in 4-5% nonfat dried milk in TBST (50mM Tris-HCl pH = 7.4, 150 mM NaCl, 0.1% Tween-20), and then probed overnight at 4°C with primary antibodies diluted in blocking buffer (anti-Eed [20], anti-Ezh2 [20], anti-Actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-H3K27me1 (Upstate, Charlottesville, VA), anti-H3K27me2 [5], and anti-H3K27me3 [5]). Subsequently, blots were washed in TBST, and then probed overnight at 4°C with appropriate HRP-conjugated secondary antibodies (Pierce, Rockford, IL). Blots were washed first in TBST and then in TBS (50mM Tris-HCl pH = 7.4, 150 mM NaCl), before detecting secondary antibodies with SuperSignal® West Dura Extended Duration Substrate (Pierce, Rockford, IL). Finally, blots were exposed to film, which was then developed.

Northern blotting

Triplicate samples of wild-type and *Eed*$^{null/null}$ ES cells were harvested and lysed directly in TRIzol® Reagent (Invitrogen, Carlsbad, CA). RNA was isolated from the TRIzol® lysate by phenol-chloroform extraction, and RNA was precipitated with isopropanol. Precipitated nucleic acids were washed in 70% ethanol and resuspended in sterile water. Isolated RNAs were electrophoresed in 1X MOPS (20 mM MOPS, 50 mM sodium acetate, 1 mM EDTA, pH=7), formaldehyde, agarose gels, and then, RNA was transferred to Nytran SuperCharge membranes (Schleicher & Schuell, Keene, NH) in 10X SSC (1.5 M NaCl, 150mM $Na_3C_6H_5O_7$, pH=7).

Radiolabeled Northern probes were generated by incubating the full length *Eed* cDNA or a fragment of the *Gapdh* cDNA with random hexamers, dTTP, dATP, dGTP, α-^{32}P dCTP, and Klenow enzyme (New England Biolabs, Beverly, MA). Labeled probes were purified from unincorporated nucleotides using ProbeQuant™ G-50 Micro Columns (Amersham Biosciences, Amersham, UK).

Blots were prehybrdized at 65°C for 1-2 hours in Church buffer (1% BSA, 1mM EDTA, 0.5M $NaPO_4$, 7% SDS) and then hydridized at 65°C overnight with radiolabeled probe diluted in Church buffer. Subsequently, blots were washed in 0.2X-2X SSC, 0.1% SDS at 65°C and then exposed to film overnight at -80°C.

RealTime RT-PCR

RNA was isolated from triplicate samples of wild-type and *Eed*$^{null/null}$ ES cells using TRIzol® Reagent (Invitrogen, Carlsbad, CA) as above, and contaminating DNA was eliminated by treating with DNase for one hour at 37°C. cDNA was prepared using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and then analyzed by RealTime PCR for *Ezh2* and *Hprt* cDNA. *Ezh2* CT values were normalized to *Hprt* CT values.

3.5. REFERENCES

1. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039-1043.

2. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev *16*, 2893-2905.

3. Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197-208.

4. Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185-196.

5. Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H., and Jenuwein, T. (2003). Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol Cell *12*, 1577-1589.

6. Pasini, D., Bracken, A.P., Jensen, M.R., Denchi, E.L., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J *23*, 4061-4071.

7. Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jenuwein, T., Otte, A.P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481-495.

8. Denisenko, O.N., and Bomsztyk, K. (1997). The product of the murine homolog of the Drosophila extra sex combs gene displays transcriptional repressor activity. Mol Cell Biol *17*, 4707-4717.

9. Schumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *384*, 648.

10. van der Vlag, J., and Otte, A.P. (1999). Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet *23*, 474-478.

11. Denisenko, O., Shnyreva, M., Suzuki, H., and Bomsztyk, K. (1998). Point mutations in the WD40 domain of Eed block its interaction with Ezh2. Mol Cell Biol *18*, 5634-5642.

12. Sewalt, R.G., van der Vlag, J., Gunster, M.J., Hamer, K.M., den Blaauwen, J.L., Satijn, D.P., Hendrix, T., van Driel, R., and Otte, A.P. (1998). Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. Mol Cell Biol *18*, 3586-3595.

13. Ebert, A., Schotta, G., Lein, S., Kubicek, S., Krauss, V., Jenuwein, T., and Reuter, G. (2004). Su(var) genes regulate the balance between euchromatin and heterochromatin in Drosophila. Genes Dev *18*, 2973-2983.

14. Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

15. Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

16. Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev *17*, 1870-1881.

17. Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science *306*, 1574-1577.

18. Dichtl, B., Aasland, R., and Keller, W. (2004). Functions for S. cerevisiae Swd2p in 3' end formation of specific mRNAs and snoRNAs and global histone 3 lysine 4 methylation. Rna *10*, 965-977.

19. Morin-Kensicki, E.M., Faust, C., LaMantia, C., and Magnuson, T. (2001). Cell and tissue requirements for the gene eed during mouse gastrulation and organogenesis. Genesis *31*, 142-146.

20. Hamer, K.M., Sewalt, R.G., den Blaauwen, J.L., Hendrix, T., Satijn, D.P., and Otte, A.P. (2002). A panel of monoclonal antibodies against human polycomb group proteins. Hybrid Hybridomics *21*, 245-252.

FIGURE 3.1. Loss of H3K27 Methylation in *Eed*$^{null/null}$ ES and TS cells

Immunofluorescence analysis of **(A and D)** H3K27me1, **(B and E)** H3K27me2, and **(C and F)** H3K27me3 in wild-type and *Eed*$^{null/null}$ ES **(A-C)** and TS **(D-F)** cells. ES and TS cell colonies indicated by arrows. Examples of wild-type irradiated fibroblast feeders, which serve as internal controls for the staining, indicated by asterisks. The specificity of the methyl-specific antibodies have been previously demonstrated [5].

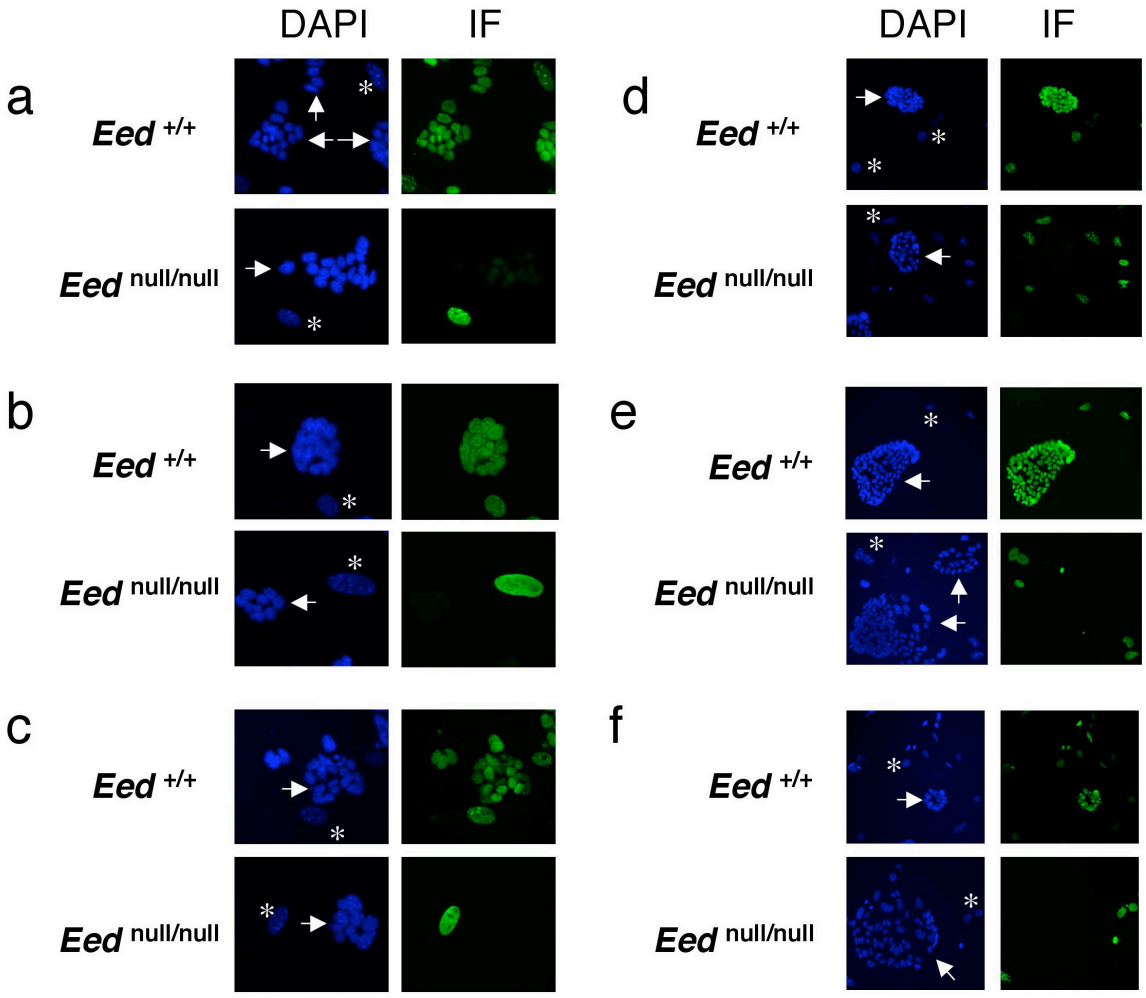

DAPI
IF
a
Eed +/+
Eed null/null
b
Eed +/+
Eed null/null
c
Eed +/+
Eed null/null
DAPI
IF
d
Eed +/+
Eed null/null
e
Eed +/+
Eed null/null
f
Eed +/+
Eed null/null

FIGURE 3.2. Reduced Eed and Ezh2 Protein Levels but Normal mRNA Levels in *Eed*$^{null/null}$ ES cells

(A) Whole cell lysates from wild-type or *Eed*$^{null/null}$ ES cells analyzed by Western blotting with antibodies detecting Eed or Ezh2. Equal loading was verified by blotting with an antibody detecting Actin. **(B)** *Eed* and *Gapdh* Northern blots. **(C)** *Eed* mRNA levels relative to *Gapdh* mRNA levels. *Eed*$^{+/+}$ values were set to 1.0. **(D)** RealTime PCR analysis of *Ezh2* mRNA. Wild-type values were set to 1.0.

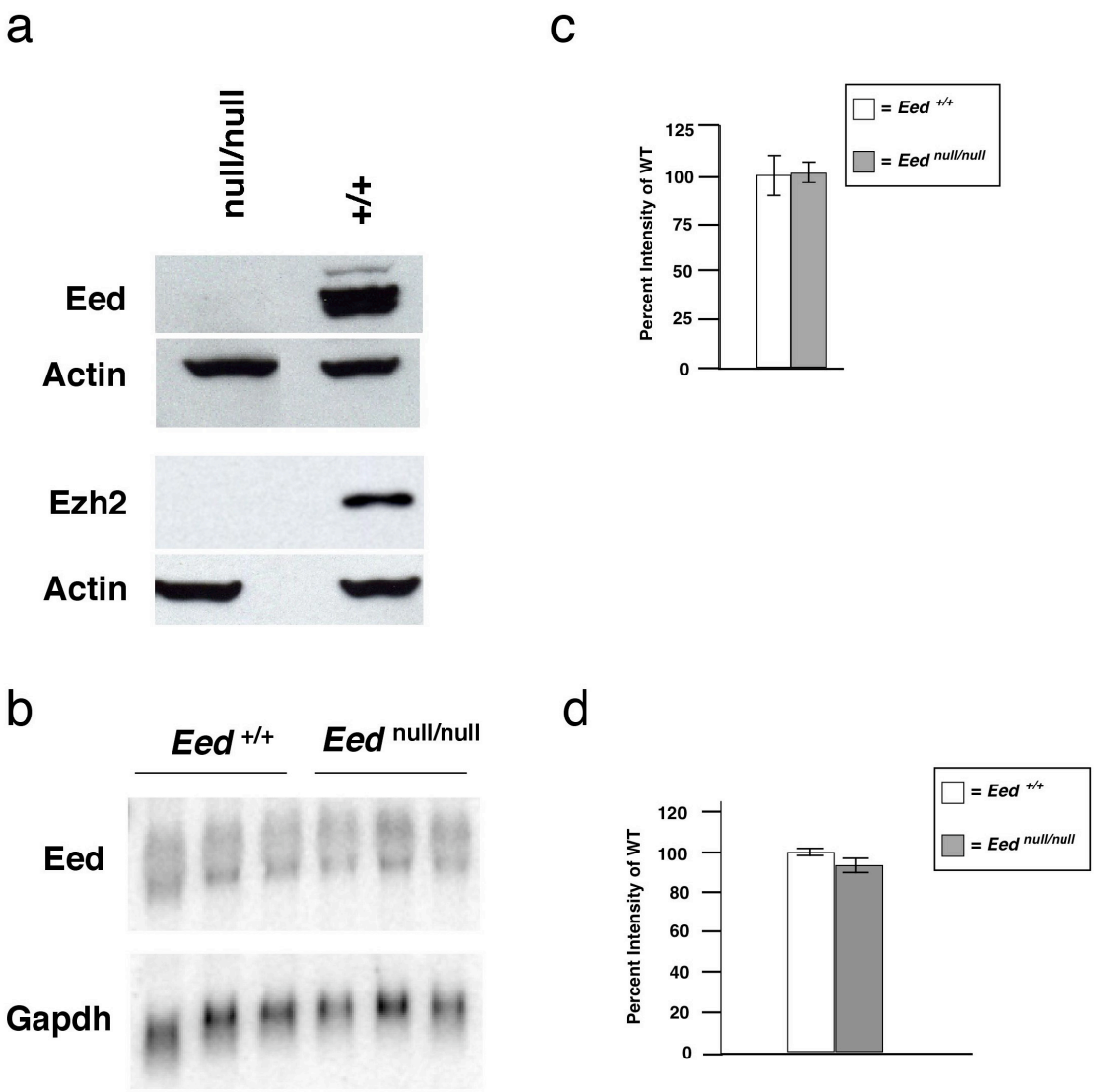

a
null/null
+/+
Eed
Actin
Ezh2
Actin
b
Eed +/+
Eed null/null
Eed
Gapdh
c
= Eed +/+
= Eed null/null
Percent Intensity of WT
125
100
75
50
25
0
d
= Eed +/+
= Eed null/null
Percent Intensity of WT
120
100
80
60
40
20
0

FIGURE 3.3. Defect in H3K27me1 Methylation in *Eed*$^{null/null}$ ES Cells is Rescued by Wild-Type *Eed*

Immunofluorescence analysis of H3K27me1 in wild-type ES cells (top panels), in *Eed*$^{null/null}$ ES cells either mock transfected (second panels) or transfected with plasmids expressing *Eed* (third panels), *Eed*^{hypo} (fourth panels), or *Eed*^{null} (fifth panels), and in *Eed*$^{null/null}$ ES cells stably expressing Eed (bottom panels).

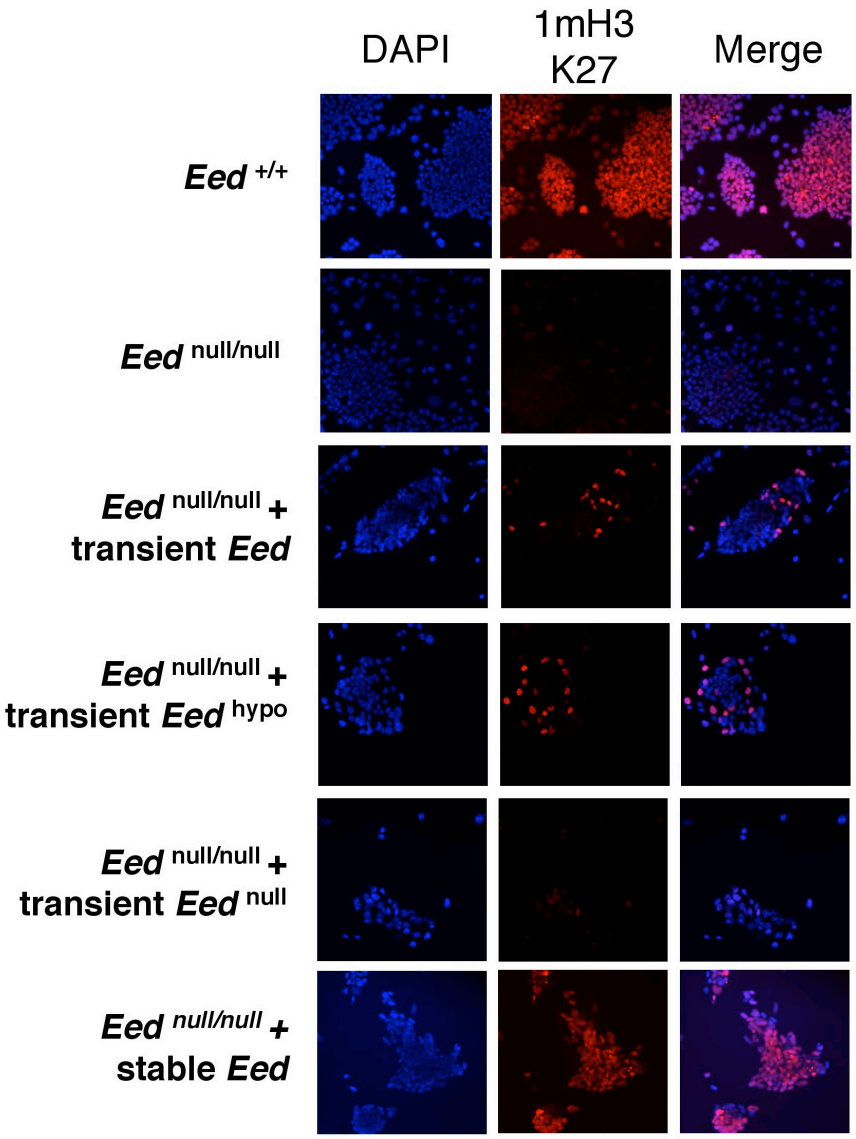
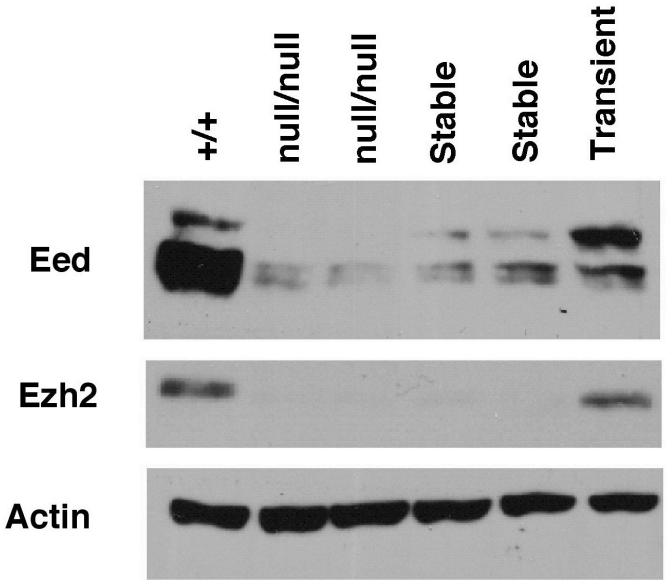

DAPI
1mH3 K27
Merge
Eed +/+
Eed null/null
Eed null/null + transient Eed
Eed null/null + transient Eed hypo
Eed null/null + transient Eed null
Eed null/null + stable Eed

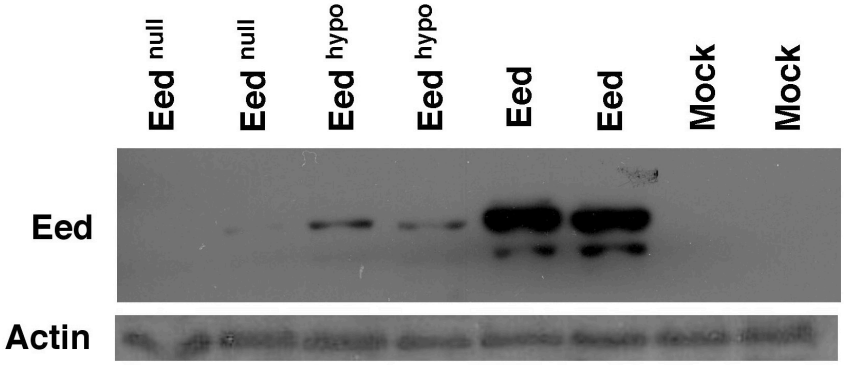
FIGURE 3.4. Eed and Ezh2 Protein Levels in Rescue Lines

(A) Whole cell lysates from wild-type ES cells (+/+), *Eed*$^{null/null}$ ES cells (null/null), *Eed*$^{null/null}$ ES cells stably expressing Eed (stable), or *Eed*$^{null/null}$ ES cells transiently transfected with an Eed expression plasmid (transient) analyzed by Western blotting with antibodies detecting Eed, Ezh2, or Actin. Low levels of Eed protein in *Eed*$^{null/null}$ ES cells may reflect a small amount of residual mutant protein or feeder contamination. **(B)** *Eed*$^{null/null}$ ES cells were cotransfected with a Gfp expressing plasmid and either mock or plasmids expressing *Eed, Eed*^{hypo}, or *Eed*^{null}. Transfections efficiencies were controlled by Gfp fluorescence, and whole cell lysates were analyzed by Western blotting with anti-Eed antibodies. Equal loading was verified by blotting with an antibody detecting Actin.

a

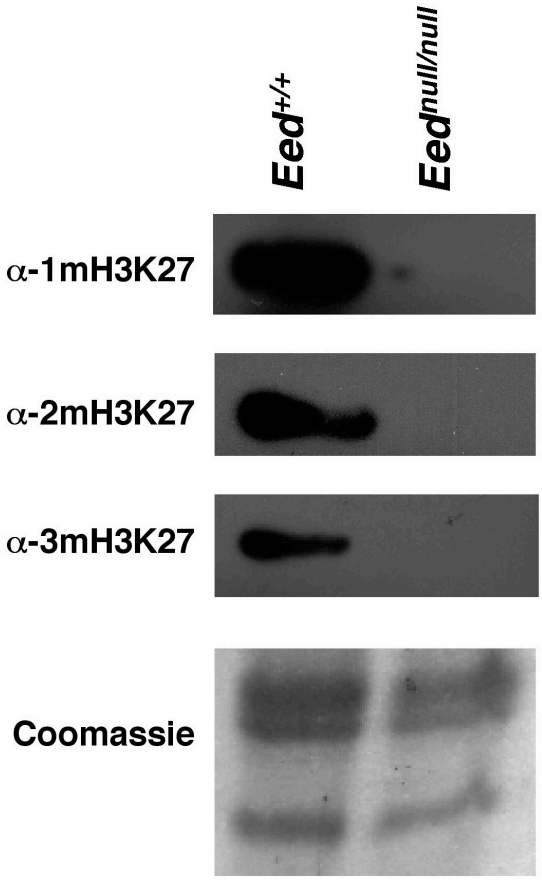

+/+
null/null
null/null
Stable
Stable
Transient
Eed
Ezh2
Actin

b


Eed null
Eed null
Eed hypo
Eed hypo
Eed
Eed
Mock
Mock
Eed
Actin

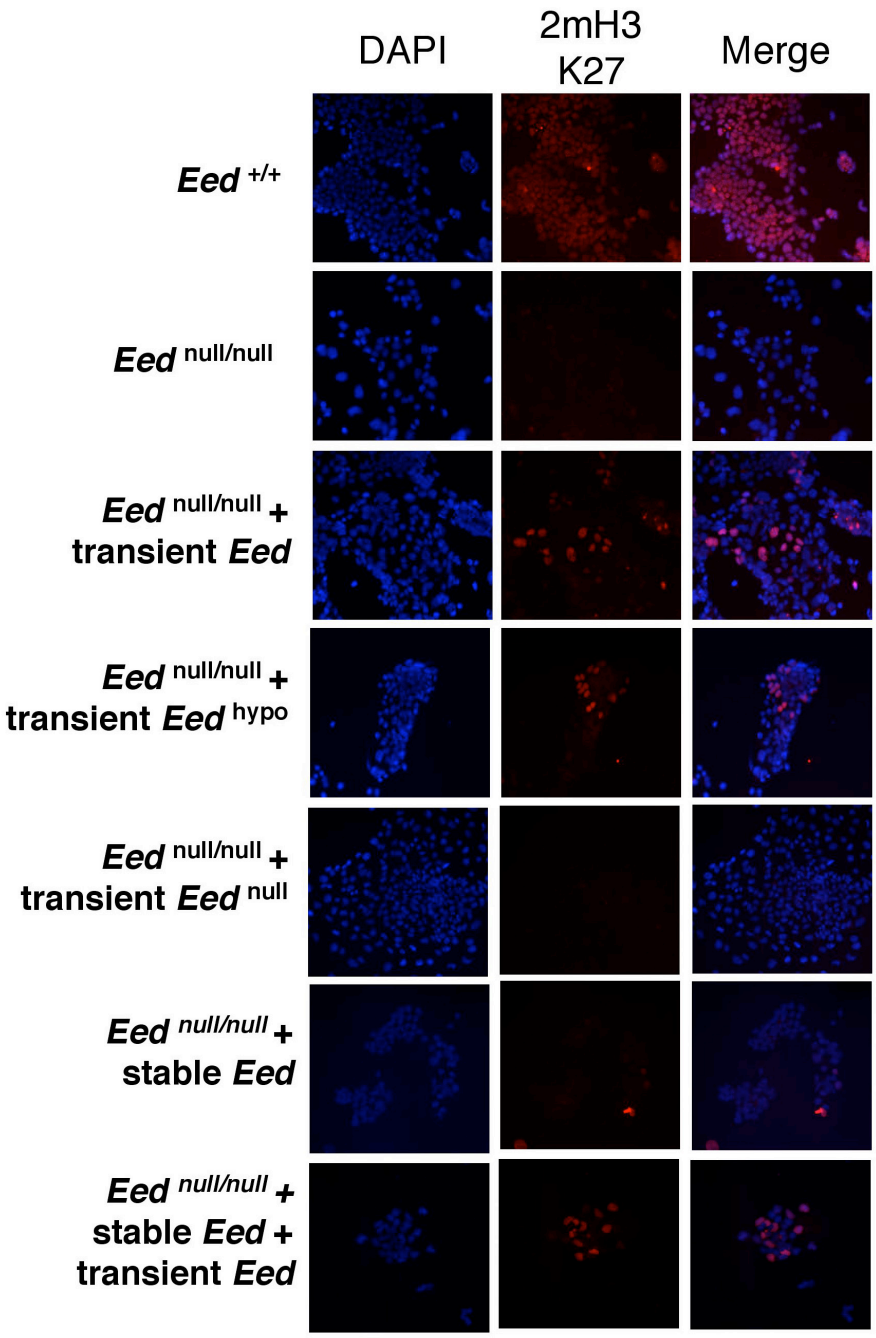
SUPPLEMENTARY FIGURE 3.1. Western Blot Confirmation of H3K27 Methylation Defects in *Eed*$^{null/null}$ ES cells

Acid-extracted histones from wild-type and *Eed*$^{null/null}$ ES cells were analyzed by Western blotting with antibodies recognizing H3K27me1, H3K27me2, and H3K27me3. Equal loading was verified by Coomassie stain.


Eed+/+
Eednull/null
α-1mH3K27
α-2mH3K27
α-3mH3K27
Coomassie

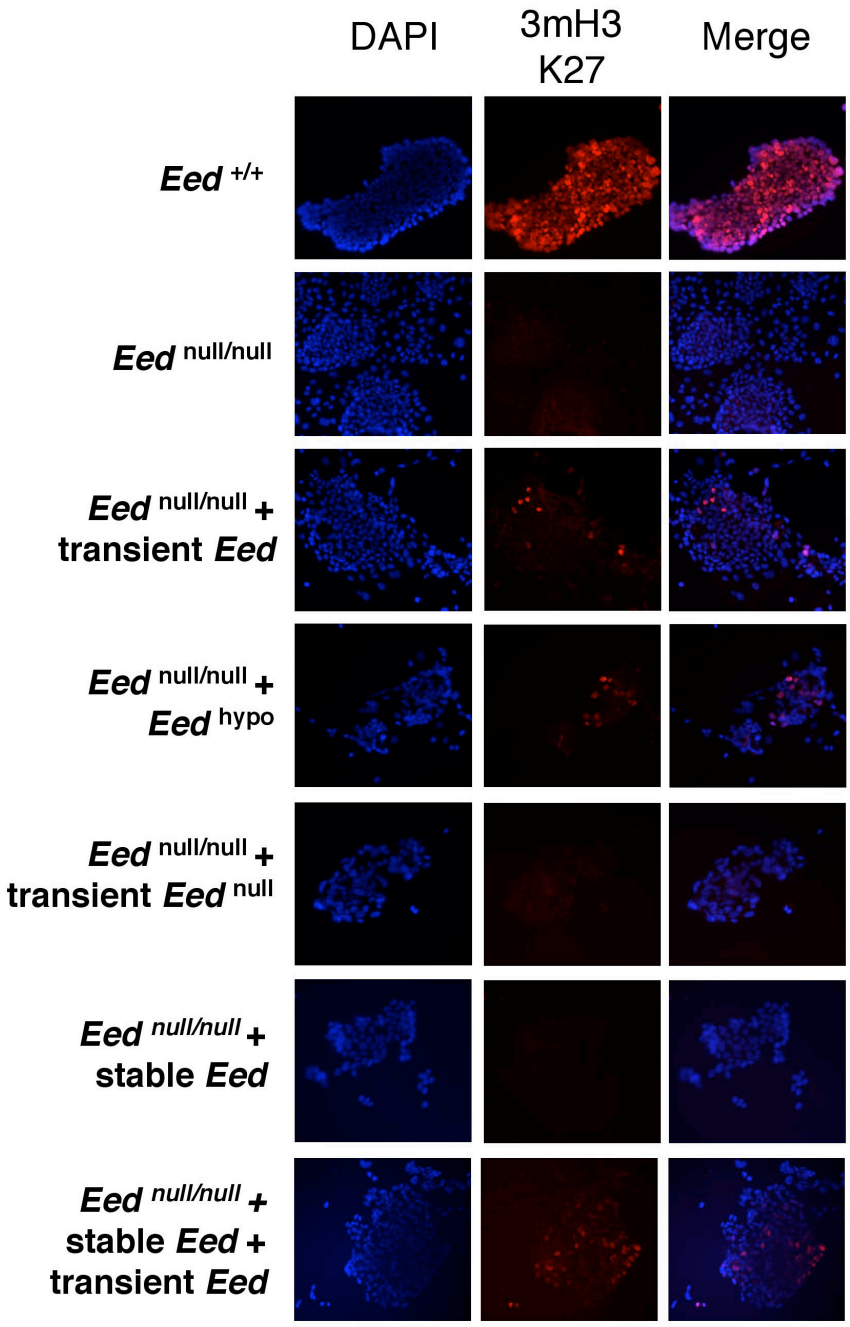
SUPPLEMENTARY FIGURE 3.2. Defect in H3K27me2 Methylation in *Eed*$^{null/null}$ ES Cells is Rescued by Wild-Type *Eed*

Immunofluorescence analysis of H3K27me2 in wild-type ES cells (top panels), in *Eed*$^{null/null}$ ES cells either mock transfected (second panels) or transfected with plasmids expressing *Eed* (third panels), *Eed*^{hypo} (fourth panels), or *Eed*^{null} (fifth panels), in *Eed*$^{null/null}$ ES cells stably expressing Eed (sixth panels), and in *Eed*$^{null/null}$ ES cells stably expressing Eed and transiently transfected with a plasmid expressing Eed (bottom panels).


DAPI
2mH3 K27
Merge
Eed +/+
Eed null/null
Eed null/null + transient Eed
Eed null/null + transient Eed hypo
Eed null/null + transient Eed null
Eed null/null + stable Eed
Eed null/null + stable Eed + transient Eed

SUPPLEMENTARY FIGURE 3.3. Defect in H3K27me3 Methylation in *Eed*$^{null/null}$ ES Cells is Rescued by Wild-Type *Eed*

Immunofluorescence analysis of H3K27me3 in wild-type ES cells (top panels), in *Eed*$^{null/null}$ ES cells either mock transfected (second panels) or transfected with plasmids expressing *Eed* (third panels), *Eed*^{hypo} (fourth panels), or *Eed*^{null} (fifth panels), in *Eed*$^{null/null}$ ES cells stably expressing Eed (sixth panels), and in *Eed*$^{null/null}$ ES cells stably expressing Eed and transiently transfected with a plasmid expressing Eed (bottom panels).


DAPI
3mH3 K27
Merge
Eed +/+
Eed null/null
Eed null/null + transient Eed
Eed null/null + Eed hypo
Eed null/null + transient Eed null
Eed null/null + stable Eed
Eed null/null + stable Eed + transient Eed

CHAPTER 4

MOLECULAR AND FUNCTIONAL MAPPING OF ISOFORM START SITES AND MOTIFS IN THE POLYCOMB GROUP PROTEIN EED

Molecular and functional mapping of EED isoform start sites and motifs required for PRC2 histone methylation

Nathan D. Montgomery[1,2,3], Della Yee[1,2], Stephanie A. Montgomery[4,5] and Terry Magnuson[1,2,3*]

(1) Department of Genetics, (2) Carolina Center for Genome Sciences, (3) Curriculum in Genetics and Molecular Biology, (4) Department of Microbiology and Immunology, (5) Carolina Vaccine Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Running Title: Molecular and functional characterization of EED

This chapter was submitted for publication to *Molecular and Cellular Biology*.

4.1. ABSTRACT

Polycomb Group (PcG) proteins represent a conserved family of developmental regulators that mediate heritable transcriptional silencing by modifying chromatin states. One PcG complex, the PRC2 complex, is composed of several proteins, including the histone H3 lysine 27 (H3K27) methyltransferase EZH2 and the WD-repeat protein EED. Histone H3K27 can be mono- (H3K27me1), di- (HeK27me2), or trimethylated (H3K27me3). However, it remains unclear what regulates the number of methyl groups added to H3K27 in a particular nucleosome. In mammalian cells, EED is present as four distinct isoforms, which are believed to be produced by utilizing four distinct, in-frame translation start sites in a common *Eed* mRNA. To assess the roles of individual EED isoforms in H3K27 methylation, we characterized three of the four EED isoform start sites and demonstrated that individual isoforms are not necessary for H3K27me1, H3K27me2, or H3K27me3. Instead, the core WD-40 motifs and the histone binding region of EED alone are sufficient to mediate all three marks, demonstrating that EED isoforms do not control the enzymatic activity of the PRC2 complex.

4.2. INTRODUCTION

Polycomb Group (PcG) proteins are a conserved family of development regulators that modify chromatin states in order to mediate heritable transcriptional silencing. PcG-mediated repression is important in diverse biological processes including X-chromosome inactivation, genomic imprinting, and segmental patterning [1-9]. One PcG complex, the PRC2 complex, is composed of several bona fide PcG proteins, including the histone H3 lysine 27 (H3K27) methyltransferase EZH2 and the WD-repeat protein EED [9-12].

Four EED isoforms are found in mammals, and these isoforms are thought to be produced by utilizing four in-frame translation start sites in the *Eed* mRNA [13]. The identities of those sites were postulated on the basis of *in vitro* translation studies, which were supported by subsequent immunoblotting experiments with antibodies raised to peptides predicted to be present in some but not other isoforms [13, 14]. However, the four putative start sites have not been demonstrated formally *in vivo*.

Although most proteins initiate translation at methionine-encoding AUG codons, an increasing number of proteins are recognized to initiate translation from non-AUG codons. Often, these alternative start sites generate upstream isoforms of proteins also translated from downstream, canonical AUG initiation codons, and typically, the alternative codons differ from the canonical AUG sequence at only one of the three nucleotide positions [15, 16]. For instance, Fibroblast Growth Factor-2 (FGF2) is present as five isoforms in mammalian cells, with four CUG initiation codons upstream of a canonical AUG start site, and Vascular Endothelial Growth Factor (VEGF) is translated from both an upstream CUG and a downstream AUG [17-21]. Translation of the putative

EED isoforms appears to be consistent with both of these trends as well. The upstream EED-1 and EED-2 are postulated to initiate translation from non-canonical GUG codons at positions 169-171 and 274-276 in the *Eed* cDNA, respectively, and the putative start sites for EED-3 and EED-4 are canonical AUG sequences at positions 451-453 and 493-495 [13, 14].

EED isoform usage is regulated developmentally, and EED-2, which has only been observed in undifferentiated stem cells and in tumors, has been proposed to be important in maintaining developmental plasticity [22]. However, definitive biochemical functions of the various EED isoforms have not been demonstrated. Previous work postulated that EED isoforms control the substrate specificity of the PRC2 complex. In those initial studies, the largest isoforms, EED-1 and EED-2 appeared to direct EZH2 methyltransferase activity towards histone H1K26, whereas EED-3 and EED-4 appeared to direct EZH2 methyltransferase activity towards H3K27 [13]. However, a second recent study failed to confirm these findings [23].

Histone H3K27 can be mono- (H3K27me1), di- (HeK27me2), or trimethylated (H3K27me3)[24]. H3K27me3 is a repressive histone modification that localizes to confirmed targets of PcG silencing, including the inactive X-chromosome[1, 2, 25-27]. H3K27me3 mediates its repressive effect by recruiting to chromatin or at least stabilizing the association of a second PcG complex, PRC1 [28-31]. Conversely, the functions of H3K27me1 and H3K27me2 are not known. Moreover, it remains unclear what regulates the number of methyl groups added to H3K27 in a particular nucleosome. Two simplistic models have been proposed to explain this specificity [32]. The first model proposes that distinct methyltransferases or distinct complexes mediate each H3K27

methylation state. In the second model, a single methyltransferase is responsible for all three methylation states but is somehow regulated so as to add one methyl group to certain nucleosomes and two or three methyl groups to others.

Because all four known EED isoforms associate with EZH2 [13, 22], we examined whether these isoforms might control the number of methyl groups added to H3K27 in a particular nucleosome. In the present study, we definitively characterize three of the four EED isoform start sites and demonstrate that individual isoforms are not necessary for H3K27me1, H3K27me2, or H3K27me3. Instead, EED's core WD-40 motifs and histone binding region alone are sufficient to mediate all three marks, demonstrating that EED isoforms do not control the enzymatic activity of the PRC2 complex.

4.3. METHODS AND MATERIALS

Cell lines and culture

CD1 murine embryonic fibroblasts (MEFs) were plated on gelatin-coated coverslips and grown to subconfluency. Wild-type embryonic stem (ES) cell line 25.5 and *Eed*$^{l7Rn5\text{-}3354SB/l7Rn5\text{-}3354SB}$ (herein referred to as *Eed* mutant or *Eed*$^{-/-}$) ES cell line 21 were grown first on irradiated fibroblast feeders before being plated on coverslips without feeders and grown to subconfluency [33]. In transfection experiments, ES cells plated on coverslips were transfected using Lipofectamine 2000™ (Invitrogen) and harvested 48 hours later.

Immunofluorescence

Cells on coverslips were permeabilized with CSK buffer (100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM PIPES [pH 6.8]) and fixed in 4% paraformaldehyde. To stain, cells were washed in phospho-buffered saline (PBS) and incubated with blocking buffer (PBS, 5% goat serum, 0.2% Tween-20, 0.2% fish skin gelatin). After blocking, samples were incubated with primary antibody (anti-H3K27me1 [Upstate], anti-3mK27me2[24], anti-3mK27me3[24], or anti-HP1-∝ [Upstate]), which had diluted 1:250 in blocking buffer. Subsequently, cells were washed in PBS/0.2% Tween-20 and then incubated with secondary antibody (Goat anti-Rabbit Alexa 594 [Molecular Probes]) diluted 1:250 in blocking buffer. Blocking and antibody incubations were always performed in a humid chamber at 37° C. Subsequently, cells were washed again in PBS/0.2% Tween-20 and mounted with Vectashield™ containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories). Stained slides were

visualized by fluorescence microscopy, and then black and white images were captured with a Spot CCD digital camera before being pseudo-colored and merged with Spot software V3.5.9 (Diagnostic Instruments Inc.).

Plasmid Construction

Constructs expressing *mes-6, esc, escl,* or *Eed* cDNAs were cloned into the shuttle TA-cloning vector pGEM®-T Easy (Promega) and then subcloned into EcoRI-digested pTarget™ (Promega) by conventional molecular biology techniques. The orientation and identity of all inserted sequences were confirmed by fully sequencing the cDNA and the cloning junctions.

Eed cDNAs were truncated by PCR, utilizing forward primers that annealed within the *Eed* cDNA and reverse primers anchored in pTarget (primer sequences available upon request). Site-directed point mutations were generated by standard methods. Briefly, primers spanning EED-3 and EED-4 start sites but harboring ATG→ATA mutations were used as forward primers with a reverse primer anchored in the pTarget. Finally, in order to engineer strong translation start sites, in frame consensus Kozak-(GCCACC)ATG 5' extensions were included on forward primers.

Western blotting

Wild-type ES cell line 25.5, wild-type ES cell line E14 [34], mock-transfected *Eed* mutant ES cell line 21, and *Eed* mutant ES cell line 21 transfected with various expression constructs were harvested in urea lysis buffer (7.5 M Urea, 0.01 M Tris [pH 8.0]), 0.1M NaH_2PO_4) 48 hours after transfection. Urea-lysates were also harvested from

mouse *Wap-T_{121}* mammary tumor tissue, which is generated by tissue-specific expression of T_{121}, a fragment of SV40 T antigen that interferes with the function of Retinoblastoma-family proteins [35]. Proteins were separated on 10% SDS-PAGE gels in Tris-Glycine running buffer and transferred to Immun-Blot PVDF membranes (Bio-Rad) in Tris-Glycine Methanol transfer buffer. Membranes were blocked in 5% non-fat dried milk (NFDM [Food Lion])/TBST (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20) and then incubated overnight at 4° C with a mouse monoclonal anti-EED antibody [36] diluted 1:400 in 3% NFDM/TBST. Membranes were vigorously washed in TBST and then incubated overnight at 4° C with HRP-conjugated goat anti-rabbit antibody (Pierce) diluted 1:3000 in 5% NFDM/TBST. Membranes were then vigorously washed in TBST and TBS (50 mM Tris HCl [pH 7.4], 150 mM NaCl), before adding SuperSignal West Dura Extended Duration Substrate (Pierce) developing reagents. Finally, blots were exposed to film and developed.

4.4. RESULTS

Distinct localization of H3K27 methylation states

Unlike mouse stem cell lines, differentiated mouse cells, such as murine embryonic fibroblasts (MEFs,) have a striking nuclear architecture, in which regions of the genome packaged as part of the pericentric heterochromatin are clearly visible by DAPI staining as DNA-rich foci. Using this characteristic DNA staining pattern to provide landmarks, we assessed the localization of H3K27me1, H3K27me2 and H3K27me3 in CD1 MEFs (Figure 4.1). As previously reported, H3K27me1 appeared to be enriched in the DNA-rich pericentric heterochromatin (Figure 4.1A and[24]). Conversely, H3K27me2 and H3K27me3 were specifically excluded from these regions, instead staining in a pattern reciprocal to that of H3K27me1 (Figures 4.1B and 4.1C). HP1-α, an established marker of pericentric heterochromatin[37-39], was also enriched in the DNA-rich foci, confirming the identity of these regions (Figure 4.1D). Finally, H3K27me2 and H3K27me3 staining patterns were distinguished by the characteristic enrichment of H3K27me3 on the inactive X-chromosome (Figure 4.1B and 4.1C).

Characterization of EED isoforms expressed in wild-type ES cells

The distinct localization patterns of the three H3K27 methylation states indicates that the number of methyl groups added to H3K27 in a particular nucleosome is a regulated process. Given the existence of three H3K27 methylation states and four EED isoforms, candidates to control this specificity include the EED isoforms themselves. To test this possibility, we aimed to rescue *Eed* mutant embryonic stem (ES) cells, which lack detectable levels of endogenous H3K27me1, H3K27me2, and H3K27m3 [32], with

constructs expressing individual EED isoforms. However, before a systematic series of rescue experiments was performed, we attempted to confirm the identities of the EED isoforms.

Previous work indicates that all four EED isoforms are present in wild-type, undifferentiated mouse ES cells[22]. However, we consistently detect only three isoforms in multiple, independently-derived embryonic stem cell lines (Figure 4.2). To confirm the identity of the three isoforms observed in our undifferentiated ES cells, we compared the isoforms present in those cells to the isoforms present in HeLa cells and in mammary tumor tissue from mouse *Wap-T_{121}* mammary tumors. Previous work has demonstrated that EED-1, EED-3, and EED-4 are expressed in HeLa cells and that EED-2 is upregulated in many mouse tumors[22]. Consistent with those reports, we observed high levels of EED-1, EED-3 and EED-4 and much lower levels of EED-2 in HeLa cells (Figure 4.2A). Similarly, EED-2, along with EED-3 and EED-4, was observed in *Wap-T_{121}* mammary tumors (Figure 4.2B). Comparison of the isoforms present in those sources with the isoforms present in our embryonic stem cells confirmed the identity of the isoforms we observe in embryonic stem cells as EED-1, EED-3, and EED-4 (Figure 4.2).

Deletion mapping of EED isoform start sites

Previous work has suggested that the four EED isoforms are generated by utilizing four distinct translation initiation sites in a common *Eed* messenger RNA[13]. In the mouse, those putative start sites correspond to GUG 169-171, GUG 274-276, AUG 451-453, and AUG 493-495, respectively (Figure 4.3A). Overall, mouse and human

EED are very similar proteins. In fact, the predicted EED-3 and EED-4 isoforms are 100% identical between the two species (37). However, the sequences between the putative EED-1 and EED-2 start sites, are much more variable [40]. Given the abrupt boundary between highly variable and nearly identical sequences, we questioned whether the actual EED-1 start site might be further 3' than previously reported.

To map EED isoform start sites, we generated a series of *Eed* expression constructs progressively truncated at the 5' end of the *Eed* cDNA (Figure 4.3A). Individual constructs were then transiently transfected into *Eed* mutant ES cells, which lack detectable endogenous EED, and EED isoform expression was assessed by western blotting. In these experiments, the furthest 5' intact translational start site was generally utilized preferentially to downstream translational start sites (Figure 4.3). This observation suggests that regulated usage of the various EED translational start sites is not simply a consequence of the interaction between *trans*-acting factors and sequences present in the message but instead may be influenced by upstream events, such as splicing. Additionally, because isoform expression could be lost either by deleting past an isoform start site or by deleting an upstream regulatory element required for translation from an intact start site, the absence of a band is uninformative in this assay. However, the continued presence of an isoform after its putative start site has been deleted is strong evidence that the actual start site must be further downstream.

Consistent with the relaxed sequence conservation between mouse and human *Eed* sequences beginning with and immediately downstream from GUG 169-171, EED-1 was expressed from constructs truncated 32 (Δ201) and even 88 (Δ257) nucleotides beyond the reported EED-1 start site, suggesting that EED-1 may not initiate translation

at GUG 169-171 as previously proposed (Figure 4.3B). EED-1 expression was lost only after deleting the 5' 312 nucleotides of the *Eed* cDNA, a deletion extending 143 nucleotides beyond the reported EED-1 start site and 38 nucleotides beyond the published EED-2 start site (Figure 4.3B). Consistent with the published identity of the EED-3 start site at AUG 451-453, this isoform was observed after deleting the 5' 417 nucleotides but not after deleting the 5' 455 nucleotides (Figure 4.3B). Finally, EED-4 was present even in the largest truncation, which deleted the 5' 455 nucleotides, consistent with the published EED-4 start site residing at AUG493-495 (Figure 4.3B).

Confirmation of EED isoform start sites

To verify that GUG 169-171 is upstream of the actual EED-1 start site, we forced expression from this codon by replacing the sequences encoding GUG 169-171 with a canonical translation start site consisting of a consensus Kozak sequence followed by an AUG initiator codon (Figure 4.4A). Consistent with the hypothesis that EED-1 translation actually initiates further downstream, the resulting product was substantially larger than EED-1 (Figure 4.4B, asterisk). A lower level of an EED-1 sized product, presumably initiating at the actual start site further downstream, was also observed after transfection with this construct (Figure 4.4B, arrow).

Given that the deletion mapping data indicates that either the true EED-1 start site or some regulatory element required for translation from the true EED-1 start site maps between nucleotides 257 and 312, we hypothesized that EED-1 may be produced by translation initiating from the predicted EED-2 start site at GUG 274-276. To test that possibility, we forced expression of a protein initiating translation at that site by replacing

GUG 274-276 with a consensus Kozak followed by an AUG initiator codon (Figure 4.4A). Consistent with EED-1 translation initiating at this location, the resulting product was the same size as EED-1 observed in mouse ES cells (Figure 4.4B).

Because EED-2 was not expressed in our wild-type ES cells or from any of our truncated expression constructs, we were unable to characterize this isoform. However, driving translation from a candidate start site at GUG 397-399 produced a protein that appears to be smaller than EED-2 (data now shown), suggesting that the EED-2 start site lies between GUG 274-276 and GUG 397-399 or that EED-2 is generated by alternative mechanisms.

Our deletion mapping data were consistent with the presumptive EED-3 and EED-4 start sites residing at AUG 451-453 and AUG 493-495, respectively. To confirm that translation of those isoforms originates at those sites, we engineered AUG→AUA site directed point mutations into the AUG 451-453 and AUG 493-495 codons (Figure 4.4A). Consistent with those codons being the initiation codons for the two smaller isoforms, constructs harboring those mutations failed to express EED-3 and EED-4 (Figure 4.4C).

Ability of EED isoforms to mediate H3K27 methyltransferase activity

To determine whether all three EED isoforms present in our ES cells are required to mediate the three H3K27 methylation states, *Eed* mutant ES cells were transiently transfected with a series of *Eed* cDNA expression constructs. This rescue assay has previously been utilized to demonstrate that protein(s) expressed from a full-length *Eed*

cDNA cassette can mediate all three H3K27 methylation states[32], although the role of individual isoforms in that rescue has not been directly assessed.

Constructs harboring site-directed mutations to eliminate EED-3 and EED-4 expression or truncated to eliminate EED-1 expression were both able to rescue all three H3K27 methylation states in *Eed* mutant ES cells (Figure 4.5D and E). Additionally, a construct retaining only the EED-4 start site also rescued H3K27me1, H3K27me2, and H3K27me3 (Figure 4.5F). As previously reported, expression of the $Eed^{I7Rn5\text{-}3354SB}$ allele, which produces an unstable and nonfunctional protein harboring a L→P substitution, failed to rescue H3K27 methylation(data not shown and [32]). Collectively, these results demonstrate that the three H3K27 methylation states are not dependent on individual EED isoforms.

Disruption of EED WD-40 motifs eliminates methyltransferase activity

EED and its homologs in other organisms are WD-repeat proteins. However, there is disagreement about the number of WD-40 motifs present in EED, with estimates varying between five and seven [41-43]. In functional studies assessing EED's ability to bind EZH2 or its ability to mediate transcriptional repression when tethered to a GAL4 DNA binding domain, only five WD-40 motifs have appeared functionally necessary [44, 45]. Those five motifs map to *Eed* cDNA sequences 721-808 (WD-40 motif 1), 1012-1105 (WD-40 motif 2), 1150-1240 (WD-40 motif 3), 1330-1444 (WD-40 motif 4), and 1672-1762 (WD-40 motif 5). To determine whether those same regions are required for EED's ability to mediate H3K27 methylation, *Eed* mutant ES cells were transiently

transfected with a series of *Eed* cDNA expression constructs progressively truncated from either the N- or C-terminus.

Deletion of any of the five putative WD-40 motifs abolished EED's ability to mediate H3K27 methylation, as demonstrated by immunofluorescence (Figure 4.6D-G). However, N-terminally truncated proteins containing all five WD-40 motifs retained H3K27 methyltransferase activity. A protein lacking the N-terminal 16 amino acids of EED-4 (Δ5' 541) was able to mediate all three H3K27 methylation states (Figure 4.6B), suggesting that the N-terminal regions in EED, including those amino acids that distinguish individual isoforms, are not required for the catalytic activity of the PRC2 complex. A second deletion, Δ5' 697 also left the five putative WD-40 motifs intact. However, protein expressed from this construct retains only 8 amino acids upstream of WD-40 motif 1. Although the expression of the Δ5' 697 construct consistently rescued the H3K27me1 defect, little if any H3K27me2 and H3K27me3 were observed (Figure 4.6C).

Nematode and fly *Eed* homologs fail to rescue H3K27 methylation defects in *Eed* mutant cells

Given that EED's WD repeats alone appear sufficient to mediate the PRC2 complex's H3K27 methyltransferase activity, we examined whether the complex's requirement for a WD-repeat protein can be satisfied by WD-repeat proteins other than EED, in particular *Eed* homologs from other organisms. To address this question, *Eed* mutant ES cells were transiently transfected with constructs expressing either the *C. elegans* homolog of *Eed*, *mes-6*, or the *D. melanogaster Eed* homologs, *esc* and *escl*.

Previous work has suggested that fly and mouse *Eed* homologs may not be functionally equivalent, as *Eed* functions in a dominant negative fashion in flies. Consistent with functional differences in these homologous proteins, *mes-6*, *esc*, and *escl* were all unable to rescue the H3K27 methylation defects in *Eed* mutant mouse ES cells (Figure 4.7), even though MES-6 and ESC normally exist in PcG complexes with H3K27 methyltransferase activity [10, 12, 46].

4.5. DISCUSSION

EED-1, EED-3, and EED-4 translation start sites map to GUG 274-276, AUG 451-453, and AUG 493-495

Mammalian EED proteins are present as four isoforms of unclear function [13, 22, 23]. Previous work has suggested that the four EED isoforms are generated by translation initiating at GUG 169-171, GUG 274-276, AUG 451-453, and AUG 493-495, respectively [13, 14]. This interpretation followed from early studies by Denisenko and Bomsztyk, who assessed EED proteins translated in a cell free system [14]. Subsequently, Kuzmichev *et al.* generated an isoform-restricted EED antibody, called αNT, which was raised to peptides encoded by *Eed* cDNA sequences from positions 258-453 (M26). Because the αNT recognized EED-1 and EED-2 but not EED-3 and EED-4, those results demonstrated that EED-1 and EED-2 must include the αNT epitope and must initiate translation upstream of M451-453, as predicted by Denisenko and Bomsztyk [14].

Here, we have directly assessed the identity of the EED translation start sites by a combination approach involving deletion mapping, forced translation from reported start sites, and site-directed mutagenesis of candidate initiation codons. These experiments definitively map EED-1, EED-3, and EED-4 start sites to GUG 274-276, AUG 451-453, and AUG 493-495, respectively. Importantly, because the informative αNT antibody utilized by Kuzmichev *et al.* recognizes amino acids that would be present not only in a hypothetical protein initiating at GUG 169-171 but also in a protein initiating at GUG 274-276, the EED-1 initiation site reported here, the immunoblotting data from Kuzmichev *et al.* are fully consistent with the results presented in the present work [13].

On other hand, these results do contradict the earlier work of Denisenko and Bomsztyk [14]. Denisenko and Bomsztyk assessed EED proteins expressed from truncated and engineered *Eed* cDNAs comparable to those utilized here. However, whereas we assessed translation from those messages in living cells, Denisenko and Bomsztyk utilized a rabbit reticulocyte cell-free system. In their assay, a truncation that would have deleted the 5' 222 nucleotides in our constructs eliminated EED-1 expression, even though a truncation extending 35 nucleotides further (Δ257) did not disrupt EED-1 expression in our assay (Figure 4.3B). The differences in our respective methodologies may account for the discrepancy in our results. The major advantage to the transient transfection assay employed in this report is that it allows direct comparison of EED expressed from expression constructs to endogenous EED expressed in the same cell type.

In their cell-free system, Denisenko and Bomsztyk observed apparent upregulation of EED-1 after mutating GUG 169-171 and flanking sequences to more closely resemble a canonical initiation sequence. Whereas a similar experiment in our assays produced a protein larger than EED-1 (Figure 4.4B), Denisenko and Bomsztyk reported production of an EED-1 sized protein. A protein initiating at GUG 169-171 is predicted to be less than 4 kDa larger than a protein initiating at GUG 274-276. In our hands, resolving these bands required large gels run at low voltage. Accordingly, differences in electrophoresis conditions could explain why the protein expressed in their assay appeared to approximate the size of EED-1.

The absence of EED-2 expression precludes its characterization

We were unable to characterize the EED-2 start site because endogenous expression of that isoform was not detectable in our ES cells nor was EED-2 expression detected from any truncated *Eed* expression construct expressed in ES cells. Previous studies demonstrated EED-2 expression in undifferentiated ES cells and in tumors [22]. It is not clear why EED-2 was not present in the ES cells utilized here. Kuzmichev *et al.* demonstrated that EED-2 is rapidly downregulated when ES cells are stimulated to differentiate [22]. Hence, the most parsimonious explanation for our failure to detect EED-2 is that the ES cells employed were beginning to differentiate when they were harvested. If so, the cells must nevertheless retain their full developmental potential, because similarly cultured cells have contributed to all three germ layers in our hands in embryonic chimera experiments [33]. In the event that the cells are beginning to differentiate, one possible explanation would be the removal of the cells from fibroblast feeders prior to harvesting in order to minimize feeder contamination in our immunoblotting assays. However, EED-2 was also not detectable in E14 ES cells (Figure 4.2), which are feeder-independent [34]. Finally, because EED-2 was observed in both mouse mammary tumors and in HeLa cells (Figure 4.2), the absence of EED-2 in our ES cells is not simply a more general inability to detect EED-2.

Regardless of the reason that EED-2 was not expressed in our ES cells, its absence precluded characterization of its start site. However, a GUG at position 397-399 fulfills the minimal requirements of a translational start site [15, 16]. As a result, we forced expression of a protein initiating at that location and compared it to EED-2 from *Wap-T*$_{121}$ mammary tumor tissue (data not shown). The observed protein was smaller than EED-2, implying that EED-2 translation must initiate upstream of GUG 397-399 but

downstream of EED-1's start site at GUG 277-279. However, because we have not observed EED-2 from an intact *Eed* mRNA, we cannot rule out that EED-2 could be generated by a post-translational modification, such as cleaving, or by alternative splicing.

EED isoforms do not regulate the number of methyl groups added to H3K27

Although the biological functions of H3K27me1 and H3K27me2 have not been defined, it is clear from localization studies that the three H3K27 methylation states are partitioned to nucleosomes associated with distinct regions of the genome (Figure 4.1 and [24]). This specificity, in turn, implies that the number of methyl groups added to a particular nucleosome is a regulated process. We hypothesized that EED isoforms could function as regulatory switches controlling H3K27 methylation states. However, none of the EED isoforms were specifically required in order to generate H3K27me1, H3K27me2, or H3K27me3 (Figure 4.5). In fact, a truncated EED protein initiating sixteen amino acids downstream of EED-4's start site appeared to mediate H3K27 methylation as robustly as the full-length protein (Figure 4.6). These results demonstrate that the N-terminal extensions discriminating EED isoforms are not required for the enzymatic activity of the PRC2 complex. Instead, we propose that the four isoforms direct PRC2 complexes to distinct targets. While such a regulatory function could involve determining the protein substrates preferred by EZH2 as suggested by Kuzmichev *et al*. [13], an equally intriguing possibility is that the isoforms function to localize EED-containing complexes to distinct regions of the genome.

EED WD-40 motifs and histone binding regions are required for H3K27 methylation

EED's ability to mediate H3K27 methylation was lost in constructs truncated to eliminate any of the protein's five WD-40 motifs (Figure 4.6). These observations are consistent with earlier studies indicating that all five WD-40 motifs are required for EZH2 binding and EED-mediated transcriptional repression [44, 45]. Interestingly, whereas the Δ5' 541 construct was able to rescue all three H3K27 methylation states, the protein expressed from the Δ5' 697 construct, which also retained all five WD-40 motifs, was only able to mediate substantial levels of H3K27me1 (Figure 4.6B and 4.6C). Recent work suggests that the N-termini of EED and of its fly homolog ESC bind to histone H3 and that the histone binding N-terminus of ESC is required for H3K27me3 [47]. The Δ5' 697 construct deletes the region of EED required for histone H3 binding. Accordingly, the reduced levels of H3K27me2 and H3K27me3 mediated by this construct suggest that, as in Drosophila, the histone H3-binding N-terminus of EED may be required for full activity of PRC2 complex. Previously, we have demonstrated a quantitative relationship between levels of EED and H3K27 methylation [32]. Whereas low levels of EED are sufficient to mediate H3K27me1, higher levels appear necessary to mediate H3K27me2 and H3K27me3. The Δ5' 697 protein is likely a severely impaired protein that retains its ability to interact with the catalytic subunit via the intact WD-40 motifs but has greatly reduced activity due to the absence of the histone-binding N-terminus. As previously proposed, the histone-binding region of EED may be required to position PRC2 complexes appropriately in order to mediate the necessary histone-complex interactions required for full methylation [47]. Consequently, the Δ5' 697

protein appears to have an intermediate level of activity sufficient to mediate low levels of H3K27 methylation but insufficient for full H3K27me3.

Fly and worm *Eed* homologs fail to rescue *Eed* mutant H3K27 methylation defects

Despite the presence of conserved functional motifs, including WD-40 motifs and a histone binding amino terminus, the fly and worm homologs of *Eed* were unable to rescue the H3K27 methylation defects in *Eed* mutant ES cells (Figure 4.7). These results extend earlier work indicating that EED and ESC and are not functionally equivalent. In Drosophila, *Eed* is unable to rescue *esc* mutant embryonic lethality and instead functions in a dominant negative fashion, enhancing a leg transformation defect [48]. More importantly, *in vitro* binding experiments fail to detect an interaction between mouse EED and fly E(Z) [48]. Mouse EED and fly ESC are 55% identical and 74% similar in the regions of the respective proteins harboring the WD-40 motifs that mediate interaction with EZH2 and E(Z) [48]. Hence, these results suggest that subtle sequence features that distinguish these proteins must be critical for functional interactions between EED and EZH2 and between ESC and E(Z).

4.6. REFERENCES

1. Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

2. Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jenuwein, T., Otte, A.P., and Brockdorff, N. (2003). Establishment of histone h3 methylation on the inactive X chromosome requires transient recruitment of Eed-Enx1 polycomb group complexes. Dev Cell *4*, 481-495.

3. Gebuhr, T.C., Bultman, S.J., and Magnuson, T. (2000). Pc-G/trx-G and the SWI/SNF connection: developmental gene regulation through chromatin remodeling. Genesis *26*, 189-197.

4. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004). Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet *36*, 1291-1295.

5. Umlauf, D., Goto, Y., Cao, R., Cerqueira, F., Wagschal, A., Zhang, Y., and Feil, R. (2004). Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. Nat Genet *36*, 1296-1300.

6. Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J.C., Nagy, A., and Magnuson, T. (2001). Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat Genet *28*, 371-375.

7. Mager, J., Montgomery, N.D., de Villena, F.P., and Magnuson, T. (2003). Genome imprinting regulated by the mouse Polycomb group protein Eed. Nat Genet *33*, 502-507.

8. Ringrose, L., and Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet *38*, 413-443.

9. Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039-1043.

10. Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell *111*, 197-208.

11. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev *16*, 2893-2905.

12. Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell *111*, 185-196.

13. Kuzmichev, A., Jenuwein, T., Tempst, P., and Reinberg, D. (2004). Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell *14*, 183-193.

14. Denisenko, O.N., and Bomsztyk, K. (1997). The product of the murine homolog of the Drosophila extra sex combs gene displays transcriptional repressor activity. Mol Cell Biol *17*, 4707-4717.

15. Touriol, C., Bornes, S., Bonnal, S., Audigier, S., Prats, H., Prats, A.C., and Vagner, S. (2003). Generation of protein isoform diversity by alternative initiation of translation at non-AUG codons. Biol Cell *95*, 169-178.

16. Tikole, S., and Sankararamakrishnan, R. (2006). A survey of mRNA sequences with a non-AUG start codon in RefSeq database. J Biomol Struct Dyn *24*, 33-42.

17. Arnaud, E., Touriol, C., Boutonnet, C., Gensac, M.C., Vagner, S., Prats, H., and Prats, A.C. (1999). A new 34-kilodalton isoform of human fibroblast growth factor 2 is cap dependently synthesized by using a non-AUG start codon and behaves as a survival factor. Mol Cell Biol *19*, 505-514.

18. Huez, I., Bornes, S., Bresson, D., Creancier, L., and Prats, H. (2001). New vascular endothelial growth factor isoform generated by internal ribosome entry site-driven CUG translation initiation. Mol Endocrinol *15*, 2197-2210.

19. Meiron, M., Anunu, R., Scheinman, E.J., Hashmueli, S., and Levi, B.Z. (2001). New isoforms of VEGF are translated from alternative initiation CUG codons located in its 5'UTR. Biochem Biophys Res Commun *282*, 1053-1060.

20. Prats, H., Kaghad, M., Prats, A.C., Klagsbrun, M., Lelias, J.M., Liauzun, P., Chalon, P., Tauber, J.P., Amalric, F., Smith, J.A., and et al. (1989). High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc Natl Acad Sci U S A *86*, 1836-1840.

21. Florkiewicz, R.Z., and Sommer, A. (1989). Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. Proc Natl Acad Sci U S A *86*, 3978-3981.

22. Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T.S., Scher, M., Kirmizis, A., Ouyang, X., Brockdorff, N., Abate-Shen, C., Farnham, P., and Reinberg, D. (2005). Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A *102*, 1859-1864.

23. Martin, C., Cao, R., and Zhang, Y. (2006). Substrate preferences of the EZH2 histone methyltransferase complex. J Biol Chem *281*, 8365-8370.

24. Peters, A.H., Kubicek, S., Mechtler, K., O'Sullivan, R.J., Derijck, A.A., Perez-Burgos, L., Kohlmaier, A., Opravil, S., Tachibana, M., Shinkai, Y., Martens, J.H., and Jenuwein, T. (2003). Partitioning and plasticity of repressive histone methylation states in mammalian chromatin. Mol Cell *12*, 1577-1589.

25. Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K., Koseki, H., Fuchikami, T., Abe, K., Murray, H.L., Zucker, J.P., Yuan, B., Bell, G.W., Herbolsheimer, E., Hannett, N.M., Sun, K., Odom, D.T., Otte, A.P., Volkert, T.L., Bartel, D.P., Melton, D.A., Gifford, D.K., Jaenisch, R., and Young, R.A. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. Cell *125*, 301-313.

26. Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., Bell, G.W., Otte, A.P., Vidal, M., Gifford, D.K., Young, R.A., and Jaenisch, R. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349-353.

27. Bracken, A.P., Dietrich, N., Pasini, D., Hansen, K.H., and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev *20*, 1123-1136.

28. Schwartz, Y.B., and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet *8*, 9-22.

29. Min, J., Zhang, Y., and Xu, R.M. (2003). Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. Genes Dev *17*, 1823-1828.

30. Fischle, W., Wang, Y., Jacobs, S.A., Kim, Y., Allis, C.D., and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. Genes Dev *17*, 1870-1881.

31. Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004). Hierarchical recruitment of polycomb group silencing complexes. Mol Cell *14*, 637-646.

32. Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942-947.

33. Morin-Kensicki, E.M., Faust, C., LaMantia, C., and Magnuson, T. (2001). Cell and tissue requirements for the gene eed during mouse gastrulation and organogenesis. Genesis *31*, 142-146.

34. Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987). HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature *326*, 292-295.

35. Simin, K., Wu, H., Lu, L., Pinkel, D., Albertson, D., Cardiff, R.D., and Van Dyke, T. (2004). pRb inactivation in mammary cells reveals common mechanisms for tumor initiation and progression in divergent epithelia. PLoS Biol *2*, E22.

36. Hamer, K.M., Sewalt, R.G., den Blaauwen, J.L., Hendrix, T., Satijn, D.P., and Otte, A.P. (2002). A panel of monoclonal antibodies against human polycomb group proteins. Hybrid Hybridomics *21*, 245-252.

37. James, T.C., and Elgin, S.C. (1986). Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. Mol Cell Biol *6*, 3862-3872.

38. James, T.C., Eissenberg, J.C., Craig, C., Dietrich, V., Hobson, A., and Elgin, S.C. (1989). Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. Eur J Cell Biol *50*, 170-180.

39. Jones, D.O., Cowell, I.G., and Singh, P.B. (2000). Mammalian chromodomain proteins: their role in genome organisation and expression. Bioessays *22*, 124-137.

40. Schumacher, A., Lichtarge, O., Schwartz, S., and Magnuson, T. (1998). The murine Polycomb-group gene eed and its human orthologue: functional implications of evolutionary conservation. Genomics *54*, 79-88.

41. Schumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *384*, 648.

42. Gutjahr, T., Frei, E., Spicer, C., Baumgartner, S., White, R.A., and Noll, M. (1995). The Polycomb-group gene, extra sex combs, encodes a nuclear member of the WD-40 repeat family. Embo J *14*, 4296-4306.

43. Ng, J., Li, R., Morgan, K., and Simon, J. (1997). Evolutionary conservation and predicted structure of the Drosophila extra sex combs repressor protein. Mol Cell Biol *17*, 6663-6672.

44. Sewalt, R.G., van der Vlag, J., Gunster, M.J., Hamer, K.M., den Blaauwen, J.L., Satijn, D.P., Hendrix, T., van Driel, R., and Otte, A.P. (1998). Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. Mol Cell Biol *18*, 3586-3595.

45. van der Vlag, J., and Otte, A.P. (1999). Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet *23*, 474-478.

46. Bender, L.B., Cao, R., Zhang, Y., and Strome, S. (2004). The MES-2/MES-3/MES-6 complex and regulation of histone H3 methylation in C. elegans. Curr Biol *14*, 1639-1643.

47. Tie, F., Stratton, C.A., Kurzhals, R., and Harte, P.J. (2007). The N-Terminus of Drosophila ESC Binds Directly to Histone H3 and is Required for E(Z)-Dependent Trimethylation of H3 Lysine 27. Mol Cell Biol.

48. Wang, J., Tie, F., Jane, E., Schumacher, A., Harte, P.J., and Magnuson, T. (2000). Mouse homolog of the Drosophila Pc-G gene esc exerts a dominant negative effect in Drosophila. Genesis *26*, 67-76.

FIGURE 4.1. Localization of Histone H3K27 methylation marks

Immunofluorescence analysis of **(A)** H3K27me1, **(B)** H3K27me2, **(C)** H3K27me3, and **(D)** HP1-α in CD1 murine embryonic fibroblasts.

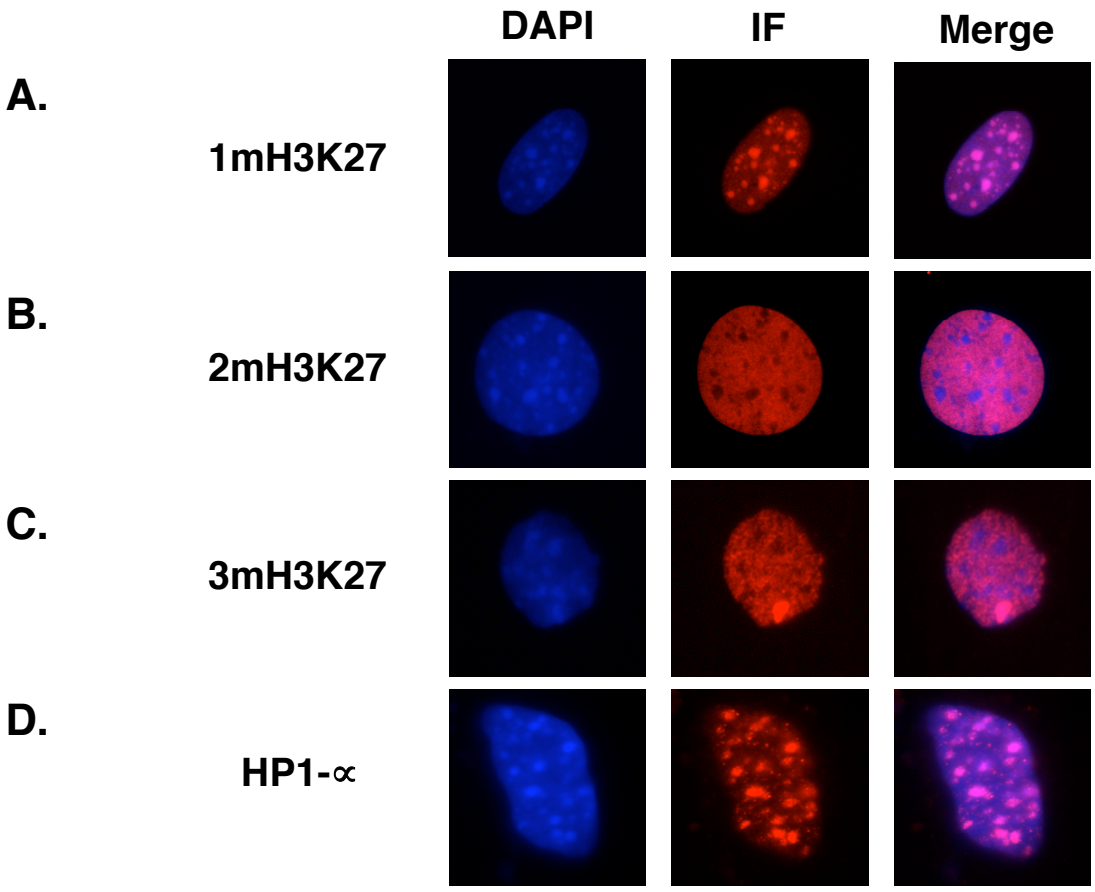

DAPI
IF
Merge
A.
1mH3K27
B.
2mH3K27
C.
3mH3K27
D.
HP1-∝

FIGURE 4.2. Confirmation of EED isoform identities

Western blot analysis of EED comparing isoforms observed in wild-type or *Eed* mutant embryonic stem cells to isoforms observed in **(A)** HeLa cells or **(B)** Mouse *Wap-T121* mammary tumors. On prolonged exposure, EED-2 becomes visible in HeLa lysates (A-right panel). EED isoforms 1-4 are indicated as 1, 2, 3, and 4.

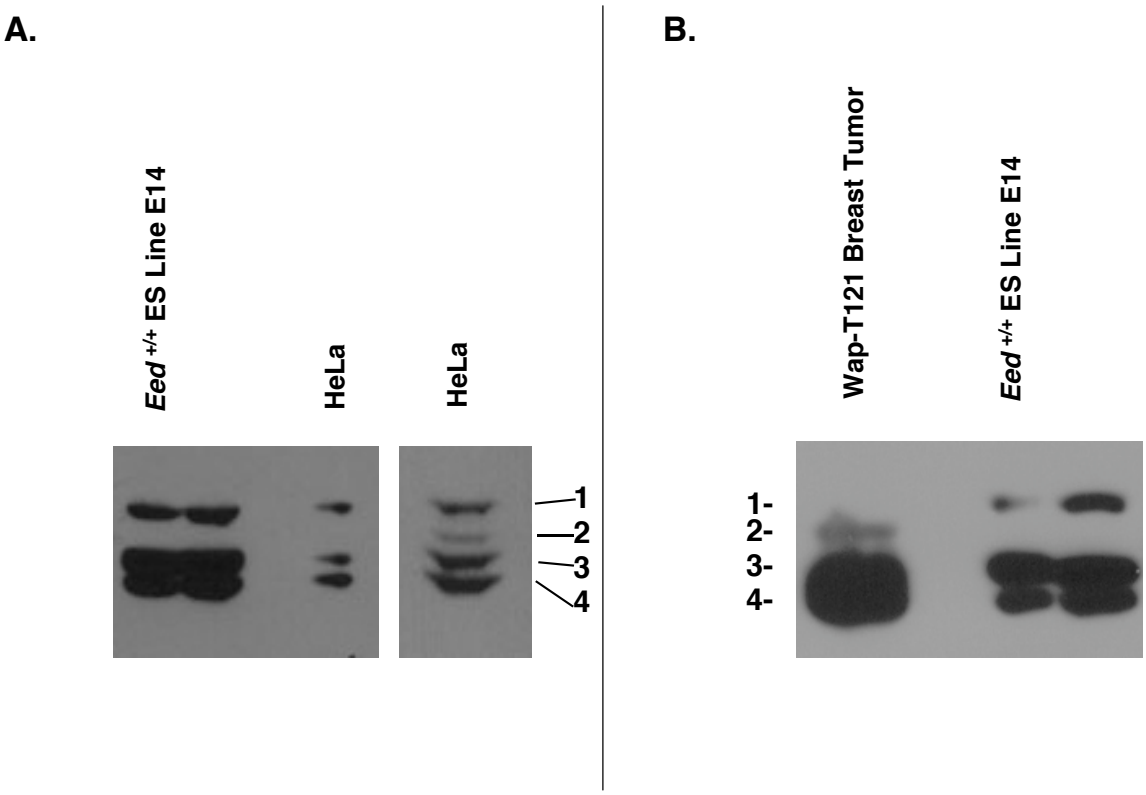

A.
Eed +/+ ES Line E14
HeLa
HeLa
1
2
3
4
B.
Wap-T121 Breast Tumor
Eed +/+ ES Line E14
1-
2-
3-
4-

FIGURE 4.3. Deletion mapping of EED translational start sites

(A) Constructs transfected into *Eed* mutant embryonic stem cell line 21 (below) are shown. Putative translation start sites for EED-1 (GUG 169-171), EED-2 (GUG 274-276), EED-3 (AUG 451-453), and EED-4 (AUG 493-495) are indicated in the schematic of the endogenous mRNA as 1,2,3, and 4 and correspond to codon locations in the mouse *Eed* transcript (Accession number: BC012966). Deletion numbers refer to nucleotides removed from the 5' end of the *Eed* cDNA (e.g. Δ210 refers to a construct expressing an *Eed* cDNA lacking the 5' 210 nucleotides of the full length message). **(B)** Whole-cell lysates from $Eed^{+/+}$ ES cell line 25.5 (Wild-type), $Eed^{-/-}$ ES cell line 21 (Mutant), or $Eed^{-/-}$ ES cell line 21 transiently transfected with the *Eed* expression constructs shown in **(A)**were analyzed by Western blotting with an antibody detecting EED.

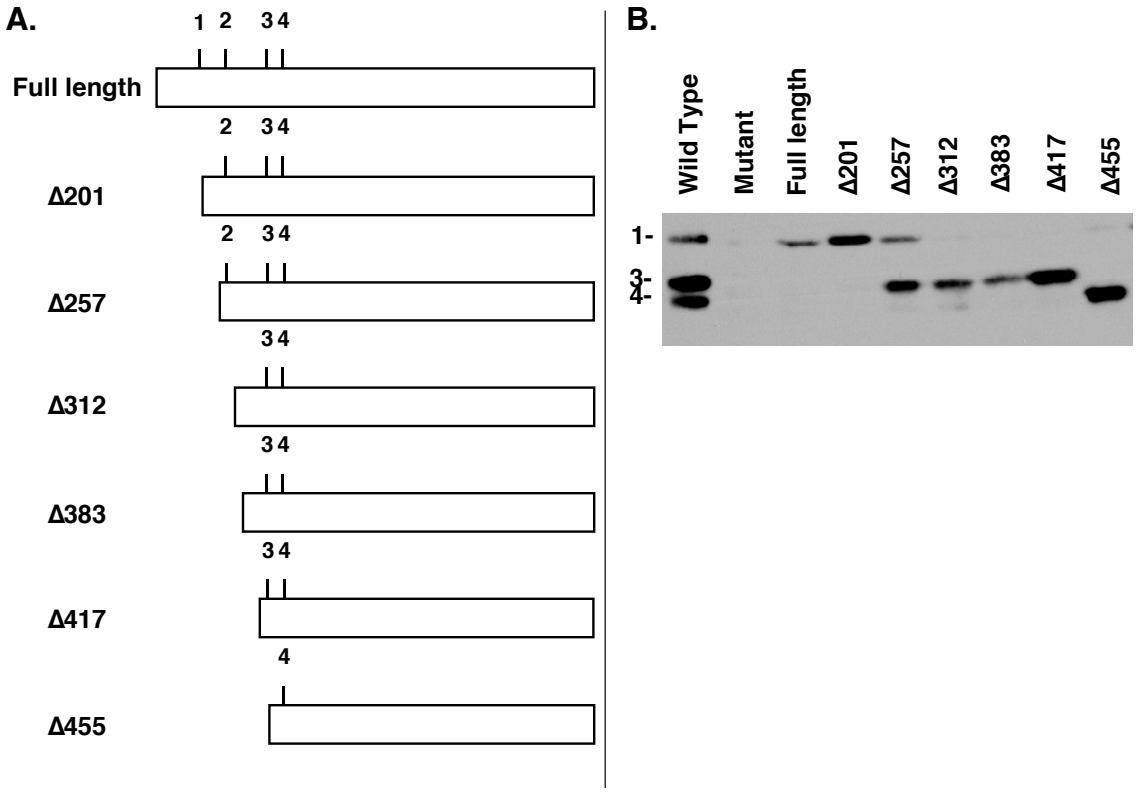

A.
1 2 3 4
Full length
2 3 4
Δ201
2 3 4
Δ257
3 4
Δ312
3 4
Δ383
3 4
Δ417
4
Δ455
B.
Wild Type
Mutant
Full length
Δ201
Δ257
Δ312
Δ383
Δ417
Δ455
1-
3-
4-

FIGURE 4.4. Verification of EED isoform start sites.

(A) Constructs transfected into *Eed* mutant embryonic stem cell line 21. 1,2,3, and 4 indicate reported EED translational start sites GUG 169-171, GUG 274-276, AUG 451-453, and AUG 493-495, respectively. Black boxes refer to strong Kozak-AUG sequences engineered into the expression construct in order to drive translation from the 169-171 codon (Kozak AUG 169-171) and from the 274-276 codon (Kozak AUG 274-276), respectively. "X" markings through the putative EED-3 and EED-4 start sites in Δ417 no3,4 and in Δ455 no4 indicate AUG→AUA mutations intended to disrupt translation intitation. **(B and C)** Whole-cell lysates from $Eed^{+/+}$ ES cell line 25.5 (Wild-type), $Eed^{-/-}$ ES cell line 21 (Mutant), or $Eed^{-/-}$ ES cell line 21 transiently transfected with the *Eed* expression constructs shown in **(A)** analyzed by Western blotting with an antibody detecting EED.

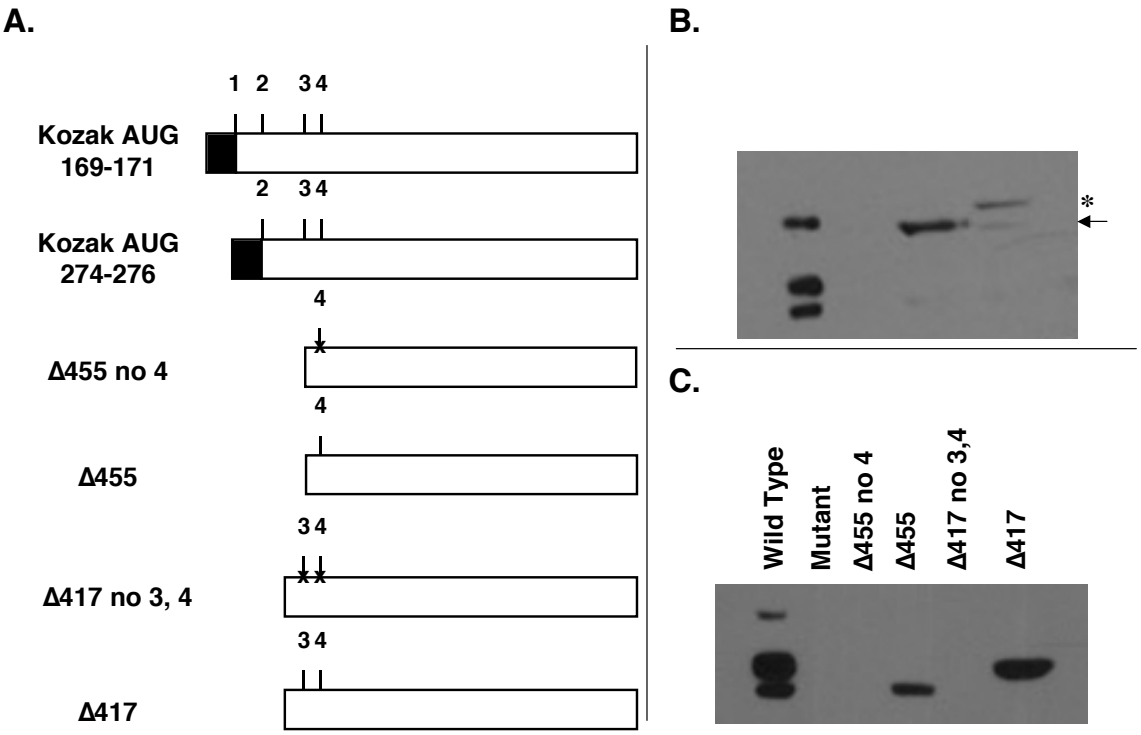

A.
1 2 3 4
Kozak AUG
169-171
2 3 4
Kozak AUG
274-276
4
Δ455 no 4
4
Δ455
3 4
Δ417 no 3, 4
3 4
Δ417
B.
*
C.
Wild Type
Mutant
Δ455 no 4
Δ455
Δ417 no 3,4
Δ417

FIGURE 4.5. Histone H3K27 methylation in cells lacking one or more EED isoforms

Immunofluorescence analysis of H3K27me1, H3K27me2, and H3K27me3 in wild-type ES cell line 25.5 (*Eed*$^{+/+}$) and in *Eed* mutant ES cell line 21 either mock transfected (*Eed* $^{-/-}$) or transiently transfected with the indicated constructs. *Eed* expression constructs transfected into ES cell line 21 are shown on the left. Isoform start sites at GUG 274-276, AUG 451-453, and AUG 493-495 are shown as 1*, 3, 4, respectively. 1* discriminates the GUG 274-276 start site for EED-1 reported here from the GUG169-171 start site reported previously(7, 18). DAPI-stained DNA is blue, and methylated histones are shown in red. "X" markings through the putative EED-3 and EED-4 starts sites in Full length no 3,4 and in Δ417 no3,4 represent AUG→AUA mutations intended to disrupt translation intitiation. EED 1036-1038 L→P refers to *Eed*$^{l7Rn5\text{-}3354SB}$, a point mutant protein previously demonstrated to lack H3K27 methyltransferase activity. In the transient transfection assay, approximately 10% of the ES cells are successfully transfected, and with constructs expressing functional EED, a similar percentage of cells are rescued.

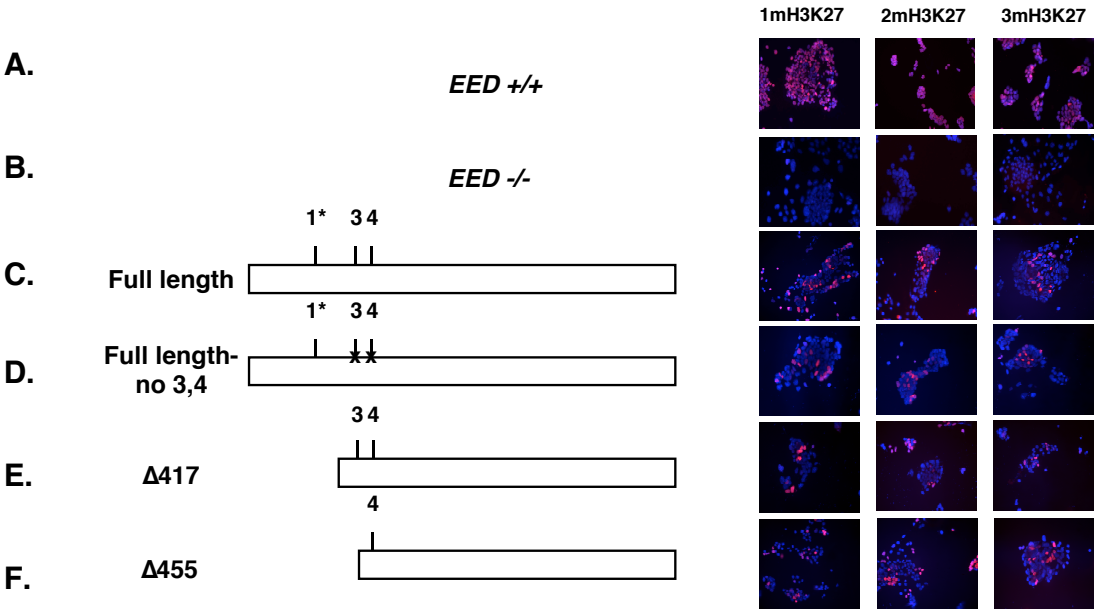

A.
EED +/+
B.
EED -/-
C.
Full length
1*
3 4
D.
Full length-
no 3,4
1*
3 4
E.
Δ417
3 4
F.
Δ455
4
1mH3K27
2mH3K27
3mH3K27

FIGURE 4.6. Functional mapping of required WD-40 motifs in EED

Immunofluorescence analysis of H3K27me3 in *Eed* mutant ES cell line 21 either mock transfected ($Eed^{-/-}$) or transiently transfected with the indicated constructs. DAPI-stained DNA is blue, and methylated histone are shown in red. Diagonally-lined boxes refer to putative WD-40 motifs encoded by cDNA sequences 721-808 (WD-40 motif 1), 1012-1105 (WD-40 motif 2), 1150-1240 (WD-40 motif 3), 1330-1444 (WD-40 motif 4), and 1672-1762 (WD-40 motif 5) (38). Black boxes refer to consensus Kozak + ATG initiator sequences engineered into the construct.

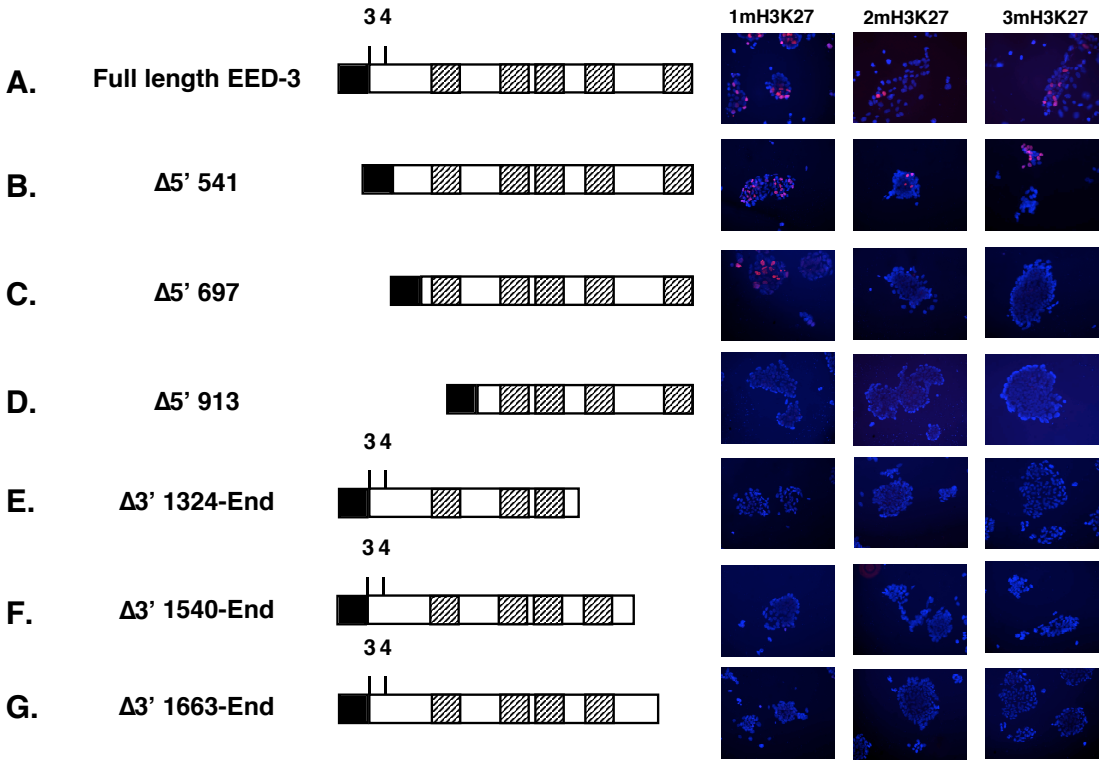
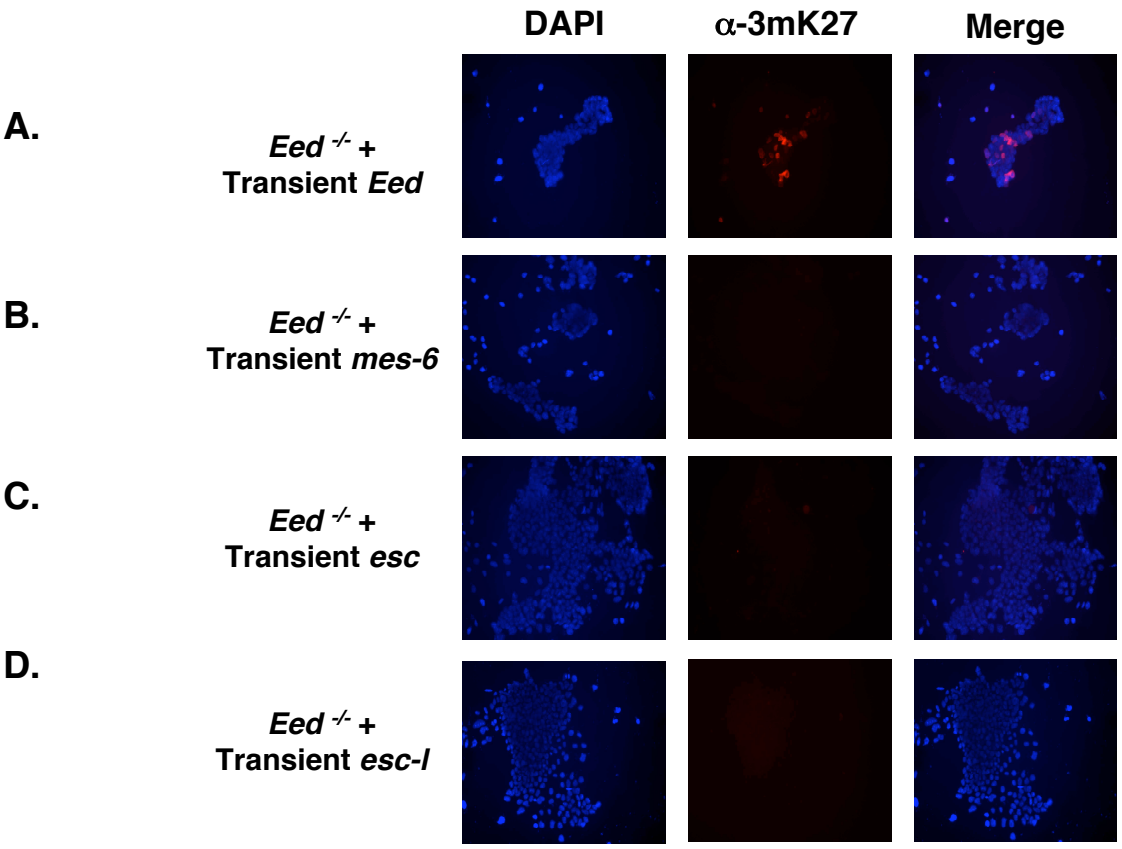

3 4
1mH3K27
2mH3K27
3mH3K27
A.
Full length EED-3
B.
Δ5' 541
C.
Δ5' 697
D.
Δ5' 913
3 4
E.
Δ3' 1324-End
3 4
F.
Δ3' 1540-End
3 4
G.
Δ3' 1663-End

FIGURE 4.7. Interspecies rescue analysis

Immunofluorescence analysis of H3K27me3 in *Eed* mutant ES cell line 21 transiently transfected with constructs expressing the **(A)** mouse, **(B)** *C. elegans* or **(C and D)** *D. melanogaster Eed* homologs.


DAPI
α-3mK27
Merge
A.
Eed -/- +
Transient Eed
B.
Eed -/- +
Transient mes-6
C.
Eed -/- +
Transient esc
D.
Eed -/- +
Transient esc-l

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

In recent decades, genetic and biochemical data in a variety of systems have begun to clarify the role of PcG proteins in transcriptional memory. However, critical details of PcG-mediated silencing remain elusive. Three particularly important questions are 1) how are PcG proteins recruited to their targets, 2) how do PcG-mediated histone modifications interplay with other epigenetic marks, and 3) how are PcG-mediated histone modifications regulated. The data presented here begin to address all of these questions. Of equal importance, these data also define critical future experimental directions that may further clarify the mechanistic details of PcG-mediated silencing. These issues are considered in detail below.

5.1. RECRUITMENT OF POLYCOMB GROUP PROTEINS

In flies, PcG proteins are often recruited by sequence-specific transcription factors to Polycomb Response Elements, which contain multiple copies of binding sites for a number of Drosophila transcription factors, including Pleiohomeotic and GAGA Factor [1]. However, similar elements have not been identified in mammals. More recent work suggests that fly PcG proteins can also be targeted by small RNAs [2]. Data presented here and elsewhere demonstrate that $Eed^{null/null}$ cells and embryos have defects in both X-chromosome inactivation and in *KvDMR* cluster imprinting (Figure 2.1-2) [3-5]. Notably, both of these processes are dependent on noncoding RNAs. These RNAs, *Xist* and *Kcnq1ot1,* have been proposed to recruit trans-acting factors in *cis* to silence genes on the Xi and in the *KvDMR* cluster, respectively[6-9]. Together, these results suggest that mammalian PcG proteins could be recruited to the Xi, to the *KvDMR* cluster, and perhaps to many other targets by noncoding RNAs like *Xist* and *Kcnq1ot1*.

Although this model is appealing, PcG proteins have not yet been demonstrated to associate directly with *Xist* or *Kcnq1ot1*, and future studies must determine whether these RNAs directly recruit PRC2 or whether they recruit intermediate molecules that subsequently recruit PcG proteins. This putative association could be assessed by electrophoretic mobility shift assays with *Xist* or *Kcnq1ot1* RNAs incubated with isolated PRC2 complexes.

5.2. INTERPLAY BETWEEN PcG-MEDIATED HISTONE MODIFICATIONS AND OTHER EPIGENETIC MARKS

Diverse mechanisms are employed to modify and remodel the chromatin template in order to influence gene expression. In addition to the vast array of covalent modifications found on histone tails, nucleosomes are also modified by the presence of histone variants and physically displaced by ATP-dependent remodeling complexes [10, 11]. In some species, including mammals, an additional layer of epigenetic control is provided by DNA methylation. Accordingly, the cell has a diverse repertoire of regulatory mechanisms at its disposal.

In many systems, stable gene expression states are produced by hierarchical recruitment of several of these epigenetic regulators. For instance, in a variety of systems, transcriptional silencing is achieved by the coordinated action of HMTases and DNA methyltransferases, often with, H3K9 methylation serving as an epigenetic mark that directly or indireclty recruits DNA methyltransferases [12-14].

In contrast, the relationship between H3K27 methylation and DNA methylation is less clear. In at least one tumor cell line, DNMT1 directly associates with PRC2, and

PRC2 is required for DNA methylation at several PcG-target genes in those cells [15]. Conversely, our own previous work has demonstrated that parent-of-origin DNA methylation is maintained at several DMRs in *Eed*$^{null/null}$ embryos, including two DMRs in the *KvDMR* cluster[4]. Given that EZH2 is lost in *Eed*$^{null/null}$ cells (Figure 3.2), these results suggest that parent-of-origin DNA methylation, unlike imprinted expression at these same loci, is not dependent on PRC2. Hence, unlike the tumor cell data, these results imply that PcG-mediated histone modifications and DNA methylation may be targeted independently.

The data presented here support that interpretation. If the functions of PcG proteins and DNA methyltransferases are interdependent, one would expect mutations in PcG genes and in DNA methylransferases to influence overlapping target genes. Here, we have demonstrated that *Eed*$^{null/null}$ TS cells have imprinting defects reciprocal to those observed in *Dnmt1*$^{null/null}$ placentas (Figure 2.7) [9]. Specifically, the three proximal genes in the *KvDMR* cluster that become biallelically expressed in *Eed*$^{null/null}$ TS cells are imprinted normally in *Dnmt1*$^{null/null}$ placentas; conversely, the central and distal genes misexpressed in *Dnmt1*$^{null/null}$ placentas are imprinted normally in *Eed*$^{null/null}$ TS cells. These results suggest that maintenance DNA methylation and PcG-mediated histone modifications have distinct targets and function independently in the *KvDMR* cluster. However, these conclusions are tempered by an important caveat. Although TS cells do represent a primitive placental precursor, we have not assessed imprinting defects in matched tissues from *Eed* and *Dnmt1* mutants. Accordingly, these conclusions may be strengthened considerably by future work comparing *KvDMR* cluster imprinting in *Eed*$^{null/null}$ and *Dnmt1*$^{null/null}$ TS cells.

Even in the absence of a direct comparison between *Eed* and *Dnmt1* mutant cells, the tissue-specficity of epigenetic defects in *Eed* mutants is notable. X-chromosome inactivation defects in $Eed^{null/null}$ conceptuses are restricted to a population of differentiating extraembryonic cells [5]. Similarly, I observed broader imprinting defects in $Eed^{null/null}$ TS cells than in $Eed^{null/null}$ conceptuses, suggesting that defects in autosomal imprinting in *Eed* mutants may also be restricted to, or at least more severe in, extraembryonic cells. In the mouse, extraembryonic tissues are DNA hypomethylated relative to embryonic tissues, because embryonic tissues, unlike extraembryonic tissues, are remethylated after implantation [16]. *Eed* may be dispensable at many loci in embryonic tissues, because DNA methylation provides a robust, alternative epigenetic memory system. According to this model, in the absence of that reinforcement in extraembryonic tissues, mutations in *Eed* have more dire consequences. This interpretation is supported by the ability to rescue $Eed^{hypo/null}$ midgestation lethality with wild-type extraembryonic tissues, and it supports the view that DNA methylation and PcG-mediated histone modifications function independently at many targets in the mouse [17].

5.3. REGULATED ADDITION OF METHYL GROUPS TO HISTONE H3K27

Histones can be mono-, di-, or trimethylated at several lysine residues, including at H3K27. However, in most cases, it is unclear what regulates how many methyl groups are added to histones associated with a particular region of the genome. Two simple models have been proposed to control this specificity [18]. In the first model, distinct mono-, di-, and trimethylating enzymes are responsible for each methyl state. In the

second model, a single enzyme mediates all three methyl states but is regulated so as to add one methyl group to certain nucleosomes and two or three to others. Regulation of the enzyme could be mediated by the presence of regulatory subunits, post-translational modifications, etc.

Here, we have demonstrated that the PcG gene *Eed* is required for all three H3K27 methylation states in our high passage ES and TS cells (Figure 3.1 and Supplementary Figure 3.1). *Eed*$^{null/null}$ cells have dramatically reduced levels of EZH2, the catalytic subunit of the PRC2 complex, suggesting that EZH2 is unstable outside of intact PRC2 complexes (Figure 3.2). Additionally, we have demonstrated that transient transfection of an *Eed* cDNA expression cassette rescues the H3K27 methylation defects in *Eed*$^{null/null}$ cells, confirming that EED can mediate H3K27me1, H3K27me2, and H3K27me3 (Figure 3.4 and Supplementary Figures 3.2 and 3.3).

Alone, these results appear to support the second model, in which a single complex mediates all three methyl states. However, unlike *Eed* mutants, embryos and cells homozygous for a gene trap mutation in the PRC2 subunit *Suz12* retain H3K27me1 [19]. *Suz12* mutants have been reported to lack EZH2, while retaining EED. These results imply that SUZ12 and EZH2, unlike EED, are dispensable for H3K27me1. Supporting this conclusion, *Eed*$^{null/null}$ cells stably expressing a low level of EED have robust H3K27me1, even in the absence of an observable rescue of EZH2 protein levels (Figures 3.3 and 3.4).

We have interpreted these results to suggest that EED mediates monomethylation independently of SUZ12 and EZH2 (Chapter 3). However, there are alternative interpretations to explain the presence of H3K27me1 in both *Suz12* gene trap mutants and

in *Eed*$^{null/null}$ cells stably expressing *Eed*. Although *Suz12* gene trap mutants appear to be null mutants as assessed both by embryonic phenotype (which is comparable to the *Eed*$^{null/null}$ mutant phenotype) and by the apparent absence of residual SUZ12 protein, gene traps do not provide a "clean" genetic lesion. That is to say, gene traps terminate transcription by providing a strong splice acceptor sequence upstream of a transcriptional termination sequence. The splice acceptor allows the cassette to "trap" spliced transcripts in order to produce truncated and often unstable proteins. As a result, gene traps will produce a true null mutation only if all transcripts splice into the trap. On the other hand, if a percentage of transcripts splice around the trap, wild-type message and wild-type protein will be produced. As a consequence, proving that a gene trap is a null allele is dependent on the detection limits of molecular assays used to identify the untrapped mRNA or protein. Hence, it is formally possible that the *Suz12* gene trap creates a severe hypomorphic but not null allele. For similar reasons, it is possible that a low level of EZH2 persists in those mutants and in our *Eed*$^{null/null}$ cells that stably express *Eed*. As a result, an alternative interpretation of our data is that H3K27me1 requires only a very low level of PRC2, and that sufficient PRC2 persists in *Suz12* gene trap mutants and in *Eed*$^{null/null}$ cells stably expressing *Eed*. This possibility cannot be formally excluded until cells harboring a deletion that removes the *Ezh2* SET domain have been analyzed for H3K27me1.

As an alternative, we proposed that PRC2 complexes containing distinct EED isoforms could be responsible for H3K27me1, H3K27me2, and H3K27me3. We have demonstrated that EED-1, EED-3, and EED-4 are produced by translation initiating at the positions GUG 277-279, AUG 452-454, and AUG 494-496 in the *Eed* mRNA,

respectively (Figures 4.3 and 4.4). However, these isoforms are not required for any of the H3K27 methylation states (Figure 4.5). Instead, a truncated form of EED containing only the protein's WD-40 and histone binding motifs is sufficient for all three marks (Figure 4.6). Hence, the mechanisms controlling the number of methyl groups added to H3K27 remain elusive. However, as an important starting point, we have identified the first protein, EED, involved in mediating all three marks.

Determining the mechanisms regulating H3K27me1, H3K27me2, and H3K27me3 may prove to be a formidable task. Because there are no known biological readouts of H3K27me1 or H3K27me2, it would be difficult to design an effective screen for genes required for these marks. In simpler organisms with more comprehensive genetic tools, such as *S. cerevisiae*, it would be possible to prepare histones from a library of mutants with mutations in all known genes and then to directly assess the status of each methyl state in each mutant by western blotting. However, neither *S. cerevisiae* nor *S. pombe*, have H3K27 methylation or orthologs of PcG proteins.

A related question is whether methyl groups are added sequentially to H3K27 or whether unmethylated histones can be directly di- or trimethylated. We have observed a quantitative relationship between EED protein levels and H3K27 methylation (Chapter 3). Additionally, in cells harboring a *Suz12* mutation that may be hypomorphic for PRC2 function, the normally H3K27 trimethylated inactive X chromosome becomes dimethylated (S. Chamberlain and T. Magnuson, submitted). These results suggest that a low level of PRC2 activity may be sufficient for lower H3K27 methylation states and that higher levels may be required for full methylation. If true, the mechanisms controlling H3K27 methylation states may simply involve regulating the stability of PRC2

association with chromatin. Regions were PRC2 is only transiently associated with histones may be monomethylated, whereas regions where PRC2 is stably tethered may be di- or even trimethylated. If true, this trend might be recapitulated by tethering PRC2 to DNA binding transcription factors with weak or strong affinity for various templates. A variation on this model is that H3K27me1 could be added to histones prior to their incorporation into chromatin and that di- and trimethylation are marks that are only added to nucleosomal substrates. This possibility could be assessed by analyzing H3K27 methylation in free histones associated with chromatin assembly complexes.

The functions of EED isoforms also remain unclear. At a mechanistic level, initial studies suggested that EED-3 and EED-4 direct EZH2 to methylate H3K27, whereas EED-1 and EED-2 direct EZH2 towards histone H1K26[20]. However, subsequent studies utilizing similar biochemical strategies failed to confirm these findings[21]. Additionally, we have demonstrated that the EED-3 and EED-4 start sites are dispensable for H3K27 methylation (Figure 4.5). As a result, mechanistic distinctions between the isoforms have not been clearly defined.

However, EED isoforms do appear to be developmentally regulated, and EED-2 is clearly upregulated in many tumors and may also be upregulated in stem cells[22]. This dynamic regulation suggests that EED isoforms are functionally significant. One intriguing possibility is that these isoforms are required to recruit PRC2 complexes to distinct target genes. However, identifying isoform-specific targets awaits the availability of robust isoform-restricted antibodies or the generation of mice harboring mutations that eliminate the expression of individual isoforms.

5.4. Conclusions

Although transcriptional silencing by PcG proteins has been co-opted to regulate diverse targets in the course evolution, the molecular mechanisms responsible for this silencing are remarkably conserved. The data presented in this dissertation reflects both the adaptability of PcG-silencing and the conservation of the molecular mechanisms responsible for that silencing. We have demonstrated a specific role for the mouse PcG gene *Eed* in genomic imprinting, but we also have uncovered what is likely to be a more general requirement for *Eed* and its homologs in histone methylation. Together, these results extend our understanding of the evolutionarily diverse roles for PcG silencing as well as the evolutionary conserved mechanisms of PRC2 function.

5.5. REFERENCES

1. Schwartz, Y.B., and Pirrotta, V. (2007). Polycomb silencing mechanisms and the management of genomic programmes. Nat Rev Genet *8*, 9-22.

2. Grimaud, C., Bantignies, F., Pal-Bhadra, M., Ghana, P., Bhadra, U., and Cavalli, G. (2006). RNAi components are required for nuclear clustering of Polycomb group response elements. Cell *124*, 957-971.

3. Wang, J., Mager, J., Chen, Y., Schneider, E., Cross, J.C., Nagy, A., and Magnuson, T. (2001). Imprinted X inactivation maintained by a mouse Polycomb group gene. Nat Genet *28*, 371-375.

4. Mager, J., Montgomery, N.D., de Villena, F.P., and Magnuson, T. (2003). Genome imprinting regulated by the mouse Polycomb group protein Eed. Nat Genet *33*, 502-507.

5. Kalantry, S., Mills, K.C., Yee, D., Otte, A.P., Panning, B., and Magnuson, T. (2006). The Polycomb group protein Eed protects the inactive X-chromosome from differentiation-induced reactivation. Nat Cell Biol *8*, 195-202.

6. Plath, K., Fang, J., Mlynarczyk-Evans, S.K., Cao, R., Worringer, K.A., Wang, H., de la Cruz, C.C., Otte, A.P., Panning, B., and Zhang, Y. (2003). Role of histone H3 lysine 27 methylation in X inactivation. Science *300*, 131-135.

7. Mancini-Dinardo, D., Steele, S.J., Levorse, J.M., Ingram, R.S., and Tilghman, S.M. (2006). Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes. Genes Dev *20*, 1268-1282.

8. Umlauf, D., Goto, Y., Cao, R., Cerqueira, F., Wagschal, A., Zhang, Y., and Feil, R. (2004). Imprinting along the Kcnq1 domain on mouse chromosome 7 involves repressive histone methylation and recruitment of Polycomb group complexes. Nat Genet *36*, 1296-1300.

9. Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R., and Reik, W. (2004). Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat Genet *36*, 1291-1295.

10. Bultman, S., Montgomery, N., and Magnuson, T. (2004). Chromatin-Modifying Factors and Transcriptional Regulation During Development. In Handbook of Stem Cells, Volume 1, 1st Edition, R. Lanza, ed. (San Diego, CA: Elsevier), pp. 63-91.

11. Montgomery, N.D., Magnuson, T., and Bultman, S. (2006). Epigenetic Mechanisms of Cellular Memory During Development. In Essentials of Stem Cell Biology, 1st Edition, R. Lanza, ed. (San Diego: Elsevier), pp. 69-80.

12. Lehnertz, B., Ueda, Y., Derijck, A.A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., Chen, T., Li, E., Jenuwein, T., and Peters, A.H. (2003). Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. Curr Biol *13*, 1192-1200.

13. Tamaru, H., and Selker, E.U. (2001). A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature *414*, 277-283.

14. Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature *416*, 556-560.

15. Vire, E., Brenner, C., Deplus, R., Blanchon, L., Fraga, M., Didelot, C., Morey, L., Van Eynde, A., Bernard, D., Vanderwinden, J.M., Bollen, M., Esteller, M., Di Croce, L., de Launoit, Y., and Fuks, F. (2006). The Polycomb group protein EZH2 directly controls DNA methylation. Nature *439*, 871-874.

16. Li, E. (2002). Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet *3*, 662-673.

17. Wang, J., Mager, J., Schnedier, E., and Magnuson, T. (2002). The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. Mamm Genome *13*, 493-503.

18. Montgomery, N.D., Yee, D., Chen, A., Kalantry, S., Chamberlain, S.J., Otte, A.P., and Magnuson, T. (2005). The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol *15*, 942-947.

19. Pasini, D., Bracken, A.P., Jensen, M.R., Denchi, E.L., and Helin, K. (2004). Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. Embo J *23*, 4061-4071.

20. Kuzmichev, A., Jenuwein, T., Tempst, P., and Reinberg, D. (2004). Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell *14*, 183-193.

21. Martin, C., Cao, R., and Zhang, Y. (2006). Substrate preferences of the EZH2 histone methyltransferase complex. J Biol Chem *281*, 8365-8370.

22. Kuzmichev, A., Margueron, R., Vaquero, A., Preissner, T.S., Scher, M., Kirmizis, A., Ouyang, X., Brockdorff, N., Abate-Shen, C., Farnham, P., and Reinberg, D. (2005). Composition and histone substrates of polycomb repressive group

complexes change during cellular differentiation. Proc Natl Acad Sci U S A *102*, 1859-1864.

APPENDIX

PERINATAL LETHALITY OF C57BL/6.CD1 (N4-N6F1) *EED*$^{HYPO/HYPO}$ MICE

6.1. INTRODUCTION

As mentioned in previous chapters, two ENU-generated *Eed* alleles, Eed^{null} and Eed^{hypo}, have been utilized to characterize PRC2 function *in vivo*. Transient transfection of constructs expressing Eed^{hypo} rescues the H3K27 methylation defects in $Eed^{null/null}$ cell lines, and H3K27me1, H3K27me2, and H3K27me3 are all present in $Eed^{hypo/hypo}$ fibroblasts (Figure 3.3, Supplementary Figures 3.2 and 3.3, and data not shown). These results indicate that unlike Eed^{null}, Eed^{hypo} retains the ability to mediate PRC2 function, at least at a low level. Instead, the phenotypes in $Eed^{hypo/hypo}$ animals are likely a consequence of the reduced stability of the EED^{hypo} protein (Figure 3.4).

$Eed^{hypo/hypo}$ animals are generally viable but runted on outbred backgrounds, with skeletal transformations due to abnormal homeotic gene expression boundaries[1-3]. The animals also appear to be subfertile, although a detailed analysis of $Eed^{hypo/hypo}$ fertility has not been performed. Many mutations in the mouse produce strain-dependent phenotypes, which are a consequence of genetic interactions between that mutation and one or more variants present at other loci[4, 5]. $Eed^{hypo/hypo}$ animals have been characterized predominantly on an outbred CD1 background[3].

The Eed^{hypo} allele is an T→A substitution that converts a nonpolar isoleucine in the third of EED's five WD-40 motifs into a polar asparagine[2]. This point mutation does not create or eliminate any known restriction enzyme recognition sites, meaning that one must utilize linked markers for genotyping. Originally, the coat color gene *Tyrosinase* (*Tyr*) was used as a linked, visible marker for maintenance of the Eed^{hypo} stock[3]. However, *Tyr* is nearly 3 Mb from *Eed*, and recombination events, though infrequent, can complicate stock maintenance. Following the publication of the mouse

genome sequence, the increasing availability of strain single nucleotide polymorphism (SNP) data facilitated the identification of an informative SNP 2.1 kb from the *Eed*^{hypo} mutation. Because the *Eed* mutant alleles were originally generated on a Balb/c background and because this SNP generates a restriction fragment length polymorphism (RFLP) in which *Nla*III cuts a C57BL/6 allele but not a Balb/c allele, it provides a useful molecular marker for *Eed*^{hypo} genotyping. In the course of backcrossing the *Eed*^{hypo} mutation onto a C57BL/6 background for genotyping purposes, we observed highly penetrant, perinatal lethality not previously reported in *Eed*$^{hypo/hypo}$ animals.

6.2.METHODS AND MATERIALS

Strain maintenance and genotyping

A Balb/c – C57BL/6 *Nla*III RFLP was identified in the Celera mouse genome database. This SNP resides 2.1 kb from the site of the Eed^{hypo} mutation.

In the first generation, sequence-confirmed $Eed^{hypo/+}$ animals were backcrossed to C57BL/6 animals. The genotypes of animals in new litters were determined as follows. First, genomic DNA was prepared from ear tissue by treatment in ear lysis buffer (50 mM Tris, pH 8.8; 1 mM EDTA; 0.5% Tween-20; 20 μg/mL proteinase K). Lysates were genotyped by PCR with primers flanking the polymorphism (Fwd: CAGGCAGTCATTTCATCCGTTC, Rev: GGAGGAGGAGGAAGATTTCAACAC), followed by digestion with *Nla*III (New England Biolabs). In subsequent backcrosses, both $Eed^{hypo/+}$ males and females were utilized to insure that both sex chromosomes were also being crossed onto the C57BL/6 background.

For intercrosses, C57BL/6 backcross generation-matched $Eed^{hypo/+}$ dams and sires were mated, and cages were checked daily for new litters. At birth, animals were first visually genotyped by eye pigmentation ($Eed^{hypo/hypo}$ animals are typically albino because *Eed* is tightly linked to *Tyr*, and the ENU-generated *Eed* alleles were generated in an albino Balb/c stock). At weaning, DNA was prepared from survivors for molecular genotyping as described above. Deviation from predicted Mendelian frequencies was assessed by chi-square testing.

For embryological analyses, timed-matings were performed, and dams were sacrificed at embryonic day 18.5 (E18.5). Embryos were dissected from maternal tissue, weighed, and genotyped by eye pigmentation.

6.3. RESULTS

As the *Eed*^{hypo} allele was backcrossed onto a C57BL/6 background, regular intercrosses were performed to generate *Eed*$^{hypo/hypo}$ animals. Consistent with previous reports, in outcrosses involving either the original, outbred stock or *Eed*$^{hypo/+}$ animals generated from one or two backcrosses to C57BL/6 (herein referred to C57BL/6.CD1 (N1), C57BL/6.CD1 (N2), etc.), *Eed*$^{hypo/hypo}$ animals were observed at predicted frequencies at weaning (Table 6.1). However, *Eed*$^{hypo/hypo}$ animals were almost never observed at weaning in litters from intercrosses involving *Eed*$^{hypo/+}$ C57BL/6.CD1 (N4-N6) animals (Table 6.1).

In order to determine whether the preweaning lethality of *Eed*$^{hypo/hypo}$ C57BL/6.CD1 (N4-N6F1) animals occurred before or after birth, intercross cages were checked daily for new litters, and pups were genotyped at birth. *Eed*$^{hypo/hypo}$ C57BL/6.CD1 (N4-N6F1) animals and corpses were recovered at Mendelian frequencies at postnatal day 1 (P1) (Table 6.2). However, half of these animals were already dead, and the remainder died by postnatal day 2 (P2).

In order to determine whether any *Eed*$^{hypo/hypo}$ embryos were dying prenatally, timed *Eed*$^{hypo/+}$ C57BL/6.CD1(N6) intercross matings were performed and litters were collected at embryonic day 18.5 (E18.5). C57BL/6.CD1(N6F1) *Eed*$^{hypo/hypo}$ E18.5 embryos were alive but runted (Table 6.3 and Figure 6.1). On average, C57BL/6.CD1(N6F1) *Eed*$^{hypo/hypo}$ embryos were 25% smaller than their wild-type littermates.

6.5. DISCUSSION

The cause of death in C57BL/6.CD1 (N4-N6F1) *Eed*$^{hypo/hypo}$ animals is unclear. Perinatal lethality can be caused by a wide range of developmental defects, and determining a primary cause of death in mutants dying at these stages can be difficult. As a consequence, in the absence of physiological details, perinatal lethality is often explained as a "failure to thrive." Although that description is mechanistically vague, it may be appropriate in discussing C57BL/6.CD1 (N4-N6F1). *Eed*$^{hypo/hypo}$ perinatal lethality.

After four to six backcrosses, the *Eed*^{hypo} mutation is expected to reside in a background where, on average, 93.75%-98.4% of unlinked loci are C57BL/6. The strain-dependent lethality indicates that one or more C57BL/6 alleles are compromising the fitness of *Eed*$^{hypo/hypo}$ animals. While this genetic interaction could reveal gene products important to *Eed* function, it is also possible that the strain-dependent lethality simply reflects the reduced vigor of an inbred mouse strain. In other words, C57BL/6.CD1 (N4-N6F1) *Eed*$^{hypo/hypo}$ animals may essentially be "sick with two, unrelated diseases," the first caused by the *Eed* genotype and the second caused by being inbred. Together, those two independent but additive disadvantages may make it difficult for these animals to compete with their siblings for their mother's resources. Supporting this conclusion, E18.5 C57BL/6.CD1 (N6F1) *Eed*$^{hypo/hypo}$ E18.5 embryos are already runted, and these smaller mutant animals are likely to be at a competitive disadvantage in the critical hours immediately following parturition. If true, C57BL/6.CD1 (N4-N6F1) *Eed*$^{hypo/hypo}$ lethality might be rescued simply by separating mutants from their wild-type siblings and providing them with a competent, outbred foster mother.

The prenatal runting in C57BL/6.CD1 (N6F1) *Eed*$^{hypo/hypo}$ embryos is itself an interesting phenotype. *Eed*$^{hypo/hypo}$ fibroblasts have proliferation defects (Y. Chen and T. Magnuson, personal communication), suggesting that this runting could be due to a developmental delay caused by slowed growth in mutant embryos. The perigastrulation arrest in *Eed*$^{null/null}$ embryos is partially rescued by a mutation in *Cdkn1c*, a cell cycle inhibitor (J. Mager and T. Magnuson, personal communication). If the prenatal runting and perinatal lethality in C57BL/6.CD1 (N4-N6F1) *Eed*$^{hypo/hypo}$ embryos is due to reduced rates of proliferation, mutations in cell cycle inhibitors may be able to rescue these defects as well.

6.5. REFERENCES

1. Rinchik, E.M., and Carpenter, D.A. (1993). N-ethyl-N-nitrosourea-induced prenatally lethal mutations define at least two complementation groups within the embryonic ectoderm development (eed) locus in mouse chromosome 7. Mamm Genome *4*, 349-353.

2. Schumacher, A., Faust, C., and Magnuson, T. (1996). Positional cloning of a global regulator of anterior-posterior patterning in mice. Nature *384*, 648.

3. Wang, J., Mager, J., Schnedier, E., and Magnuson, T. (2002). The mouse PcG gene eed is required for Hox gene repression and extraembryonic development. Mamm Genome *13*, 493-503.

4. Pearson, H. (2002). Surviving a knockout blow. Nature *415*, 8-9.

5. Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., and et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science *269*, 230-234.

C57BL/6 Backcrosses	*Eed*$^{+/+}$	*Eed*$^{hypo/+}$	*Eed*$^{hypo/hypo}$	$\chi 2$	P value
0-2	7 (8.5)	19 (17)	8 (8.5)	0.53	0.767
4-6	13 (9.5)	24 (19)	1 (9.5)	10.21	0.006

TABLE 6.1. Strain-dependent lethality in *Eed*$^{hypo/hypo}$ animals

C57BL/6.CD1 (N0-N2) or C57BL/6 (N4-N6) *Eed*$^{hypo/+}$ animals were intercrossed, and progeny were molecularly genotyped at weaning. Observed numbers of animals of genotype are indicated with expected numbers shown in parentheses. Deviation from expected ratios was assessed by chi square analysis, assuming one degree of freedom.

	Wild-type	*Eed*$^{hypo/hypo}$
P1	18	6*
P2	18	3**

TABLE 6.2. Perinatal lethality of C57BL/6.CD1 (N4-N6F1) *Eed*$^{hypo/hypo}$ animals

C57BL/6.CD1 (N4-N6) *Eed*$^{hypo/+}$ animals were intercrossed, and progeny were genotyped at P1 or P2 by scoring eye pigmentation. Because *Eed*^{hypo} is tightly linked to a nonfunctional *Tyr* allele, animals lacking eye pigmentation will generally be *Eed*$^{hypo/hypo}$ and animals with pigmented eyes will be *Eed*$^{+/+}$ or *Eed*$^{hypo/+}$.

* 3 of 6 albino animals recovered at P1 were dead.

** 3 of 3 albino animals recovered at P2 were dead.

	Wild-type	*Eed*$^{hypo/hypo}$
Mass (g)	1.30 ± 0.04	0.98 ± 0.13

TABLE 6.3. Prenatal runting in C57BL/6.CD1 (N6F1) *Eed*$^{hypo/hypo}$ embryo

Timed intercross matings were conducted between C57BL/6.CD1 (N4-N6) *Eed*$^{hypo/+}$ animals, and litters were sacrificed at E18.5. Embryos were weighed and then genotyped by scoring eye pigmentation as explained above. Means and standard deviations are shown. The difference in mean mass between wild-type and mutant embryos is statistically significant, as assessed by an unpaired t-test ($P < 0.0001$).

FIGURE 6.1. Prenatal runting in C57BL/6.CD1 (N6F1) *Eed*$^{hypo/hypo}$ embryos

C57BL/6.CD1 (N6F1) *Eed*$^{hypo/hypo}$ (left) and wild-type (right) E18.5 embryos from C57BL/6.CD1 (N6) *Eed*$^{hypo/+}$ intercrosses. On average, mutants are 25% smaller than their wild-type littermates (Table 6.3).