Published in final edited form as:

Biochemistry. 2012 July 10; 51(27): 5379–5386. doi:10.1021/bi3004318.

PHC3 SAM linker allows open-ended polymerization of PHC3 SAM

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Abstract

Sterile alpha motifs (SAMs) are frequently found in eukaryotic genomes. An intriguing property of many SAMs is their ability to self-associate, forming an open-ended polymer structure whose formation has been shown to be essential for the function of the protein. What remains largely unresolved is how polymerization is controlled. Previously, we had determined that the stretch of unstructured residues N-terminal to the SAM of a *Drosophila* protein called Polyhomeotic (Ph), a member of the Polycomb Group (PcG) of gene silencers, plays a key role in controlling Ph SAM polymerization. Ph SAM with its native linker created shorter polymers compared to Ph SAM attached to either a random linker or no linker. Here, we show that the SAM linker for the human Ph ortholog, Polyhomeotic homolog 3 (PHC3), also controls PHC3 SAM polymerization but does so in the opposite fashion. PHC3 SAM with its native linker allows longer polymers to form compared to when attached to a random linker. Attaching the PHC3 SAM linker to Ph SAM also resulted in extending Ph SAM polymerization. Moreover, in the context of full-length Ph protein, replacing the SAM linker with PHC3 SAM linker, intended to create longer polymers, resulted in greater repressive ability for the chimera compared to wild-type Ph. These findings show that polymeric SAM linkers evolved to modulate a wide dynamic range of SAM polymerization abilities and suggest that rationally manipulating the function of SAM containing proteins through controlling their SAM polymerization may be possible.

The sterile alpha motif (SAM) is a ~ 70 residue, alpha helical, modular domain that is highly prevalent in many different eukaryotic proteins ranging from kinases, transcription factors and even membrane associated proteins^{1, 2}. Many SAMs have the unusual ability to polymerize. They can self-associate utilizing two binding surfaces on each SAM. Polymerization is mediated through the binding of one of these surfaces, e.g., the midloop (ML) surface, to the end-helix (EH) binding surface of a different SAM thereby creating a polymer organized in a head-to-tail manner (Fig. 1A). Remarkably, even despite little sequence identity between some of the SAMs that polymerize, all polymeric SAM structures determined thus far have in common a left-handed helical polymer architecture^{3, 4, 5, 6, 7, 8, 9} (Fig. 1B). Nearly 700 SAMs are predicted to polymerize in this fashion¹⁰. This shared architecture found amongst a diverse collection of proteins suggests a common functional trait for SAM polymers.

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In each case studied thus far, SAM polymerization has been shown to be closely correlated to the function of proteins, often involving dynamic cellular processes. For example, for SAM containing gene silencing proteins, the ability to repress transcription is dependent on SAM polymerization while derepression of target genes occurs with SAM depolymerization^{6, 11, 12}. Additionally, SAM polymerization has been shown to be required for endocytosis of a clathrin adaptor protein⁹ as well as the localization of a kinase⁸. Given the close relationship between function and polymerization, it may be possible to modulate the function of a SAM protein by controlling the dynamic polymerization of the SAM. Knowledge of how SAM polymerization is regulated would be required to accomplish this, but this remains unresolved.

We had shown previously that the stretch of unstructured residues adjacent to a SAM can influence the degree to which the SAM polymerizes¹². Polyhomeotic (Ph) is a *Drosophila* protein and member of the Polycomb Group (PcG), a family of chromatin associated gene silencing proteins that epigenetically regulate the gene expression program. Ph contains three structured domains, the HD1 (homology domain 1), FCS (Phe-Cys-Ser), and the C-terminal SAM that are conserved in all three human Ph orthologs (Fig. 1C). Even though Ph SAM can polymerize⁴, microscopy studies of the multi-protein complex that Ph is a member of called Polycomb Repression Complex 1 (PRC1), showed no evidence of long filaments that would be consistent with polymerization^{13, 14}. A possible explanation for these differences came from studying the regions surrounding Ph SAM. The region of Ph encompassing the linker residues between the FCS and SAM and including the SAM, forms much smaller polymers than just the isolated Ph SAM¹². Studies with an unrelated, random linker (RLink) composed of residues frequently found in intrinsically disordered proteins showed that it too could hinder open-ended polymerization, though not as well as the Ph SAM native linker. As with other SAM polymer proteins, Ph function was shown to be correlated to Ph SAM polymerization as overexpression of Ph with RLink, expected to form longer polymers than wild-type, exhibited greater growth suppressive ability than wild-type.

We sought to determine if other polymeric SAM proteins are similarly influenced by their SAM linkers. Ph has three human orthologs: human Polyhomeotic homolog 1, 2 and 3 (PHC1, PHC2 and PHC3 also referred to as hPh1, 2 and 3). Like Ph, PHC1 and 2 control expression of HOX genes¹⁵ and PHC1 has been shown to be important for regulating stem cells^{16, 17}. PHC3 plays a potential tumor suppressor role in osteosarcomas^{18, 19}. While it remains unclear how the three orthologs are distinct from each other, each appears to function in a non-overlapping manner as suggested by their presence in different multi-protein assemblies^{20, 21}. In this study, we have identified a different functional role between PHC3 and Ph. In our investigation of PHC3, we found that its SAM linker also affects PHC3 SAM polymerization but does so in the opposite fashion as Ph SAM linker: PHC3 SAM linker does not hinder open-ended SAM polymers like Ph SAM linker or RLink but rather allows long polymers to form. Our results show that SAM linkers can have a wide-ranging effect on the length of SAM polymers.

Experimental Procedures (Materials and Methods)

Bacterial two hybrid

PHC3 SAM (residues 914 – 983) were cloned as either C-terminal fusion to lambda-CI in the pBT plasmid or as a C-terminal fusion to RNA polymerase α in the pTRG plasmid. Mutations were introduced using the QuikChange Site Directed Mutagenesis Kit (Agilent Technologies). Chemical competent BacterioMatch reporter cells (Stratagene) were transformed with miniprep DNA and plated out onto LB agar plates with 100 μ g/ml ampicillin, 50 μ g/ml tetracycline and 25 μ g/ml chloramphenicol. Images of the agar plates were taken after a 16 hour incubation at 37°C.

Protein preparations

All proteins prepared for in vitro studies are summarized in Table 1. MBP fused genes were sub-cloned into pBADM-41+ (EMBL), transformed into ARI814 cells²², and expression induced with 0.2% arabinose. All other constructs were cloned into a modified pET-3c vector (Novagen) and induced using 1 mM IPTG. Typically, bacterial cells from 1 L cultures were resuspended with 10 ml of 50 mM tris pH 8.0, 100 mM NaCl, 25 mM imidazole pH 7.5, 1 mM PMSF, and 10 mM β ME, lysed by sonication and purified using Ni affinity chromatography. Further purification was performed using ion exchange chromatography. When required, TEV protease digestion was carried out to remove the leader sequence with a subsequent second Ni affinity chromatography purification. All protein samples were dialyzed into appropriate buffers for either the analytical ultracentrifugation (AUC) or NMR experiments.

Analytical ultracentrifugation

All protein samples were prepared in 10 mM tris pH 8.0, 50 mM NaCl, 1 mM TCEP. Velocity sedimentation experiments were conducted at the *Center for Analytical Ultracentrifugation of Macromolecular Assemblies* (CAUMA) at the University of Texas Health Science Center at San Antonio using a Beckman Optima XL-I analytical ultracentrifuge and resulting data analyzed with the Ultrascan program²³ (version 9.9 release 1282, <http://www.ultrascan.uthscsa.edu>). The experimental designs and the analyses were performed as previously described¹². All sample concentrations and rotor speeds are indicated in the appropriate figures and legends.

NMR

The isotopically labeled ¹⁵N PHC3 SAM linker (PHC3 817–909) was prepared to a final concentration of 1.2 mM in 20 mM Na phosphate buffer pH 6.0, 50 mM NaCl. The unlabeled PHC3 SAM EH mutant L971R (PHC3 914–983 L971E) was prepared in the same buffer to a concentration of 2.1 mM. The appropriate amounts of PHC3 914–983 L971 was added to the labeled linker solution matching 1 and 2 molar equivalents of the labeled PHC3 817–909. A {¹H}-¹⁵N HSQC spectrum was measured after each addition on a Bruker 700 MHz spectrometer at 302.5 K.

Transcription assay

The luciferase reporter transcription assay was carried out as previously described¹² using 50 ng of the fulllength *polyhomeotic-proximal* (*ph-p*, also called *ph*) gene cloned in pPacFlag (kind gift from Dr. Albert J. Courey) expression plasmid and 3.75 ng of the *lacZ* gene expression plasmid (a kind gift from Dr. Yuzuru Shiio) to normalize the signals for transfection efficiency differences. The Dual-Light Combine Reporter Gene Assay System (Applied Biosystems) was used to measure both luciferase and β -galactosidase activities. The data are presented as the ratio of the two enzyme activities.

Results

PHC3 SAM forms a polymer

While the high sequence identity shared by the SAMs of Ph and PHC3 (64% over 70 residues, Fig. 1E) suggests that PHC3 SAM also polymerizes, this had not yet been shown. We first wished to establish that PHC3 SAM indeed does polymerize. For SAMs that polymerize, mutations at either the ML or the EH surface can disrupt polymerization. However, a SAM with a mutation on one binding surface is still be able to bind a SAM with a mutation on the opposite binding surface because complementary native binding surfaces are still present on each of the different surface mutants (Fig. 1A). We tested whether this was the case for PHC3 SAM using a bacterial two hybrid binding assay²⁴ where binding between two proteins is indicated by expression of the β -lactamase reporter gene conferring ampicillin resistance. The PHC3 SAM (PHC3 914–983) ML surface double mutant L953R/H958R (see Fig. 1D and E for structures of the Ph SAM-Ph SAM interface and the sequence alignment of all the proteins used in this study) showed no interaction with itself but many bacterial colonies were observed when co-expressed with the EH PHC3 SAM mutants L967R or L971R (Fig. 2A). Similarly, the lack of colonies on the PHC3 SAM L967R and PHC3 SAM L971R co-transformed bacteria indicate no interaction between the two EH mutants. This result would be expected if PHC3 SAM forms a head-to-tail polymer utilizing the ML and EH binding surfaces as with Ph SAM. We further obtained evidence that PHC3 SAM is a polymer through expression of PHC3 SAM in bacteria. When expressed in bacteria, wildtype PHC3 SAM was found almost entirely in the insoluble lysate. While typical of many heterologously expressed proteins in bacteria, we have found that for many SAMs, polymerization is the cause of the insolubility. In such cases, mutating a SAM-SAM binding surface can induce soluble expression. Expression of the EH mutant where Leu 971, predicted to be at the center of the SAM-SAM interaction (Fig. 1D), is mutated to a charged Glu residue completely reverses the expression pattern where nearly all of PHC3 SAM L971E is now expressed in the soluble fraction. Together with the bacterial two hybrid data, we conclude that PHC3 SAM is a polymer utilizing the same ML and EH binding surfaces to mediate the SAM-SAM interactions required for polymerization as Ph SAM. Additional experiments showing PHC3 SAM polymerization were obtained using sedimentation velocity analytical ultracentrifugation. These results are described in detail below corresponding to the results shown in Fig. 4C.

PHC3 SAM linker allows formation of longer polymers

Motivated by the discovery of the functional role of the Ph SAM linker of hindering polymerization, we examined whether PHC3 SAM with its N-terminal linker (PHC3 817–983) would also result in shorter polymers. Bacterially expressed PHC3 817–983 is insoluble, a result opposite to what would be expected if the PHC3 SAM linker hinders polymerization. Interestingly, however, attaching either the Ph SAM linker or RLink to PHC3 SAM produced soluble protein whose polymerization we quantitated using sedimentation velocity (SV) analytical ultracentrifugation (Fig. 3A). The Ph SAM linker and RLink affects PHC3 SAM polymerization in the same manner they influence Ph SAM. The van-Holde Weischet²⁵ sedimentation profile of Ph SAM linker attached to PHC3 SAM (Ph 1397–1484 PHC3 901–983) exhibited an *s*-value of 3 throughout the boundary fraction indicating shorter polymers compared to PHC3 SAM attached to RLink (RLink PHC3 901–983). Interestingly, the 3 *s* value for Ph 1397–1484 PHC3 901–983 chimera is identical to that of Ph 1397–1484 attached to its own Ph SAM¹². The effect of PHC3 SAM linker on Ph SAM polymerization could not be assessed because this chimera could not be obtained in a soluble form. We were, however, able to obtain soluble chimeric proteins by attaching maltose binding protein (MBP) to the N-terminus of the chimeric proteins. The sedimentation profiles of the MBP fused chimeras show the identical results: PHC3 SAM linker (PHC3 817–890) attached to either Ph or PHC3 SAM results in a larger distribution of *s*-values indicating larger aggregates (Fig. 3B). Together, these results show that PHC3 SAM linker induces aggregation when attached to either Ph or PHC3 SAM. While possible that the PHC3 SAM linker promotes non-specific aggregation rendering any protein attached to it to be aggregated, for the reasons stated below, this appears not to be the case. Rather, the large *s* value distributions of SAMs attached the PHC3 SAM linker appears to be the result of forming longer polymers.

PHC3 SAM linker is unfolded and does not induce aggregation

To investigate whether the PHC3 SAM linker alone promotes non-specific aggregation, we expressed just the PHC3 SAM linker (PHC3 817–909) in bacteria. Like the isolated Ph SAM linker¹², the PHC3 817–909 expresses highly and nearly all in the soluble fraction (Fig. 4A). We prepared isotopically labeled ¹⁵N PHC3 817–909 to high concentrations for NMR studies. The {¹H}-¹⁵N HSQC spectrum (Fig. 4B, left) shows the backbone N-H signals all clustered in the range expected for a random coil (7.9 – 8.7 ¹H PPM) indicating that PHC3 817–909 is not structured. Furthermore, PHC3 817–909 does not aggregate as indicated by the lack of broadening and disperse nature of the backbone N-H signals.

Even though the isolated PHC3 SAM linker (PHC3 817–890) does not aggregate, it remains possible that when attached to the SAM, non-specific aggregation still occurs. If indeed the aggregation observed for either Ph or PHC3 SAM attached to PHC3 817–890 is due to polymerization and not to the inherent aggregating inducing property of PHC3 817–890, then mutations at the SAM-SAM interface in the context of PHC3 817–983 should produce smaller polymers compared to wild-type. If instead PHC3 817–890 induces non-specific aggregation, then SAM-SAM interface mutations would likely not effect aggregation. To test this, we introduced putative PHC3 SAM-SAM interface mutations in the context of the MBP fused PHC3 817–983. PHC3 L953R is equivalent to Ph SAM L1547R in the sequence

alignment (Fig. 1E). In Ph SAM, L1547R is at the edge of the SAM-SAM interface (Fig. 1D) and results in hindering polymerization but does not completely abolish it^{4, 12}. PHC3 L971 is equivalent to Ph L1565 (Fig. 1E) and is predicted to be at the center of the SAM-SAM interface (Fig. 1D). Ph SAM L1565R produces even shorter polymers than the L1547R mutation. The same results are observed in the context of MBP PHC3 817–983. The van-Holde-Weischet (vHW) analysis of the SV AUC results of MBP PHC3 817–983 L953R shows a smaller distribution of sedimentation coefficients than that of the wild-type and greater than that of the L971E mutant (Fig. 4C). Together, this data supports the idea that the large s-value distributions observed for the SAMs attached to PHC3 SAM linker is not due to the inherent aggregating properties of PHC3 SAM linker but rather due to polymerization. The data also further supports the findings presented in Fig. 2 showing that PHC3 SAM polymerizes utilizing the same binding surfaces as Ph SAM.

Amino acid content is important for controlling polymerization

In our previous study¹², Ph SAM was shown to be able to bind its native linker in trans raising the possibility that intramolecular interactions between the linker and SAM could play a role in controlling SAM polymerization. Using NMR, we tested whether PHC3 SAM linker could also contact PHC3 SAM in trans. The isotopically labeled ¹⁵N PHC3 817–909 shows no perturbation of its backbone N-H resonances with the in trans addition of a soluble polymer deficient PHC3 SAM mutant (PHC3 914–983 L971E) (Fig. 4B, right). The complementary experiment where non-labeled PHC3 817–909 is added to ¹⁵N labeled PHC3 914–983 could not be performed because of the poor quality of the HSQC spectrum of the PHC3 SAM.

Amino acid content was also shown previously to be important for the polymer influencing function of the Ph SAM linker as a scrambled Ph SAM linker resulted in forming the same length Ph SAM polymers as the native linker. We prepared several chimeric SAM proteins where the amino acids of the linkers were arranged in a scrambled manner and attached to either Ph or PHC3 SAMs. MBP was attached at the N-terminus of these proteins in order to obtain soluble proteins and to measure the level of polymerization using SV. The SAMs attached to the scrambled linkers show similar sedimentation profiles compared to the native, non-scrambled linkers (Fig. 5). When the scrambled PHC3 SAM linker sequence (PHC3 sc817–900) was attached to either PHC3 SAM or Ph SAM, the s-value distributions were above 15 s indicating formation of long polymers. This is similar to the long polymers formed by the non-scrambled PHC3 SAM linker (Fig. 3B). In contrast, the scrambled Ph SAM linker (Ph sc1398–1484), which hinders open-ended polymerization of Ph SAM¹², also produced shorter polymers when attached to PHC3 SAM. This data along with the lack of binding between the PHC3 SAM linker and PHC3 SAM suggests a mechanism by which the PHC3 SAM linker influences PHC3 SAM polymerization is due to the inherent properties of the linker determined by its amino acid composition and not through direct contacts between the linker and SAM.

Ph with PHC3 SAM linker has enhanced repressive ability

Like all polymeric SAM proteins, the function of Ph is dependent on the ability of its SAM domain to polymerize. Increasing Ph SAM polymerization, then, might enhance the

repressive ability of Ph. Previous attempts at detecting repressive ability presented a significant challenge because wild-type Ph appeared to be at the detectable limit for a repressor in the context of our transcription assay (Fig. 6A) and thus even a better polymer forming Ph derivative may not show better repression of the reporter gene. We reasoned that if the repressive ability of wild-type Ph could be weakened through disruption of the SAM-SAM interaction, that the greater polymerization afforded by the linker could then be detected through rescue of the repressive ability. We attempted this strategy using the Ph SAM mutation L1547R. L1547R is at the edge of the Ph SAM-Ph SAM interface (Fig. 1D). While the mutation does hinder polymerization, it still retains some ability to self-associate^{4, 12}. Ph L1547R targeted to the promoter controlling expression of the *luciferase* reporter gene does show reduced ability to repress transcription compared to wild-type Ph. In the L1547R context, we altered the SAM linker in order to see if the greater polymerization, as expected for both RLink and PHC3 SAM linkers (Fig. 3) would then exhibit enhanced repressive ability through greater polymerization (Fig. 6B, C). The results of the transcription assay correlate precisely with the expected level of polymerization. Ph L1547R with RLink replacing the native SAM linker (Ph L1547R (1398–1484) RLink) shows a slight rescue of the repressive ability compared to Ph L1547R and Ph with the PHC3 SAM linker (Ph L1547R (1398–1484) PHC3 817–900) shows the greatest repressive ability. These results suggest that it is indeed possible to enhance the repressive ability of Ph through its increased polymerization of its SAM domain.

Discussion

The main findings of this study are the following. 1. The SAM of PHC3, the human ortholog of *Drosophila* Ph, can self-associate into a polymer. 2. Like the unstructured linker sequence N-terminal to Ph SAM, the PHC3 SAM linker is also unstructured and also influences PHC3 SAM polymerization. 3. However, unlike the Ph SAM linker which hinders open-ended polymerization, PHC3 SAM linker allows longer PHC3 SAM polymers to form. 4. The amino acid composition of the linker is important for controlling polymerization. 5. The expected greater polymerization ability from replacing the Ph SAM linker with that of the PHC3 SAM linker results in an enhanced transcription repressive ability.

What remains unresolved is the mechanism by which the unstructured linkers influence SAM polymerization. The lack of a uniform structure or requirement of a specific sequence of the SAM linker implies that polymerization is controlled in a passive manner. That is, polymerization is driven solely by the SAM-SAM binding affinity and the PHC3 SAM linker simply stays out of the way. Similarly for the Ph SAM linker, the linker conformations may be biased toward being in regions that are near either the ML or EH binding surfaces. If so, then what factors bias the distribution of the unstructured linker conformations? One possibility is suggested by the electrostatic properties of the two different linkers that have opposite effects on Ph SAM polymerization. Ph SAM and PHC3 SAM linkers have large differences in charge (Ph 1398–1484 pI = 4.00; PHC3 817–890 pI = 10.75). Interestingly, RLink, which allows intermediate length polymers to form has a charge near neutral (pI = 7.96). It could be possible that a specific charged patch on the surface of the SAM polymer influences how the linker conformations are distributed which in turn alters polymer formation ability.

How chromatin structure is affected by the longer PHC3 SAM polymers is not known. While the Ph SAM linker and its polymer hindering role likely contributes to the *Drosophila* PRC1 not forming long filaments^{13, 14}, the polymer forming ability of human PRC1 equivalents has yet to be determined. If human multi-protein complexes that contain PHC3 can form long polymers, it may represent a way to spread PcG mediated repression along chromatin. If, however, human PHC3 complexes are also limited in polymerization, regions of PHC3 protein other than the SAM linker, or even other proteins, could play a role in hindering open-ended SAM polymerization. A human PcG protein performing the same function as a different *Drosophila* PcG protein is not unprecedented. In *Drosophila*, PRC1 member Psc and its paralog Su(Z)2 function to compact chromatin into a structure inaccessible to chromatin remodeling enzymes^{13, 26}. The region of Psc that performs this function is predicted to be unstructured and is not conserved amongst its mammalian orthologs. However, a similar functioning intrinsically disordered region was found to exist within an altogether different PcG protein²⁷.

We present data showing two different SAM linkers having very different effects on SAM polymerization. The most novel and unexpected finding of our study is that despite two different SAM linkers being unstructured, which could easily imply that the linkers influence all SAM polymers in the same manner; they differentially alter SAM-SAM affinity leading to profoundly different effects on protein function. While additional studies will be required to determine the mechanism by which SAM linkers control polymerization and to see if the observed changes in polymerization is transferrable to full-length proteins, our current findings suggest that, because SAM polymerization is linked to the function of the protein, SAM linkers are capable of inducing a wide activity range onto the protein.

Acknowledgments

The authors gratefully acknowledge the support of the Cancer Therapy and Research Center at the University of Texas Health Science Center San Antonio, an NCI-designated Cancer Center.

This work was supported by the American Heart Association (0830111N), the American Cancer Society (RSG-08-285-01-GMC) and the Department of Defense Breast Cancer Research Program (BC075278) (CAK). Support for the NMR facility was provided by UTHSCSA and NIH-NCI P30 CA54174 (CTRC at UTHSCSA).

Abbreviations

Ph	Polyhomeotic
PHC3	human Polyhomeotic homolog 3
PcG	Polycomb Group
SAM	sterile alpha motif
PRC1	Polycomb Repression Complex 1
ML	mid-loop binding surface
EH	end-helix binding surface
RLink	random linker

SV	sedimentation velocity
AUC	analytical ultracentrifugation
NMR	nuclear magnetic resonance
HSQC	heteronuclear single quantum correlation
vHW	van Holde-Weischet
MBP	maltose binding protein

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Figure 1.

A. SAM polymer illustration. **B.** Surface representation of the Ph SAM polymer⁴ (PDB ID: 1kw4). Alternating SAM polymer units are shaded differently. The N-terminal residue of the SAM is highlighted in black to indicate where the linker would stem from. **C.** The domain structure of Ph and PHC3. The numbered residues indicate the boundary positions of some of the constructs used in this study. **D.** A close up of the Ph SAM-Ph SAM interface of 1B. The equivalent residues of PHC3 are in parentheses. **E.** Sequence alignment of all the proteins used in this study. All numbering referring to the *Drosophila* Ph are italicized. The

beginning of the SAM domain is marked. The highlighted PHC3 SAM-SAM interacting residues Leu 953, His 958, Leu 967 and Leu 971 correspond to the *Drosophila* Ph residues Leu 1547, His 1552, Leu 1561 and Leu 1565, respectively.

Figure 2. PHC3 SAM is a polymer. **A.** Results of the bacterial two hybrid assay. **B.** SDS PAGE showing the insoluble (I) and soluble (S) lysates of the indicated PHC3 SAM protein.

Figure 3. PHC3 SAM linker (PHC3 817–890) induces formation of large SAM polymers. **A, B.** Sedimentation velocity van-Holde Weisheit (vHW) combined distribution plot of the proteins indicated. Rotor speeds were 40 and 30 kRPMs for **A** and **B**, respectively.

Figure 4.

PHC3 SAM linker is soluble and does not aggregate. **A.** SDS PAGE showing the insoluble (I) and soluble (S) lysates of PHC3 SAM linker expressed in bacteria. **B.** Left: $\{^1\text{H}\}$ - ^{15}N HSQC of PHC3 SAM linker alone and with 2 molar equivalents of PHC3 SAM L971E added in trans (right). **C.** vHW combined distribution plot of MBP PHC3 817–983 with mutations at the SAM-SAM interface. The wild-type sedimentation profile from Fig. 3B is re-plotted here for better direct comparison.

Figure 5. Scrambled linkers similarly affect SAM polymerization as the native linkers. SV van Holde-Weischet combined distribution plot of MBP fused scrambled (sc) linker SAM chimeras. All proteins were prepared to 10 μ M. The rotor speed was 30 kRPMs.

Figure 6.

Ph with PHC3 SAM linker shows enhanced transcription repressive ability. **A.** Illustration of the luciferase reporter transcription assay carried out in *Drosophila* S2 cells. **B.** Ph constructs used in the assay. **C.** Results of the transcription assay. The error bars indicate the standard deviation of the results from three independent experiments performed simultaneously.

Table 1
Protein constructs used for in vitro experiments

All PHC3 sequences correspond to NCBI accession number NM_024947. Ph sequences correspond to the gene product of *Drosophila* proximal *ph* (*ph-p*). AUC = analytical ultracentrifugation, NMR = nuclear magnetic resonance.

Experiment	Figures	N-terminal leader sequence	protein	C-ter sequence
Bacterial expression	1B	MEKTR	PHC3 914–983	RHHHHHH
Bacterial expression,	1B, 4B	MEKTR	PHC3 914–983, L971R	RHHHHHH
NMR	3A	MHHHHHHGVDSPSAELDKKAENLYFQ*GTR	Ph 1397–1484 PHC3 901–983	RH
AUC	3A	MHHHHHHGVDSPSAELDKKAENLYFQ*GTR	RLink PHC3 901–983	RH
AUC	3B	MBP–SSS	PHC3 817–983	RH
AUC	3B	MBP–SSS	Ph 1397–1484 PHC3 901–983	RH
AUC	3B	MBP–SSS	RLink PHC3 901–983	RH
AUC	3B	MBP–SSS	PHC3 817–890 Ph 1485–1577	RRH
AUC	4B	MHHHHHHGVDSPSAELDKKAENLYFQ*GTR	PHC3 817–909	
NMR	5	MBP–SSS	PHC3 sc817–900 901–983	RH
AUC	5	MBP–SSS	PHC3 sc817–900 Ph 1485–1577	RRH
AUC	5	MBP–SSS	Ph sc1398–1484 PHC3 901–983	RH

* = TEV cleavage site. For MBP fusion constructs, the 5' end of the genes were subcloned into the SacI site of pBADM-41+ (EMBL) and corresponds to the SSS residues in the sequence.

The “sc” designation of proteins refers to the indicated residues arranged in a scrambled manner. See Figure 1E for the RLink sequence.