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A mutation in the E(Z) methyltransferase that increases trimethylation of histone H3 lysine 27 and causes inappropriate silencing of active Polycomb target genes

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ABSTRACT

Drosophila Polycomb Repressive Complex 2 (PRC2) is a lysine methyltransferase that trimethylates histone H3 lysine 27 (H3K27me3), a modification essential for Polycomb silencing. Mutations in its catalytic subunit, E(Z), that abolish its methyltransferase activity disrupt Polycomb silencing, causing derepression of Polycomb target genes in cells where they are normally silenced. In contrast, the unusual *E(z)* mutant allele *Trithorax mimic* ($E(z)^{Trm}$) causes dominant homeotic phenotypes similar to those caused by mutations in *trithorax* (*trx*), an antagonist of Polycomb silencing. This suggests that $E(z)^{Trm}$ causes inappropriate silencing of Polycomb target genes in cells where they are normally active. Here we show that $E(z)^{Trm}$ mutants have an elevated level of H3K27me3 and reduced levels of H3K27me1 and H3K27me2, modifications also carried out by E(Z). This suggests that the $E(z)^{Trm}$ mutation increases the H3K27 trimethylation efficiency of E(Z). Acetylated H3K27 (H3K27ac), a mark of transcriptionally active genes that directly antagonizes H3K27 methylation by E(Z), is also reduced in $E(z)^{Trm}$ mutants, consistent with their elevated H3K27me3 level causing inappropriate silencing. In 0–4 h $E(z)^{Trm}$ embryos, H3K27me3 accumulates prematurely and to high levels and does so at the expense of H3K27ac, which is normally present at high levels in early embryos. Despite their high level of H3K27me3, expression of *Abd-B* initiates normally in homozygous $E(z)^{Trm}$ embryos, but is substantially lower than in wild type embryos by completion of germ band retraction. These results suggest that increased H3K27 trimethylation activity of $E(Z)^{Trm}$ causes the premature accumulation of H3K27me3 in early embryogenesis, "predestining" initially active Polycomb target genes to silencing once Polycomb silencing is initiated.

Introduction

The Polycomb group (PcG) proteins were first identified for their role in the transcriptional silencing of the *Drosophila* homeotic genes. During early embryogenesis, the spatially restricted patterns of homeotic gene expression are initially established by the actions of transiently expressed transcriptional activators and repressors encoded by the gap and pair rule segmentation genes (Casares and Sanchez-Herrero, 1995; Harding and Levine, 1988; Kuziora and McGinnis, 1988; Reinitz and Levine, 1990), a process that does not require PcG proteins. By the completion of germ band elongation (~5.3 h), these transient factors have disappeared and PcG proteins have become required for subsequent maintenance of repression of the homeotic genes in cells outside of their spatially restricted expression domains. In loss-of-function PcG mutants, the homeotic genes are initially expressed in their normal spatially restricted domains, but they become ectopically derepressed as germ band elongation is completed (Celniker et al., 1989; Kuziora and McGinnis, 1988; Simon et al., 1992). At the same time, Trithorax (TRX) and other Trithorax Group (TrxG) proteins become required to prevent Polycomb silencing and maintain transcriptionally active states of the homeotic genes in cells where they were initially activated (Breen and Harte, 1993; Petruk et al., 2001; Sedkov et al., 1994).

Besides the homeotic genes, recent studies have shown that PcG proteins are also directly required for the repression of hundreds to thousands of other genes that are involved in a wide variety of biological processes, including cell fate determination, stem cell pluripotency, cell growth and proliferation, differentiation, regeneration, DNA repair, and others (Bracken and Helin, 2009; Conerly et al., 2011; Margueron and Reinberg, 2011; Martinez and Cavalli, 2006; Schwartz and Pirrotta, 2007).

More than a dozen PcG proteins have been identified. Many have been evolutionarily conserved throughout the animal and plant kingdoms, and their functions in transcriptional silencing have been similarly conserved (Margueron and Reinberg, 2011; Schwartz and Pirrotta, 2007). Several distinct multi-protein PcG complexes have been characterized, each possessing unique activities (Francis et al., 2004; Kavi and Birchler, 2009; Klymenko et al., 2006; Lagarou et al., 2008; Ng et al., 2000; Saurin et al., 2001; Savla et al., 2008;

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Scheuermann et al., 2010; Schuettengruber et al., 2009; Shao et al., 1999; Tie et al., 2001; Tie et al., 2003). Polycomb Repressive Complex 2 (PRC2) is a lysine methyltransferase that methylates lysine 27 of histone H3 (H3K27) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). Its catalytic activity resides in the SET domain of its E(Z) subunit. The various members of the SET domain methyltransferase family differ not only in their specificity for particular lysines in their polypeptide substrates, but also in their ability to use unmodified, mono- and dimethylated lysines as substrates, and their resultant yields of mono-, di- and trimethylated products (Cheng and Zhang, 2007).

Methylated H3K27 exists in vivo in mono-, di- and trimethylated forms. In *Drosophila*, H3K27me3 has been estimated to comprise only 5–10% of total H3, while H3K27me2 (50%) and H3K27me1 (10–20%) are considerably more abundant (Ebert et al., 2004). E(Z) appears to be responsible for all H3K27 di- and trimethylation and much if not all monomethylation, since loss of E(Z) function results in loss of H3K27me3, H3K27me2 and H3K27me1 (Cao et al., 2002; Kurzhals et al., 2008; Tie et al., 2009). Only H3K27me3 is associated with Polycomb silencing. In embryos and S2 cell lines, H3K27me3 is distributed in broad domains spanning entire silenced homeotic genes and other Polycomb-regulated genes (Beisel et al., 2007; Negre et al., 2006; Schuettengruber et al., 2009; Schwartz et al., 2006; Schwartz et al., 2010). Deposition of H3K27me3 at the promoter regions of target genes may be particularly crucial for silencing, perhaps serving to recruit another PcG complex, PRC1 (Beisel et al., 2007; Chopra et al., 2011; Enderle et al., 2011; Mito et al., 2007; Papp and Muller, 2006; Schuettengruber et al., 2009; Schwartz et al., 2006, 2010). In *E(z)* mutant embryos, H3K27 methylation is lost (Cao et al., 2002), and the homeotic genes are ectopically derepressed (Jones and Gelbart, 1990; Simon et al., 1992).

Loss-of-function mutations in *E(z)* and in the genes encoding other PRC2 subunits (*esc*, *escl*, *Su(z)12*, *Pcl*) cause characteristic PcG mutant phenotypes, including transformations of segmental identities due to ectopic derepression of the homeotic genes. In contrast, the unusual dominant *E(z)* mutation *Trithorax mimic* ($E(z)^{Trm}$) causes homeotic phenotypes similar to those caused by mutations in *trithorax* (*trx*), a key antagonist of Polycomb silencing (Bajusz et al., 2001). In *trx* mutants, the expression levels of the homeotic genes are initially normal, but by completion of germ band retraction are substantially lower than normal in cells where they were initially active (Breen and Harte, 1993; Sedkov et al., 1994). This results, at least in part, from default Polycomb silencing of initially active homeotic genes, when the anti-silencing activity of TRX is absent (Ingham, 1985; Ingham and Whittle, 1980; Klymenko and Muller, 2004; Pirrotta et al., 1995). The dominant *trx*-like phenotypes of $E(z)^{Trm}$ suggested that it causes inappropriate silencing of Polycomb target genes in cells where they are normally active (Bajusz et al., 2001), apparently overriding the anti-silencing effects of TRX and other TrxG proteins. Given that E(Z) is the catalytic subunit of the PRC2 methyltransferase complex, we suspected that $E(z)^{Trm}$ might be a hypermorph, producing a "hyperactive" mutant enzyme.

The $E(z)^{Trm}$ mutation causes a single amino acid substitution (R741K) in the catalytic SET domain (Bajusz et al., 2001). This same mutation has been independently isolated twice, indicating that its *trx*-like phenotypes are indeed due to the R741K substitution (Bajusz et al., 2001). This residue has been highly conserved in E(Z) orthologs, including those from plants (Fig. 1), arguing that it is likely to be functionally important. Structural and functional studies of other SET domain proteins have shown that this region of the SET domain contains residues that project into the catalytic pockets of these enzymes. Some of these residues are invariant in all SET domains (e.g., Y740) and are critical for catalysis. Others vary among different SET domain proteins but are conserved among orthologs, including a so-called "switch" residue (corresponding to F738 in E(Z)), which affects the number of methyl groups each enzyme can add to a substrate lysine (Collins et al., 2005; Zhang et al., 2003) (see Discussion). The residue at the position corresponding to R741 has not been previously implicated in the catalytic activity of any SET domain methyltransferase or shown to affect the relative yields of mono-, di- and tri-methyl reaction products. However, its conservation and proximity to other catalytically important residues suggest that the $E(Z)^{Trm}$ protein might have altered methyltransferase activity and product yields.

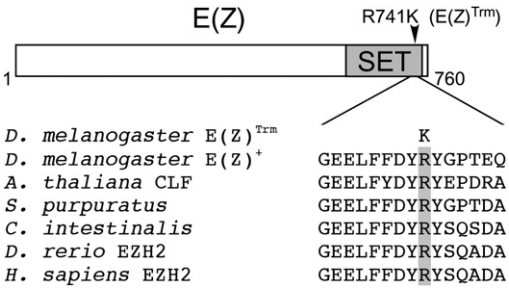

Fig. 1. Alignment of sequences surrounding the R741K $E(Z)^{Trm}$ mutation in E(Z) orthologs. The SET domain of E(Z) is located in the C-terminal portion of the protein. R741 (highlighted in gray) has been widely conserved in E(Z) homologs from both animal and plant kingdoms. Examples: plants (*A. Thaliana* CLF), invertebrates (sea urchin, *S. pupuratus*); protochordates (tunicate, *Ciona intestinalis*); vertebrates (fish, *Zebra danio*) EZH2; mammal (*H. sapiens* EZH2).

Here we report that $E(z)^{Trm}$ mutants have elevated levels of H3K27me3 and reduced levels of H3K27me2 and H3K27me1. They also have reduced levels of H3K27ac, an alternative H3K27 modification associated with transcriptionally active genes that directly antagonizes H3K27 methylation by E(Z) at transcriptionally active Polycomb target genes. We also show that $E(z)^{Trm}$ mutant embryos prematurely accumulate high levels of H3K27me3, and do so at the expense of the H3K27ac normally present in the early embryo. While *Abd-B* expression is initiated normally in $E(z)^{Trm}$ embryos, after completion of germ band elongation the level of ABD-B protein becomes much lower than in wild type embryos. These results strongly suggest that the $E(Z)^{Trm}$ mutant enzyme has increased trimethylation efficiency and that the resulting higher level of H3K27me3 (and lower level of H3K27ac) is responsible for the dominant *trx*-like mutant phenotypes of $E(z)^{Trm}$ mutants. We discuss the implications of the inappropriate silencing of initially active Polycomb target genes in this unusual *E(z)* mutant for the initiation and regulation of Polycomb silencing in the early embryo.

Results

$E(z)^{Trm}$ affects Abd-B expression in the early embryo

The dominant *trx*-like phenotypes of $E(z)^{Trm}$ heterozygous adults include partial transformations of posterior abdominal segment identities to more anterior segment identities, similar to those seen in *Abd-B* mutants. These transformations are more pronounced in $E(z)^{Trm}$ hemizygotes, which develop into pharate adults, and are even more extreme in rare pharate adult homozygotes (Bajusz et al., 2001; data not shown). This increasing severity of mutant phenotypes with increasing mutant gene dosage is a hallmark of hyperactivity mutants. Since the effect of $E(z)^{Trm}$ on homeotic gene expression has not been directly determined previously, we first examined the effects of $E(z)^{Trm}$ mutation on ABD-B protein expression in staged homozygous embryos, which should exhibit the most severe effects.

In wild type embryos, *Abd-B* transcripts can first be detected 2.5 to 3 h after fertilization, prior to completion of cellularization of the blastoderm (Harding and Levine, 1988; Kuziora and McGinnis, 1988; Reinitz and Levine, 1990). ABD-B protein is first detected around 4 h after fertilization, in stage 9, during germ band elongation,