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14. ABSTRACT

The Polycomb Group (PcG) of gene silencers are chromatin associated multi-protein complexes that maintain cell identity by regulating the expression of genomic programming genes. In stem cells, PcG complexes occupy hundreds of genomic sites repressing genes required for differentiation. We aim to investigate the role of a disulfide bond that forms within a member of the PcG called Ph. We predict that the formation of the disulfide bond functions as a redox trigger that is part of the normal functional activity of Ph. Our proposed studies will provide insights into the molecular events that underlie stem cell function by shedding light on a potential target of reactive oxygen species. Greater knowledge of these processes will undoubtedly be helpful not only in expanding our understanding of how the PcG functions but also in the design of therapeutics targeted specifically to cancer stem cells.

15. SUBJECT TERMS
Polycomb, reactive oxygen species, cysteine oxidataion

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Table of Contents

	Page
Introduction…….……..…..	4
Body……..	4
Key Research Accomplishments…….……..	6
Reportable Outcomes……	6
Conclusion……	6
References…….	7
Appendices……	7

INTRODUCTION

Polycomb Group (PcG) proteins maintain stem cells, including cancer stem cells, by repressing genes that would otherwise promote differentiation. Understanding the role of the PcG in cancer and fulfilling the promise that stem cells hold for regeneration therapy will require understanding the molecular mechanism of the PcG. The goal of this proposal was to determine whether the SAM domain of a PcG protein called Polyhomeotic (Ph) functions as a redox trigger through disulfide formation between two Ph SAM (sterile alpha motif) domains. Using funds provided by the DoD CDMRP, we have gathered data which does suggest Ph function is regulated by manipulating its oxidation state. Our findings could provide significant advances toward understanding cancer stem cells and treatment strategies to treat all cancers including breast cancer.

BODY

Background

SAM domains are found in over 3000 proteins and are involved in a diversified array of binding reactions[1]. Ph, a PcG protein, contains a C-terminal SAM domain. Like many other SAM domains, Ph SAM is able to self-associate into polymeric architecture[2] (Fig. 1A). This structure was solved at pH 4.0 which is a very low pH condition and likely to result in the protonation of any Cys residues that are present. Not surprisingly, Ph SAM residue Cys 1528 (C1528) is in the reduced state and found buried in the core of the protein (Fig. 1B). A second crystal structure of Ph SAM was determined bound to the SAM domain of another PcG protein called Scm[3] (Fig. 2A). This structure was determined at a more neutral pH condition and intriguingly, C1528 is now oxidized and forms a disulfide bond with the equivalent Cys residue of a second Ph SAM domain.

While initially believed to be an artifact of crystallization, there are reasons to believe that formation of this disulfide bond can actually play a role in Ph function. First, there is a high propensity for the disulfide to form. C1528 must undergo an energetically unfavorable conformational change from being buried in the core of the structure to being exposed in order to form the disulfide bond (Fig. 2B). Despite this energy barrier, the disulfide still forms. Second, there is extensive buried surface area between the two cross-linked Ph SAM molecules (1090 Å2) which is larger than many well-established protein-protein interactions suggesting this interaction is not the result of chance. Third, the Cys residue that undergoes the oxidation is conserved in all Ph orthologs suggesting a conserved role for the Cys residue. Fourth, a possible functional role is suggested by the structure of the disulfide linked Ph SAM dimer. An overlay of the reduced Ph SAM polymer structure on either of the two disulfide linked Ph SAM structures reveals steric clashes that would preclude Ph SAM polymerization (Fig. 2C, D). This would be a novel mode of regulating SAM domain polymerization. Finally, despite the reducing environment, disulfide bond redox triggers do occur in cells[4]. Consistent with these points, a recent study demonstrated a specific role played by reactive oxygen species in promoting the differentiation of *Drosophila* hematopoietic stem cells which resulted in down regulating Ph activity[5].

Result 1. Cys 1528 Leu (C1528L) is the most stable Ph SAM non-oxidizable mutation

Our first objective was to identify the most stable, non-oxidizable mutant in order to be able to compare it to wild-type (WT) Ph which we hypothesize acts as a redox trigger important for Ph function. We prepared a number of Ph SAM proteins mutated at C1528 and performed thermal denaturation experiments to determine their melting temperatures (Fig. 3A). Of all the C1528 mutations we studied (A, S, T, V, and L), the C1528L mutant Ph SAM domain was observed to have the highest melting temperature (T_m ~ 47.5 °C) and closest to that of WT (T_m ~ 52.5 °C). This result is perhaps not surprising because the equivalent position in the closely related Scm SAM structure is also a leucine (Fig. 3B). It is important to note that the T_m of the C1528L mutant is far above room temperature (~20 - 25 °C) in order to be assured that this mutant SAM domain will be properly folded and functional at the temperatures where *Drosophila* are grown (see **Result 5**).

Result 2: Ph SAM C1528L polymerizes

The Ph C1528L mutation was intended to preserve all molecular functions of the reduced wild-type Ph SAM domain but be void of the ability to oxidize and form the disulfide bond. Most notably, the reduced form of wild-type Ph SAM can polymerize *in vitro*[2]. We assessed the level of polymerization using analytical ultracentrifugation in collaboration with Dr. Borries Demeler and the UTHSCSA Center for Analytical Ultracentrifugation of Macromolecular Assmeblies (CAUMA). The van-Holde-Weischet combined distribution plots of the velocity sedimentation experiments for WT and C1528L Ph SAM show increasing sedimentation coefficient values with increasing boundary fraction indicative of increased self-association with increasing

protein concentration (Fig. 4A). This behavior is consistent with polymerization which we have observed for a variety of polymeric SAM domain constructs. There is a slight increase in the S-value of the C1528L mutant which we interpret as stemming from the propensity of WT Ph SAM to undergo oxidation which would hinder polymerization and result in a lower S-value for the WT. The result of the AUC experiment indicates that Ph SAM C1528L does indeed polymerize.

Result 3: Ph SAM C1528L binds Scm SAM like WT Ph SAM

In addition to Ph SAM self-association leading to polymerization, Ph SAM can also bind the SAM domain of another PcG protein called Scm[3, 6, 7] (Fig. 2, 4B). A GST pull-down experiment carried out with Ph SAM polymer deficient mutants that all have the C1528L mutation, bind to Scm SAM mutants with the identical binding pattern of WT Ph SAM polymer deficient mutants (Fig. 4C). These biochemical studies including those from **Result 2** show that the molecular functions of WT Ph SAM are preserved in Ph SAM C1528L.

Result 4: Ph C1528L represses transcription of reporter gene like WT Ph

The function of Ph and that of all PcG proteins is to repress transcription. We designed a transcription reporter assay carried out in *Drosophila* S2 cells to test the ability of a variety of Ph proteins to repress transcription (Fig. 5A). In this assay, the DNA binding domain of zif268 is fused to the N-terminus of Ph constructs allowing the chimeric proteins to be targeted to zif268 binding elements just upstream of a metallothionine promoter (MTp) that controls expression of the *luciferase* reporter gene. Zif268 fused wild-type Ph is able to repress *luciferase* expression compared to Ph that is not fused to zif268 (Fig. 5B). A SAM domain deleted Ph protein is unable to repress transcription showing that Ph requires the SAM domain for its repressive function. Polymer deficient Ph proteins (L1547R/H1556R and L1565R mutants) are unable to repress expression of the *luciferase* gene as compared to wild-type. These results, for the first time, show that Ph SAM polymerization is required Ph mediated repression. The repressive ability of the C1528L mutant is identical to wild-type Ph (Fig. 5C). This result indicates the C1528L mutation does not disrupt the Ph SAM structure or its ability to polymerize as both are required for repression (Fig. 5B).

It is reasonable to expect that the non-oxidizable Ph C1528L mutant may be less apt to repress transcription if a redox trigger is required for Ph function. One possibility for the observed equal repression for both WT Ph and Ph C1528L is that S2 cells are derived from *Drosophila* embryos which, given their early developmental stage, likely have low levels of reactive oxygen species which would help maintain Ph SAM in the reduced state and be similar to the structure of the non-oxidizable Ph C1528L mutant. Alternatively, oxidation of Ph SAM may trigger Scm binding (see below). In S2 cells, Scm binds weakly to Ph[8] which we predict to also be the case for the polymeric Ph C1528L. Together, we predict both WT Ph and Ph C1528L would behave similarly in S2 cells as is the case in our repression assay.

Result 5: Expression of Ph C1528L hinders Drosophila wing disc development

While **Results 1 - 4** show that polymerization and binding to Scm functions are preserved in the Ph C1528L mutant. We also hypothesize that oxidation of WT Ph SAM also plays some important role in Ph function. However, creating a more oxidizing environment to test the differences between WT Ph and Ph C1528L has proved to be challenging. For instance, we have carried out the transcription assay (Fig. 5A) in the presence of H_2O_2 to create a more oxidizing environment but observed little difference in the repressive abilities between WT Ph and Ph C1528L which we feel is a reflection of the technical difficulties of performing the assay and not a true reflection of the functional differences between WT Ph and Ph C1528L. If indeed the Ph SAM disulfide bond does play a role in Ph function, the consequence of this function would likely require precise temporal and spatial regulation of reactive oxygen species which would be difficult to recapitulate in more *in vitro* settings. We thus turned to *in vivo* experiments by creating transgenic flies. These experiments were performed in collaboration with Dr. Donald G. McEwen here at UTHSCSA. The obvious advantage to using *Drosophila* would be that if oxidation is required for proper Ph function, the flies would be able to naturally provide such environments when required in the normal course of development of the fly. Moreover, differences between WT Ph and the non-oxidizable Ph C1528L could be easily detected in phenotype changes.

Despite the same *in vitro* behavior of WT Ph SAM and the C1528L mutant, expression of these proteins in *Drosophila* resulted in completely different phenotypes (Fig. 6). Overexpression of wild-type Ph in the wing disc showed normal development (Fig. 6A) while the wing discs isolated from the larvae expressing the non-oxidizable Ph C1528L mutant were significantly smaller in size and exhibited a smaller posterior compartment (Fig. 6B). This result suggests that a molecular function in addition to polymerization is required for proper Ph

function *in vivo*. Given that the C1528L mutation retains the polymerization function but is incapable of forming the disulfide bond, this data supports the hypothesis that a redox trigger is indeed present in Ph.

Future work/New hypothesis:

The results of the transgenic fly experiments in **Result 5** strongly suggest that Ph SAM disulfide formation is important for Ph function. What remains to be determined, and what we will focus our future efforts on, is to determine the molecular consequence of this disulfide bond formation. We had previously proposed that the disulfide bond hinders polymerization because overlay of the Ph SAM polymer over the disulfide cross-linked Ph SAM dimer results in steric clashes that would preclude polymerization (Fig. 2C, D). While we believe this is still the case, our recent work on determining the factors that limit Ph SAM polymerization has brought forth a new hypothesis which we intend to test.

Our recent unpublished work on Ph SAM polymerization (also funded in part by the DoD, CDMRP) indicates that Ph SAM polymerization is inhibited by intra molecular interactions resulting in limiting polymerization to four to six Ph SAM units. That is, Ph SAM, which encompasses residues 1507 - 1577, is polymeric while Ph 1397 - 1577 is limited in its polymerization. This finding would be incompatible with an earlier proposed model of the Ph SAM/Scm SAM co-polymer structure where the two polymers unite at a single junction[3]. Our findings that Ph SAM polymerization is limited would also preclude extension of the polymer by the addition of Scm SAM and thereby make formation of the co-polymer structure less likely. However, upon oxidation of the Ph SAM C1528 to form the disulfide bond, Ph SAM polymerization is disrupted and would simultaneously allow interaction with Scm SAM. Therefore, we now hypothesize that the Ph SAM redox trigger is a mechanism regulating Ph SAM interaction with Scm.

There are previously reported observations consistent with this hypothesis[7]. In that study, Ph isolated from *Drosophila* embryos show little interaction with Scm. However, when recombinant proteins are co-expressed in Sf9 cells, Scm co-purifies to a much greater extent with Ph. Given the early developmental stage of *Drosophila* embryos and thus greater content of stem cells, it would be expected that they would have lower concentrations of reactive oxygen species[9] compared to Sf9 cells which are derived from ovarian tissues of the more developed pupal stage. Therefore, it is tempting to speculate that the increased reducing environment of embryos would promote the Ph SAM polymer state that limits interaction with Scm while the increased oxidation environment in tissues promotes Ph SAM disulfide formation and greater interaction with Scm. We intend to test the redox trigger/Scm interaction hypothesis using immunoprecipitation assays.

KEY RESEARCH ACCOMPLISHMENTS

1. We have identified a stable non-oxidizable, C1528L mutant for comparision to WT.
2. Ph C1528L polymerizes and possesses the same binding properties and transcription repression ability as WT.
3. Over expression of C1528L causes developmental defects in *Drosophila* while WT appears normal.

REPORTABLE OUTCOMES

N/A

CONCLUSION

We have proposed that the disulfide bond that can form between two Ph SAM domains is an important regulator of Ph function. Our results to date include the identification of a non-oxidizable Ph SAM mutant whose molecular binding functions are the same as WT but yet, when expressed *in vivo*, results in developmental defects that is absent with WT. These results provide strong rationale for continued investigation of the Ph SAM redox trigger hypothesis. Recent reports have discovered that breast cancer stem cells have lower levels of reactive oxygen species compared to non-tumorigenic cells which may contribute to greater resistance of the cancer stem cells to radiation therapy[9]. Identification of specific molecular events that are targets of reactive oxygen species may lead to the design of alternative therapies and chemotherapeutics which could target Ph SAM and ultimately be more effective in treating breast cancer.

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APPENDICES

N/A

SUPPORTING DATA

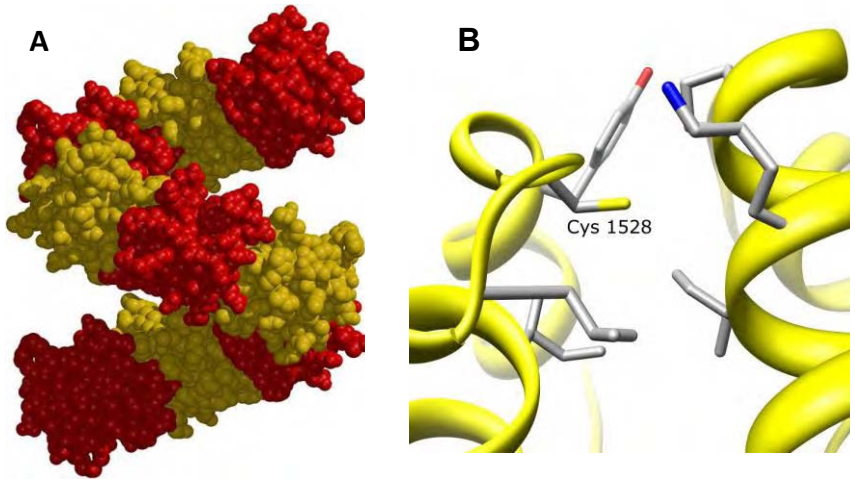


Fig. 1. Ph SAM polymer. A. Nine units of the Ph SAM polymer are shown in alternating colors[2]. C1528 is completely reduced in this structure and buried in the core of the protein (**B**).

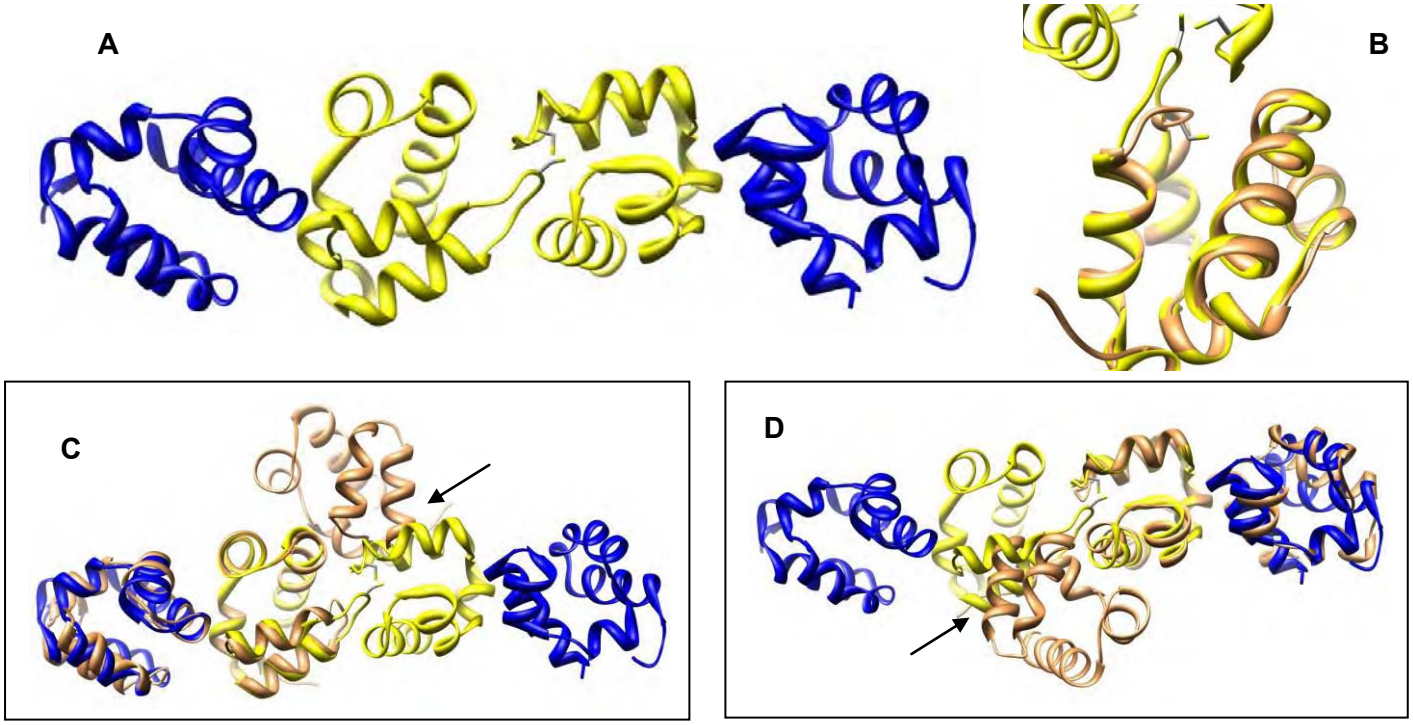


Fig. 2. Oxidized Ph SAM bound to Scm SAM. A. The asymmetric unit of the Ph SAM/Scm SAM co-crystal structure[3]. Yellow is Ph SAM, blue is Scm SAM. The two Ph SAM domains are covalently attached to each other through a disulfide linkage (side chains shown). **B**. The structure of Ph SAM with reduced C1528, polymer structure (beige), overlaid on the oxidized form (yellow) showing the conformational change one of the C1528 residues must undergo in order to form the disulfide bond. C1528 side chains are shown. **C**, **D**. Three units of the Ph SAM polymer (beige) overlaid on either of the two oxidized Ph SAMs. Steric clashes that would hinder polymerization are indicated by the arrows.

A

C1528 mutation	Tm (°C)
Cys (WT)	52.2
Leu	47.5
Val	42.5
Ala	42.5
Ser	32.5
Thr	32.5

B

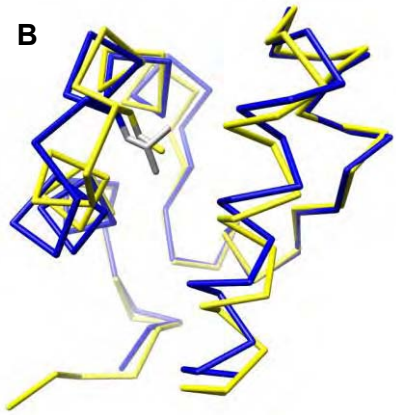
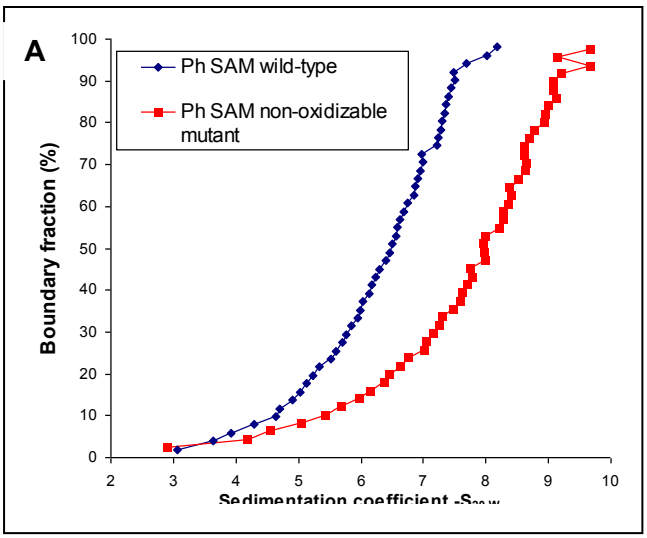
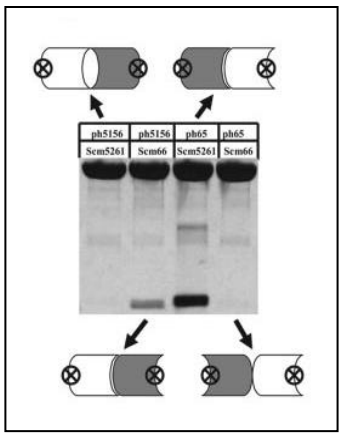
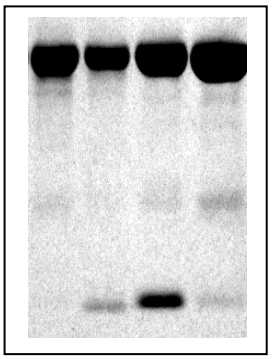


Fig. 3. C1528 mutated to Leu is the most stable non-oxidizable mutant. A. Table of melting temperatures (T_m) measured using CD spectroscopy. The values were obtained from monitoring the decrease of the 222 nm alpha helix signal with increase in temperature. **B**. Overlay of the Scm SAM with the reduced Ph SAM showing the Leu residue in Scm in the equivalent position of C1528 in Ph SAM.





B



C

Fig. 4. Ph SAM C1528L behaves similarly to WT Ph SAM. A. van-Holde Weischet combined distribution plot of WT Ph SAM (blue) and Ph SAM C1528L (red). Both show significant concentration dependent self-association (increasing S-value with increasing boundary fraction) consistent with polymerization. The smaller average S-value for the WT likely stems from its high propensity to form the disulfide bond which would hinder polymerization. **B**. A figure from the publication describing the interaction between Ph and Scm SAM domains[3]. The illustrations indicate the various polymer deficient mutants used in the GST pull-down assay and the binding mode tested in each lane of the gel. The gray shaded figures are representative of Scm SAM domains while the white figures are the Ph SAM domains. Circled X is representative of mutations on either of the two binding surfaces on the SAM domains which mediate the head-to-tail polymerization. The higher MW bands in the gel are the GST fused SAM domains while the lower MW bands are the individual SAM domains. Presence of the individual SAM domain bands is indicative of binding. **C**. The same as in **B** but using Ph SAM C1528L mutant SAM domains.

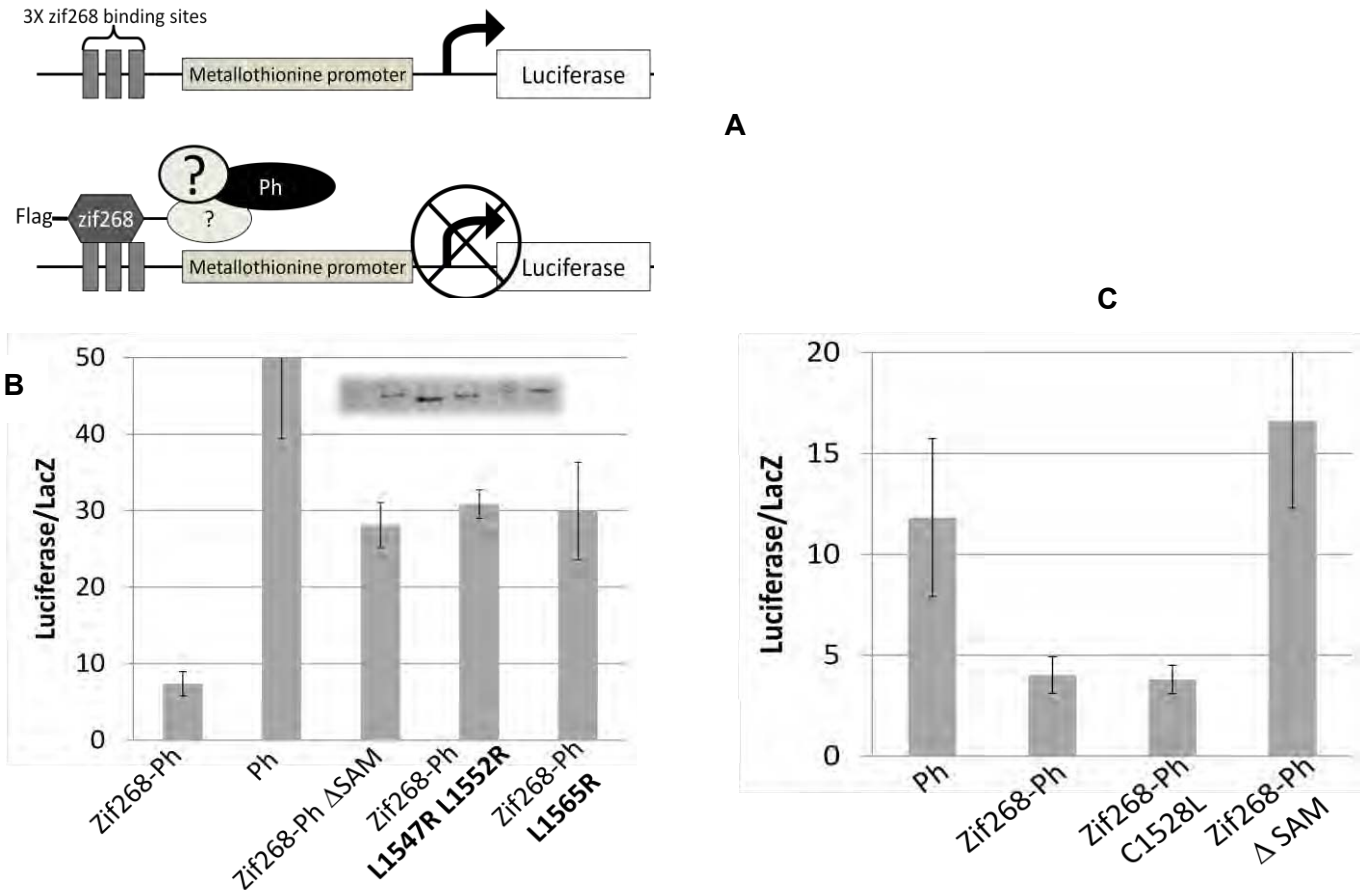


Fig. 5. Ph mediated transcription repression. A. *Luciferase* transcription reporter assay used in *Drosophila* S2 cells. **B**. Results of the assay testing Ph SAM polymerization deficient mutants. Transcription of *luciferase* from the metallothioine promoter (MTp) is measured and normalized for transfection efficiency with *LacZ* activity. Ph L1547R/H1552R disrupts one of the two binding surfaces required for polymerization while L1565R alters the other. Inset: Immunoblot of the Flag tagged Ph constructs used in the assay. Lane 1 is S2 cells alone and thus no signal is present. The remaining lanes correspond to the order of the proteins on the X-axis. **C**. Results of the assay showing Ph C1528L represses equal to that of WT. Due to the lower amounts of DNA used in this particular experiment, we were unable to detect a signal for the proteins in the immunoblot.

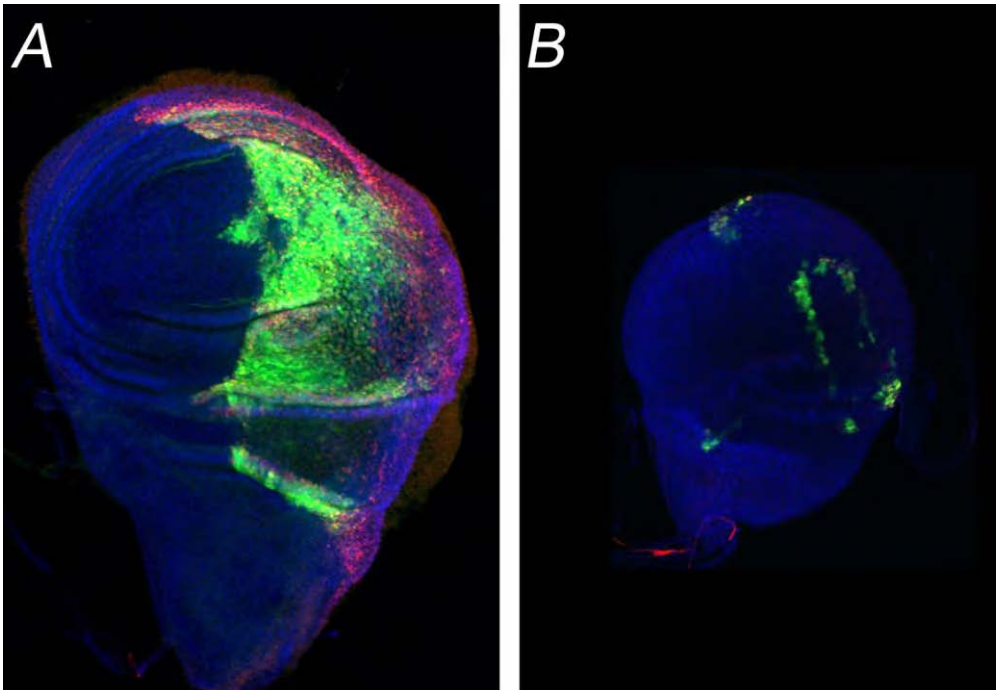


Fig. 6. Expression of Ph C1528L shows abnormal wing disc phenotype. Flag-tagged versions of WT Ph (**A**; red) and Ph C1528L (**B**; red) were expressed in the posterior compartment of the developing *Drosophila* wing disc using the engrailed Gal4 UAS-GFP driver line (green). Larvae were allowed to develop to the wandering 3rd instar stage then processed for confocal immunofluorescent microscopy. **A** and **B** are presented at identical magnification settings. The decrease in disc size for Ph C1528L (**B**) may reflect a lack of proliferation, premature cell differentiation, or an increase in programmed cell death. Further studies will be required to distinguish between these possibilities.