An Unexpected Regulatory Cascade Governs a Core Function of the *Drosophila* PRC1 Chromatin Protein Su(z)2

Son C. Nguyen, Stephanie Yu, Elaine Oberlick, and Chao-ting Wu¹

Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115

ORCID ID: 0000-0003-2187-1027 (S.C.N.)

ABSTRACT Polycomb group (PcG) proteins are major chromatin-bound factors that can read and modify chromatin states to maintain gene silencing throughout development. Here we focus on a close homolog of the PcG protein Posterior sex combs to better understand how these proteins affect regulation. This homolog, called Suppressor 2 of zeste [Su(z)2] is composed of two regions: the N-terminal homology region (HR), which serves as a hub for protein interactions, and the C-terminal region (CTR), which is believed to harbor the core activity of compacting chromatin. Here, we describe our classical genetic studies to dissect the structure of Su(z)2. Surprisingly, we found that the CTR is dispensable for viability. Furthermore, the core activity of Su(z)2 seems to reside in the HR instead of the CTR. Remarkably, our data also suggest a regulatory cascade between CTR and HR of Su(z)2, which, in turn, may help prioritize the myriad of PcG interactions that occur with the HR.

KEYWORDS PRC1; Psc; Su(z)2; intramolecular regulation; regulatory cascade

THE Polycomb group (PcG) proteins are epigenetic modifiers whose contribution to stable silencing belies the dynamic nature and diversity of their function. One set of PcG proteins forms the Polycomb Repressive Complex 1 (PRC1), which can compact chromatin *in vitro* into a state that is refractory to remodeling and transcription (King *et al.* 2002, 2005; Francis *et al.* 2004; Grau *et al.* 2011; Beh *et al.* 2012; Simon and Kingston 2013). PcG proteins are also believed to effect silencing via the post-translational modification of histones, such as the methylation of histone H3 on K27 as well as both the ubiquitylation and deubiquitylation of histone H2A on K118/9 (Cao *et al.* 2002; Wang *et al.* 2004; Lagarou *et al.* 2008; Scheuermann *et al.* 2010; Schwartz and Pirrotta. 2013; Grossniklaus and Paro 2014). While maintaining the silenced state of targeted genes, some PcG proteins have residency times on chromatin that last less than a single cell cycle, binding and dissociating from different loci at different rates (Ficz *et al.*

2005; Ren *et al.* 2008; Fonseca *et al.* 2012; Vandembunder *et al.* 2014). These observations attest to the dynamic equilibria of PcG proteins and the maintenance of their different, and sometimes opposing, chromatin states.

Here we describe our use of classical genetics to better understand how the disparate functions of PcG proteins might be coordinated. In particular, we have focused on the genetic dissection of a protein domain, called the homology region (HR), which was originally defined for its conservation among the PcG *Drosophila* *Suppressor 2 of zeste* [Su(z)2] gene, its paralogous *Posterior sex combs* (Psc) gene, and their mammalian homologs Bmi-1 and Mel-18 (Brunk *et al.* 1991; van Lohuizen *et al.* 1991). The HR, located at the N-terminal end of Su(z)2 and Psc, is believed to house the protein-protein interaction subdomains responsible for interactions with the other subunits of the PcG complexes PRC1 and dRAF, the H2AK118/9 ubiquitylation complex (Kyba and Brock 1998; King *et al.* 2005; Lagarou *et al.* 2008; Lo *et al.* 2009; Schwartz and Pirrotta 2013; Simon and Kingston 2013; Kang *et al.* 2015; Wani *et al.* 2016). The C-terminal regions (CTRs) of Su(z)2 and Psc have been considered the core of these proteins as they are the main contributors of PRC1-mediated silencing; they can, on their own, recapitulate the inhibition of chromatin remodeling, compaction of chromatin, and transcriptional repression exhibited

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¹Corresponding author: Department of Genetics, Harvard Medical School, 77 Avenue Louis Pasteur, NRB 264, Boston, MA 02115. E-mail: twu@genetics.med.harvard.edu

by PRC1 *in vitro* (King *et al.* 2002, 2005; Francis *et al.* 2004; Lo *et al.* 2009; Beh *et al.* 2012). This is consistent with the fact that, while the mammalian orthologs do not have the CTR, the function of the CTR has been retained in mammals in a stretch of positively charged residues found in the Pc subunit of the PRC1 (Grau *et al.* 2011). Thus, it is believed that the HR serves as the hub of protein–protein interactions which, in part, help direct the CTR to genomic regions requiring regulation by PcG proteins. While characterization of the CTR has been driven by the genetics of both *Su(z)2* (Wu and Howe 1995; Emmons *et al.* 2009; Lo *et al.* 2009) and *Psc* (Francis *et al.* 2004; King *et al.* 2005), the *Su(z)2* locus has been more amenable to the genetic dissection of the HR (Wu and Howe 1995; Emmons *et al.* 2009; Lo *et al.* 2009). Thus, we have been continuing our study of *Su(z)2* to characterize and analyze the functions of HR.

One allele of *Su(z)2*, named *Su(z)2¹*, is caused by a truncation mutation that deletes nearly all of the CTR while keeping the HR intact (Wu *et al.* 1989; Wu and Howe 1995; Emmons *et al.* 2009). As expected, this allele is recessive lethal, which is in line with *in vitro* evidence suggesting that the CTR is essential, providing the compaction mechanism that is necessary for transcriptional silencing (Wu and Howe 1995; King *et al.* 2005; Lo *et al.* 2009). Despite lacking the CTR, however, *Su(z)2¹* exhibits several gain of function phenotypes (Wu and Howe 1995; Emmons *et al.* 2009), suggesting that some of the functions of *Su(z)2* are compartmentalized within the HR alone. Thus, *Su(z)2¹* provides us with the opportunity to isolate and study the HR.

An interesting illustration of the gain-of-function nature of *Su(z)2¹* is its capacity to interfere with the viability of a class of hypomorphic alleles that are hemizygous viable (Emmons *et al.* 2009). This is apparent with four missense mutations of the HR (Figure 1A, e26, s36, s20, and s21), which show varying levels of viability when heterozygous with a deletion of *Su(z)2*, but are inviable when heterozygous with *Su(z)2¹* (Emmons *et al.* 2009). These findings suggested that *Su(z)2¹* is more detrimental than a complete deletion of *Su(z)2* and, therefore, may interfere with essential functions provided by the HR alleles. For example, *Su(z)2¹* might interact directly with mutant HR proteins or compete with them such that they are no longer functional, perhaps by the HR of *Su(z)2¹* acting as a “sink” and outcompeting mutant HR proteins for interactions with other factors (Figure 1B). This interpretation is particularly intriguing because the HR contains domains implicated in protein–protein or other types of interactions. These domains include a ring finger (RF) (residues 35–73) (Brunk *et al.* 1991; van Lohuizen *et al.* 1991), the core sequence of the conserved subregion 1 (CSR1), henceforth called the conserved patch (CP) (residues 97–103) (Buchwald *et al.* 2006; Emmons *et al.* 2009; Bentley *et al.* 2011), and the ubiquitin-like domain (UL) (residues 137–228) (Figure 1A) (Sanchez-Pulido *et al.* 2008; Bezsonova *et al.* 2009; Wang *et al.* 2010).

Here, we describe our genetic studies clarifying how the specific domains within the HR contribute to *Su(z)2* function. Specifically, we conducted a screen for mutations that would suppress the lethality of *Su(z)2¹* when it is heterozygous with an HR allele called s36, which disrupts the RF (Figure 1B). Remarkably, all of the isolated lines had mutations in the RF,

CP, and UL domains of *Su(z)2*. Furthermore, they demonstrated that contrary to expectations, the CTR may not be essential and that the HR, alone, can support viability, arguing that the core activity of *Su(z)2* may actually reside in the HR. Interestingly, our data reveal a potential cascade of intramolecular regulation between different domains of *Su(z)2*. We discuss how our findings may explain the potential coordination of disparate PcG functions that occur through the HR interface of *Su(z)2*.

Materials and Methods

Drosophila stocks and culture conditions

Stocks were maintained and crosses were conducted at 25° at 80% humidity on standard *Drosophila* cornmeal, yeast, sugar, and agar media with p-hydroxybenzoic acid methyl ester added as a mold inhibitor. Crosses were carried out with approximately four males and four females and brooded daily to prevent crowding. All *Su(z)2* alleles were maintained over CyO-19 (Flybase ID FBba0000315), a GFP-bearing balancer. The *Su(z)2* deficiency (Df), generously provided by Jun-yuan Ji, was generated via transposase-mediated deletion using PBac{RB}*Su(z)2*[e00448] and P{XP}[d02486].

Mutagenic screen for the generation of *Su(z)2¹* derivatives

Male *w⁻*; *Su(z)2¹/w⁺*, CyO-19 flies were fed EMS as previously described (Wu and Howe 1995) and crossed to *w⁻/w⁻*; *Su(z)2^{s36}/w⁺*, CyO-19 females. A total of 57,906 F₁ progeny were scored for white eyes and normal wings (*Su(z)2¹*/s36*), with 100 exceptional progeny isolated. These flies were backcrossed to the s36 stock to test for transmission of mutation. The 12 lines that showed transmission were then isogenized for chromosome 2.

Molecular characterization of *Su(z)2¹* derivatives

Embryos homozygous for the second chromosome were collected by setting up an overnight egg lay and selecting GFP⁺ embryos. These embryos were subjected to proteinase-K digestion to release DNA (Gloor *et al.* 1993) to be used for sequencing (Emmons *et al.* 2009). Exonic regions of *Su(z)2* were amplified by PCR, gel purified with the QIAQuick Gel Extraction kit (QIAGEN, Valencia, CA; no. 28704), and sequenced at the Harvard Medical School Biopolymers Facility. Primers for PCR and sequencing are listed in (Supplemental Material, Table S2) and ordered from Integrated DNA Technologies (Coralville, IA).

Inter se crosses and calculation of viability

Inter se crosses were performed by crossing the parental lines (*Su(z)2* mutant allele/*w⁺*, CyO-19) to the *Su(z)2¹* derivative lines. Percent viability was calculated assuming equal transmission of each allele and lethality for homozygosity of the CyO balancer, in which case 33% of progeny would be expected to be heterozygous for both *Su(z)2* alleles. We calculated a viability score as the observed number of non-CyO progeny/total progeny, normalized to the expected frequency

Figure 1 (A) Structure of *Su(z)2* and previously extant alleles (Wu and Howe 1995; Emmons *et al.* 2009). *Su(z)2* is divided into two main regions: the HR and the CTR. The HR is composed of three domains, the RF (red), the CP (gray), and the UL domains (blue). The alleles differ from wild-type either by point mutation (○), truncation, and/or frameshift mutation (white region denotes the novel amino acid sequence generated by the frameshift). (B) The competition hypothesis between *Su(z)2*¹ and *s36*. *Su(z)2*¹ outcompetes *s36* for essential protein interactions because the HR of *s36* is compromised (○), while mutation of the HR of *Su(z)2*¹ (●) restores essential interactions with *s36*.

of 33%. We report the average viability among the replicate crosses. SPSS software (IBM; Armonk, NY) was used for one-way ANOVA calculation and *post hoc* Dunnett's test to compare all crosses with the control cross with Df.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains are available upon request.

Results and Discussion

A screen to generate point mutations in domains within *Su(z)2*

Based on our model of competition between *Su(z)2*¹ and *Su(z)2*^{s36} (Figure 1B), we predicted that reversion of the

dominant negativity of *Su(z)2*¹ would reveal residues or domains within *Su(z)2* (Figure 1B) or perhaps even within other genes that are important for *Su(z)2* function. To this end, we mutagenized males carrying *Su(z)2*¹ with EMS and then crossed them to females carrying *Su(z)2*^{s36} to identify mutations that would suppress the lethality of the *Su(z)2*¹/*Su(z)2*^{s36} progeny. We screened a total of 57,906 progeny in three independent mutageneses and recovered 100 *Su(z)2*¹/*Su(z)2*^{s36} flies. These exceptional progeny were crossed back to flies carrying *Su(z)2*^{s36} to test for fertility and transmission of the mutation, and 12 lines survived this process. All mutations were mapped to chromosome 2, on which *Su(z)2* is located, and sequencing of the exons of *Su(z)2* confirmed that each line retained the original *Su(z)2*¹ mutation while gaining either a missense or nonsense mutation within the HR (Figure 2A). Henceforth, we will refer to these alleles collectively as "*Su(z)2*¹ derivatives."

Figure 2 (A) The structure of *Su(z)2¹* derivatives. (B) The viability of progeny resulting from crosses between *Su(z)2¹* derivatives, averaged between reciprocal crosses and grouped according to the affected domains (RF, RF-CP, and UL) and either *s36*, *e26*, *s20*, or *s21*. Average viabilities of individual derivatives are provided in Table S1. Viability of *Df* and *Su(z)2¹* are also shown. The R93Q allele ([†]) and the R228GΔ allele ([‡]) show lethality when paternally contributed and crossed with *e26* and *s21*, respectively (see Table S1).

In brief, our screen to suppress the lethality between *Su(z)2¹* and *Su(z)2^{s36}* recovered only mutations in the HR of *Su(z)2¹*, which houses the protein interaction domains of *Su(z)2*.

Point mutations define functional domains of *Su(z)2*

Two mutations, *D49N* and *I60V*, affect single residues within the RF of *Su(z)2¹* (Figure 2A). As these derivatives are viable with *s36* (66–87%, Figure 2B, see *Materials and Methods* for calculation of viability), they demonstrate that disruption of the RF is sufficient to revert the dominant negativity of *Su(z)2¹*. In addition, three mutations were found downstream of the RF and close to the CP, wherein two are missense mutations (*R93Q* and *I96N*) and one is a nonsense mutation that truncates the protein just before the CP and UL domains (*R93**) (Figure 2A). The remaining seven mutations affect the UL, of which four are missense and three are nonsense: *Q105¹*, *Q105²*, *S141F*, *Q151**, *C165Y*, *C214Y*, and *R228GΔ* (Figure 2A); although *Q105¹* and *Q105²* contain the same *Q105** change and are phenotypically similar to each other, they are considered distinct alleles, as they do not behave identically and may therefore be associated with alterations in the unsequenced regions of *Su(z)2* or changes elsewhere in the genome. Note that the recovery of mutations not only in the RF domain but also within the CP and UL domains suggest that these latter two domains may indirectly impact or cooperate with the functions of the RF. Indeed, the RF and the CP have been shown to engage in the same interaction with the PcG protein Ring1b *in vitro* (Buchwald *et al.* 2006; Bentley *et al.* 2011).

Suppression of dominant negativity is not allele specific

To determine whether the suppression of dominant negativity by the *Su(z)2¹* derivatives is general or, instead, limited to the specific amino acid change in the RF of the *s36* mutation, we crossed the derivatives to another RF mutation, called *e26* (Figure 1A), which disrupts a conserved histidine and is also completely lethal when heterozygous with *Su(z)2¹* (Figure 2B). Like *s36*, *e26* is also hemizygous viable, although its viability is significantly less than that of *s36* (52% vs. 93%, $P < 0.01$, Student's *t*-test, respectively, Figure 2B). Ten of the 12 derivatives are viable when heterozygous with *e26*, confirming that the capacity of the derivatives to suppress the dominant negativity of *Su(z)2¹* is not allele specific with respect to *s36*. The inviability of two derivatives (*D49N* and *I60V*) when heterozygous with *e26* was not surprising, given the phenotypic severity of *e26* (Figure 2, A and B). Interestingly, the viabilities of the derivatives when heterozygous with *e26* was more variable than that observed with *s36* (0–103% for *e26* vs. 66–105% for *s36*, Figure 2B and Table S1), revealing potential functional differences between the *Su(z)2¹* derivatives. Overall, these data show that the derivatives generally suppress the dominant negativity of *Su(z)2¹* with one other RF mutation.

We further assessed whether the dominant negativity of *Su(z)2¹* could also be suppressed when the derivatives are in *trans* to mutations other than those in the RF by crossing the derivatives to the *s20* and *s21* alleles, which are located within or near the CP and UL domains, respectively, and show strong or complete lethality with *Su(z)2¹* (Figure 2B)

Figure 3 (A) The range of viabilities of the different classes of *Su(z)2¹* derivatives when heterozygous with *s84*, *h29*, or *e26*. The crosses with *e26* are separated based on the allele coming from the mother (♀) or father (♂). Average viabilities of individual derivatives are found in Table S1. The green and red shading highlights the viabilities that were used to help classify the *Su(z)2¹* derivatives. While all *Su(z)2¹* derivatives are hemizygous inviable, class I alleles are defined by their strong viability with *s84* and *h29*, and class II and III alleles are distinguished by their different viabilities with *e26*. (B) A model proposing a cascade of regulation. The HR is proposed to harbor a CTR-independent function that requires the RF and CP (arrow 1), but is suppressed by the UL domain (arrow 2). Suppression of the UL domain by the CTR (arrow 3) allows the CTR-independent function to persist.

(Emmons *et al.* 2009). Only one of the derivatives, UL mutation *R228GA*, maintained lethality with *s21*, and only when the *s21* allele was contributed maternally (Table S1). This suggests a possible involvement of maternal effects, although we have yet to investigate this further. The remaining 11 of the 12 *Su(z)2¹* derivatives were robustly viable when heterozygous to either *s20* or *s21* (Figure 2), further arguing the significance of their impact on *Su(z)2*.

Zygotic contribution of the CTR is not essential

Over the course of our studies, we made some unexpected observations of the CTR, leading us to question the presumed essential nature of the CTR. Here, we took advantage of two alleles of *Su(z)2*, called *s84* and *h29* (Figure 1A), both of which harbor nonsense mutations that remove the CTR as well as the UL (Emmons *et al.* 2009) and are lethal when hemizygous (Figure 3A). As these alleles are extreme hypomorphs or nulls, we expected that heterozygosity of either with any allele also lacking the CTR would not be viable. Surprisingly, however, two of the *Su(z)2¹* derivatives, *C165Y* or *R228GA*, were viable when heterozygous with *s84* or *h29*. While the viability ranged from as low as 4–55%, it was observed across all our replicate crosses (Figure 3A). These observations suggest that the CTR is not necessary for viability when there are compensatory mutations within the UL domain. An alternative explanation for the viability we observed suggests read through of the nonsense mutations of these alleles. However, *h29* results from a frameshift mutation wherein read through

would only produce novel sequence followed by additional nonsense mutations, and the sequence signatures of the nonsense mutations of *s84*, *C165Y*, and *R228GA* (stop codon identity and the downstream base) are the least associated with read through (Chao *et al.* 2003; Jungreis *et al.* 2011). Of the 10 remaining *Su(z)2¹* derivatives, 4 were inconsistently and weakly viable, and 6 were completely lethal when heterozygous with *s84* or *h29* (Figure 3A and Table S1). To distinguish the two exceptional alleles, *C165Y* or *R228GA*, from the other derivatives of *Su(z)2¹*, we have placed them into their own category, which we call class I.

Our novel findings reveal a cascade of regulation between the domains of *Su(z)2* that govern its core function (Figure 3B). In our model, viability is dependent on the RF and CP domains, as observed with *s84*, *h29*, and the class I derivatives (Figure 3B, arrow 1). An intact UL domain, such as the one in *Su(z)2¹*, may suppress the RF and CP domains (Figure 3B, arrow 2), unless the UL domain is, in turn, suppressed by the CTR (Figure 3B, arrow 3) or mutations within the UL itself, as exhibited by the class I derivatives. Thus, this model proposes that, rather than the CTR encoding the core function of *Su(z)2*, the CTR acts to release the core function which is encoded, instead, in the HR. Note, not all UL mutations display CTR-independent viability, possibly because they destabilize *Su(z)2* or do not fully disrupt the suppression by the UL domain. In this light, class I mutations may be highlighting new subdomains within the UL responsible for this suppression (Figure 3B).

It is possible that our observations of CTR-independent viability reflect rescue by the *Su(z)2* homolog, *Psc*, and/or a maternal effect by the heterozygous mothers carrying a wild-type *Su(z)2*. This interpretation must be tempered, however, by the fact that neither *Psc* nor a maternally contributed *Su(z)2* function are sufficient for viability (Figure 3A) (Wu and Howe 1995; Emmons *et al.* 2009). Furthermore, we have evidence suggesting that the maternal contribution of the CTR of *Su(z)2* is not essential, as females that are heterozygous for *h29* and the class I derivative R228GΔ can produce progeny; females that are genotypically null for the CTR of *Su(z)2* were crossed to males from either parental strain, and while the yield of progeny was low (<15 progeny per cross, 66 progeny total scored), we obtained viable progeny, suggesting that maternal contribution of the CTR is not essential. Interestingly, of the 64 progeny scored, 2 were also genotypically null for the CTR, suggesting that both zygotic and maternal contributions of the CTR may be dispensable for viability. In summary, our data show that at the very least, neither maternal contribution nor somatic contribution of the CTR of *Su(z)2* are essential for viability.

Release of an essential function by mutations in the HR

The viability of the class I derivatives in spite of their lack of a CTR prompted us to determine whether these derivatives can contribute to viability in other contexts. To this end, we compared them to a simple deficiency (*Df*) of *Su(z)2*. In particular, we compared their viability when heterozygous with *e26* (class I/*e26*) to that of *Df/e26* (Figure 3A, gray shading) noting separately the viabilities associated with either maternal or paternal inheritance of the *e26* allele. While the *Su(z)2*¹ derivatives are hemizygous inviable, the viability of class I/*e26* is similar to or significantly greater than that of *Df/e26* (Figure 3A). *C165Y* shows a slight, though significant, increase in viability when paternally inherited (82% vs. 46%, $P = 0.017$), whereas *R228GΔ* shows a significant increase in viability when inherited either maternally or paternally (103% vs. 58%, $P < 0.001$, and 103% vs. 46%, $P < 0.001$, respectively). These results further support the ability of class I alleles to contribute to viability through specific mutations in the UL domain.

Interestingly, the three *Su(z)2*¹ derivatives that delete much if not all of the UL domain, but retain full RF and CP domains, can result in increased viability when heterozygous with *e26*, and we have categorized them into a new group called class II. Specifically, class II/*e26* is more viable than *Df/e26* when *e26* is maternally inherited (Figure 3A, 79–105% vs. 46%, respectively, $0.001 < P < 0.04$, one-way ANOVA). Note, however, that the increased viability is not seen in the reverse direction, that is, when *e26* is paternally inherited (Table S1, 46–68% vs. 58%, $P > 0.37$). We do not have an explanation for this observation, but maternal or paternal effects may play a role. Regardless, these data support the prediction of the cascade model that disruption of the UL domain, either by class I or class II mutations, can uncover a function that supports viability in the absence of the CTR (Figure 3B).

Figure 4 Potential mechanisms for the observed regulation of the HR by the CTR. (A) Post-translational modifications (green diamonds) lead to suppression of the UL domain. (B) Intramolecular inhibition of the UL domain by the CTR. (C) Protein X binds the UL and inhibits the RF and CP, whereas protein Y binds the CTR and the UL, allowing the RF and CP to engage in other interactions. (D) Chromatin compaction causes inhibition of the UL domain, allowing the RF and CP to perform its activities.

Finally, the remaining seven alleles, which are unable to increase viability when heterozygous with *e26*, were placed into a final group called class III. This class includes the two UL point mutations, *S141F* and *C214Y*, which, compared to the *Df*, are significantly less viable when heterozygous to *e26* (2–7% vs. 46–58%, $P \leq 0.006$). It also includes all six RF and CP mutations; five of the alleles are significantly less viable than *Df/e26* in all crosses (0–5% vs. 46–58%, $P < 0.001$), and *I96N/e26* is significantly less viable than *Df/e26* only when *e26* is paternally inherited (20% vs. 58%, $P < 0.001$) (Figure 3A). One possible interpretation of the data are that these class III alleles retain some residual dominant negativity of *Su(z)2*¹ that is responsible for the lack of viability.

Overall, the marked differences in viability between the different classes with *e26* aligns with our proposed model of regulation between the *Su(z)2* domains (Figure 3B). For example, the class III mutations that disrupt the RF or CP domains and preserve the UL domain fail to increase viability with *e26*, consistent with the prediction that the RF and CP domains house a core function of *Su(z)2* that may be suppressed by the UL domain. Conversely, loss of the UL domain would eliminate the suppression and release this core function, as reflected in the results of class II truncation alleles. Our data also suggest that disruption of the UL domain by point mutation may have the same effect, as the switch from residual dominant negativity of the class III UL mutations to the increase in viability by the class I mutations suggest that only certain point mutations can disrupt the UL domain sufficiently to release the function of the RF and CP domains. Overall, the crosses with *e26* have allowed us to dissect the functional relationship of the different domains of *Su(z)2*, and these results are consistent with a model of intramolecular regulation between the domains.

Potential mechanisms of *Su(z)2* regulation

In summary, our studies suggest that the core function of *Su(z)2* is encoded in the HR, and the role of the CTR may be to help release this function. This interpretation is based on our

surprising discovery that, in some genetic backgrounds, the CTR, which had been thought to encode the core function for Polycomb-based silencing, is actually dispensable. Specifically, the lethality seen with loss of the CTR can be rescued by mutations within the HR of *Su(z)2*. Thus, this study presents a new perspective on the relationship between the CTR and HR. In particular, CTR may function primarily to regulate the activities of the HR and, thus, it may be the HR that facilitates stable silencing by PcG proteins. This model would explain how our mutations within the UL domain can phenocopy a potential role of the CTR and permit CTR-independent viability.

There are multiple, but not mutually exclusive, scenarios that reflect the regulatory cascade between the CTR and the HR (Figure 4). The CTR may be subject to post-translational modifications, as seen by the phosphorylation of the CTR of *Su(z)2* (Bodenmiller *et al.* 2008), which may alter the overall function and/or stability of *Su(z)2* (Figure 4A). The CTR may also interact with the HR directly (Figure 4B), or engage in its own interactions that may inhibit, modulate, or participate with those that occur with the HR (Figure 4C). Indeed, the CTR of Psc is necessary and sufficient to bind cyclin B as part of a complex that also requires the HR (Mohd-Sarip *et al.* 2012). Alternatively, the propensity of the CTR and other proteins, such as M33/Cbx2, to bring nucleosomes together *in vitro* (Francis *et al.* 2004; Lo and Francis 2010; Grau *et al.* 2011) may actually reflect a novel function *in vivo*, such as a sampling and “reading” of the chromatin landscape and/or the associated chromatin-bound factors, providing an opportunity for the local regulation and prioritization of the varied PcG interactions that occur with the HR (Figure 4D) (Gao *et al.* 2012; Junco *et al.* 2013; Kassis and Brown 2013; Morey *et al.* 2013). Overall, our genetic analysis reveals a novel interaction between the CTR and HR and further study should elucidate the impact of this intramolecular regulation of *Su(z)2* on PcG function.

Our findings may also explain the absence of a CTR among the mammalian homologs of *Su(z)2*, Bmi-1, and Mel-18 (Brunk *et al.* 1991; van Lohuizen *et al.* 1991; Grau *et al.* 2011; Beh *et al.* 2012), as it is possible that mammalian HRs have evolved in such a way as to compensate for the absence of a CTR. On the other hand, it may be that the CTR function of chromatin compaction, first discovered with *Drosophila* proteins *in vitro* (Francis *et al.* 2004), still exists in mammals but is found within a different PcG member, M33/Cbx2 (Grau *et al.* 2011). Interestingly, this protein interacts with Bmi-1 and Mel-18 (Gao *et al.* 2012), preserving an evolutionary link *in trans* between compaction and the HR. Thus, our observation of CTR-independent viability may reflect a core function of the HR of *Su(z)2* that is evolutionarily conserved and intimately associated with protein interactions.

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