Yan polymerization contributes to active repression and thus contributes to precise gene expression

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Yan polymerization contributes to active repression and thus contributes to precise gene expression.

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Abstract:

Downstream of the receptor tyrosine kinase (RTK) pathway in *Drosophila*, a conserved network mediates the robust regulation of genes involved in developmentally important processes such as cell proliferation and differentiation. This regulation is accomplished by a bistable switch, termed the Yan-Pointed (Pnt) switch, in which Yan functions as a transcriptional repressor while Pnt activates the transcription of target genes. Both the mechanism of Yan's repression as well as any importance of its ability to self-associate, via an N-terminal protein-protein interaction sterile alpha motif (SAM) domain, remain unknown. As Yan exists as an oligomer *in vivo* and *in vitro* it is hypothesized that higher order structures, resulting from SAM mediated polymerization, form and favor stable the formation of active complexes contributing to active repression. In the present study and to test this hypothesis, a genetic approach was taken in tandem with an *in vivo* expression analysis. Polymeric, dimeric, and monomeric constructs of Yan were examined in lethality assays and in target gene expression analyses. Both the dimeric and monomeric constructs revealed decreased rescue ability as well as increased expression of target genes in comparison to the polymer. These studies strongly suggest a higher order structure beyond a dimer is required for robust transcriptional repression by Yan and thereby provide a direct relationship between the SAM domain and the

mechanism of the Yan repression.

Introduction:

Proper development and survival requires that gene expression be robust in the face of biological noise arising from stochasticity and fluctuating environmental conditions. An inability to maintain this precision ultimately leads to developmental defects and potentially a failure to develop. As gene expression is the sum of transcription, translation and protein stability, it is likely that mechanisms will exist to favor such robustness at each of these steps.

In *Drosophila melanogaster,* a specific network of transcription factors act to regulate development downstream of the receptor tyrosine kinase (RTK) pathway (Zhang et al 2010). This regulation is accomplished by the coordinated interplay between the transcription factors Yan and Pointed, which form a bistabe Yan-Pnt switch, where Pointed activates and Yan represses the transcription of target genes (Webber et al 2013). The network displays switch-like behavior, transitioning from an inactive “Yan-state” to an active “Pnt-state” following pathway activation. Thus in unstimulated cells, Yan is bound to DNA where it acts to repress target gene expression holding the cell in an inactive precursor state. Following activation of the RTK pathway, Yan is removed from DNA and ultimately degraded allowing Pnt to bind and activate genes previously repressed by Yan.

Regulation by Yan is essential for proper development in *Drosophila*, as loss-of- function mutants are embryonic lethal. Further, recent work has shown

that animals heterozygous for *yan* display defects in heart development and an increased expression of the transcriptional target even-skipped, a gene essential for proper heart development (Webber and Rebay 2013).

The ability of Yan to self-associate through its SAM domain has led to speculation that polymerization of Yan would allow higher order structures to spread along DNA which would perhaps impart robustness and sensitivity to upstream signaling (Qiao et al.,Kim et al. Tran et al). Missense mutations within the SAM domain that block polymerization in vitro and in Drosophila culture cells have been used to show that Yan monomers while retaining DNA binding ability are not able to repress transcription. (Webber and Rebay 2013).

In order to test whether these higher order structures form and contribute to active repression in the fly, I made use of two missense mutations in the SAM domain which I will refer to as midloop (ML) and end-helix (EH). Expression of a single mutant will restrict the polymer to its monomeric form. Alternatively co-expression of both the ML and EH mutants enables the formation of a dimer but will prevent the formation of a higher order structure. These constructs and their capacity to regulate development were studied in several contexts including their ability to rescue a *yan* null allele, transcriptional repression strength, adult survival, and cardiac function. If a Yan higher order structure beyond a dimer is required for transcriptional repression in vivo then dimeric Yan would fail to fully rescue a *yan* null mutant resulting in lethality or disrupted gene expression.

Materials and Methods:

Fly strains and genetics

yan null mutant embryos complemented by Yan in the genetic background were created for the genetic rescue assays by carrying out the following crosses:

i) yan^{833}/balancer;yan^{WT} X yan^{ER443}/balancer;yan^{WT}= yan^{833}/yan^{ER443};yan^{WT} to create the full length Yan polymer,

ii) yan^{833}/balancer;yan^{A86D}Xyan^{ER443}/balancer;yan^{V105R}= yan^{833}/yan^{ER443};yan^{V105R}/yan^{A86D} to create the Yan dimer,

iii) yan^{833}/balancer;yan^{V105R}Xyan^{ER443}/balancer;yan^{V105R}= yan^{833}/yan^{ER443};yan^{V105R} to create the Yan monomer, and

iv) yan^{833}/balancer;yan^{A86D} X yan^{ER443}/balancer;yan^{A86D}= yan^{833}/yan^{ER443};yan^{A86D} to create the Yan monomer all of which are complementary to the *yan* null mutation.

The balancer chromosome used the *CyO, twist-Gal4, UAS-GFP* system. About 300 GFP-negative embryos for each genotype were selected and the numbers of hatched embryos were counted.

Immunostaining

Embryos were collected for two hours on apple juice plates and aged to stage 11 at 25^0C. Embryos were then dechorinated in 50% bleach and fixed in a 1:1 solution of heptane and 4% formaldehyde at room temperature for 10 min with vigorous shaking. The vitellin membrane was removed by replacing the formaldehyde solution with methanol. Embryos were washed three times in methanol and then three times in PBS (Phosphate-buffered saline) after which

they were blocked in PNT (PBS, 1% normal goat serum, 0.1% Triton X-100) for 1 hour at room temperature and stained with anti-Eve MAb 3C10 (1:10) overnight at 4^0C. After the primary staining, embryos were rinsed three times for ten minutes each in PBST (PBS, 0.1% Triton X-100) and then stained overnight with the secondary antibody Cy3-conjugated goat anti mouse.

Mounting

Embryos were rinsed three times for 10 min each in PBST and then mounted in normal goat serum (NGS). The light sensitive slides were stored at -20^0C in the dark.

Quantification of Eve Expression

Embryos were genotyped according to their GFP negative phenotype. Using a Zeiss LSM 510 confocal microscope and corresponding software, 0.8 micrometer z sections were obtained in order to determine the relative intensity of Eve expression. ImageJ was used to calculate the relative mean intensity of Eve expression in mesodermal clusters of *yan*$^{ER443/833}$; Yan^{WT}, *yan*$^{ER443/833}$; Yan^{V105R}, *yan*$^{ER443/833}$; Yan^{A86D}, *yan*$^{ER443/833}$; and *yan*$^{ER443/833}$, Yan$^{V105R/A86D}$ embryos by subtracting the mean background pixel intensity from the cluster (measured using a box of defined size) and normalized by dividing the average mean pixel intensity for Eve expression in polymeric embryos. A sample size ranging from 8-10 was obtained for each genotype.

Genetic rescue assays:

Genetic rescue to larval stage

Embryos were collected overnight at 25^0C and hand selected according to their GFP negative phenotype from the following crosses: (i) *yan^{ER443}/CyO, twist-Gal4, UAS-GFP (CTG) X yan^{E833}/CTG, (ii) yan^{ER443}/CTG; Yan^{WT} X yan^{E833}/CTG;Yan^{WT}, (iii) yan^{ER443}/CTG; Yan^{A86D}X yan^{E833}/CTG;Yan^{A86D}, (iv) yan^{ER443}/CTG; Yan^{V105R}X yan^{E833}/CTG;Yan^{V105R}, (v) yan^{ER443}/CTG; Yan^{V105R}X yan^{E833}/CTG;Yan^{A86D}* using *yan* null embryos as a control. Selected embryos were then stored overnight at 25^0C. Hatched embryos were scored as having been rescued to the larval stage.

Genetic rescue to adult stage

Drosophila for each of the previously mentioned crosses were aged to the adult stage in bottles. The absolute numbers for both animals with the balancer chromosome and animals homozygous for the mutations were recorded. Homozygous flies were genotyped according to their straight winged phenotype whereas heterozygous animals, which retained the balancer chromosome, were genotyped according to the dominant curly winged phenotype of the balancer chromosome. Absolute numbers were recorded daily for 10 days to prevent the inclusion of data from the F2 generation.

Longevity of adult flies

One day old adult male flies, genotyped according to their straight winged phenotypes, were hand selected from the previously mentioned crosses using

light CO_2 anesthetization and placed into standard vials containing food with a maximum of 10 flies per vile. Flies were collected for 10 days to prevent including data from the F2 generation. The collected vials were dated and the number of deaths was recorded daily for 1 month.

Negative geotaxis assay:

One day old adult male flies, genotyped according to their straight winged phenotypes, were hand selected from the previously mentioned crosses using light CO_2 anesthetization and placed into a standard vial containing food with a maximum of 10 flies per vial. Flies were then kept at 25^0C for two hours to allow for recovery from anesthetization. After recovering, a climbing assay was conducted. In order to monitor the climbing ability of the flies a line one cm from the top of the food was drawn around the vial, vials were gently tapped knocking all of the flies to the base of the vile, after 15 seconds flies remaining below the 1 cm line were counted and recorded as having a reduced climbing ability. The assay was conducted for 10 minutes with gentle tapping every 30 seconds and recording every 30 seconds after the first 15 seconds. After these 10 minutes the flies were subjected to a heat stress at 35^0C for 30 minutes and monitored again for 10 minutes.

Flight Assay:

One L graduated cylinders were coated with mineral oil and designated with four quadrants labeled from top to bottom with 4,3, 2, or 1. Selected flies from one of the crosses were tapped into the graduated cylinder through a funnel. The ability of the *Drosophila* to fly was measured on the basis of which quadrant they

landed in. Flies which were unable to fly landed at the bottom of the cylinder and were designated with a score of zero. Flies which landed on the top quadrant were designated with a score of four, correspondingly flies which landed in the middle quadrants were designated with a two or a three respectively. This process was repeated for each of the genotypes.

Results:

Dimer displays intermediate genetic rescue ability.

Given *yan* is an essential gene and animals mutant for *yan* die prior to hatching, I first tested the ability of polymeric, dimeric, or monomeric Yan to rescue a *yan* null mutant. If polymers are the functional unit then dimer and monomer should fail to rescue. Conversely, if dimers are sufficient for Yan function, then dimers should fully rescue a yan null allele. I made use of fly lines that were null for yan and also carried either wild-type yan or recombineered versions of yan containing missense mutations in the ML and EH regions. The control wild-type Yan fully complemented the yan null allele with rescue rates of 94%. Restricting Yan to a monomeric form in contrast resulted in a reduced rescue ability with only 59% (ML mutation) or 60% (EH mutation) surviving past embryogenesis. Restricting Yan to a dimeric form had intermediate results with an increased rescue rate relative to monomeric Yan but not at the level of wild-type (Fig. 1).

Next I investigated the capacity of polymeric, dimeric, or monomeric Yan to survive to adulthood. Animals were genotyped and cultured at 25C. The number of animals that survived to adulthood were collected and counted. Only

escaper flies (straight wing phenotype with one copy of the mutation) were observed for the monomeric mutants. In contrast, a greater percentage of adult flies (homozygous for the mutation curly wing phenotyped) were observed to eclose in flies rescued with Yan dimer compared to monomeric Yan (Fig. 2).

While I was able to obtain a few escaper flies that were rescued with monomeric Yan, these flies died shortly after eclosure (Fig 3). To determine whether there was a consequence of restricting Yan to a dimer to overall fitness of the animal, I assessed lifespan of animals rescued with either polymeric or dimeric Yan (Fig. 3). I found that the survival rates of the dimer were comparable to those of the monomer, dropping below 20% survival two weeks after eclosure, while over 50% of animals rescued with the polymer were still alive at the same timepoint. Further, animals rescued with polymeric Yan continued to display significantly higher survival rates, remaining above 30% survival after 3 months, while only 10% of the monomer and dimer rescued animals were still alive by the middle of the 3rd and 4th week (Fig. 3) Together, these data suggest that a higher order structure beyond a dimer is required for full biological function.

Yan dimers are less effective repressors of *eve* transcription than Yan polymers.

In order to determine if the adverse biological consequences of restricted polymerization actually resulted from a failure to repress the transcription of target genes I looked at the expression of a known Yan target, *even-skipped (eve),* in dimeric Yan and polymeric Yan rescued animals. If full length Yan is

required for precise transcriptional repression then restricted Yan polymers will display increased expression of target gene *eve*.

Eve is expressed in 11 clusters of cells in the mesoderm that correspond to pericardial and muscle precursors. Since Yan has previously been shown to regulate eve in these cells, they provide a valid context in which to test whether a higher order structure beyond a dimer is required for proper regulation of eve.

Embryos from yan null animals rescued with either polymeric or dimeric Yan were fixed and stained with an anti-Eve antibody as previously reported (ref Webber et al., 2013 genetics paper). Eve expression in the mesodermal clusters was then quantified for each genotype. I found an increase in Eve expression in animals rescued with dimeric Yan relative to polymeric Yan rescued animals (Fig 4A, B).. In addition to an increase in overall intensity of Eve expression, dimeric Yan rescued animals also display an increased variability in Eve expression as represented by the larger error bars (Fig 4B). This increased variability and mean intensity of expression in dimeric Yan rescue animals suggest that Yan dimers are less effective repressors of *eve* transcription than polymers.

Dimeric Yan rescued animals exhibit both a reduced flight ability and a reduced ability to exert a climbing response.

Fitness assays were used as an indirect means to investigate if the specific inability of restricted polymers to repress the transcription of *eve* actually resulted in biological consequences such as impaired heart development. If an organism has impaired heart development then that organism will be less efficient at transporting oxygen through the body leading to a decreased ability to

perform activities requiring efficient oxygen transport such as climbing and flying. The inability of dimeric Yan rescued animals to perform these activities would be consistent with having impaired heart development.

I first assessed the flight ability of polymeric or dimeric Yan rescued animals. Briefly, animals were tapped into a measuring cylinder coated with mineral oil. Any animals capable of flight would land on the side of the cylinder, with height proportional to flying ability. In contrast animals incapable of flight would fall through to the bottom of the cylinder. I found that a greater percentage of the polymeric Yan rescued population were able to occupy the upper flight quadrants whereas a greater percentage of dimeric Yan rescued animals occupied the lower flight quadrants consistent with a reduced flight ability (Fig. 5).

In addition to the flight assay, a negative geotaxis assay was used to examine the ability of the animals to climb upwards in response to being startled. Animals were tapped to the bottom of a culture vial and allowed to climb back up to the top of the vial for 15 seconds. Any animals that failed to climb were scored.This was repeated every 30 seconds for 10 minutes prior to and following a heat shock. I found that dimeric Yan rescued animals displayed a decreased ability to exert a negative geotaxis response prior to heat shock. This was exacerbated following application of heat stress. (Fig. 6). Together these assays indicate that dimeric Yan rescued animals are not as fit as polymeric rescued animals supplying further evidence of a requirement of a Yan higher order structure beyond that of a polymer. Given that these assays have been

used previously as a means to assess heart development, and taken together with the increased expression of *eve* observed in dimeric Yan rescued animals, my data are consistent with a defect in heart development and/or function. Further work to assess the heart rate in developing animals and in the dimeric and polymeric Yan rescued adult flies would provide further insight.

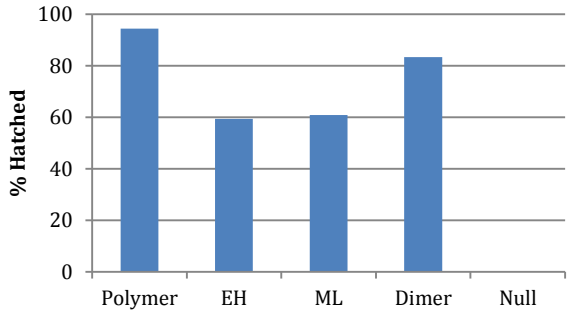

Figure 1. Dimer displays intermediate ability to rescue a *yan* null mutant to larval stages. GFP negative embyros were hand selected from the previously mentioned crosses. Hatched embryos were scored as having been rescued to the larval stage and the percentage of the population that was rescued is denoted on the y-axis. The x-axis denotes the Yan construct that rescued the animal from the *yan* null mutation.

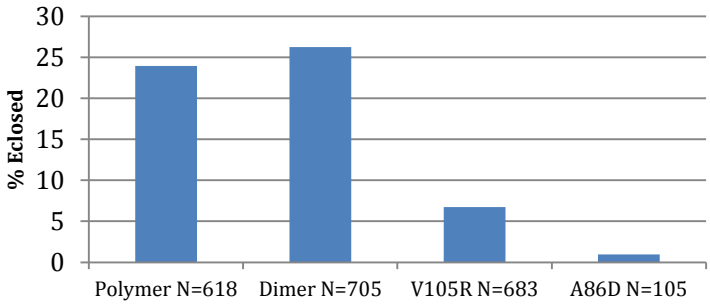

Figure 2. Dimeric Yan displays increased ability to rescue a *yan* null mutant through eclosure. Rescued flies were genotyped according to their curly winged expression. The y-axis denotes the percentage of the eclosed population that was rescued. The x-axis denotes the Yan construct that complemented the lethality of the *yan* null mutation.

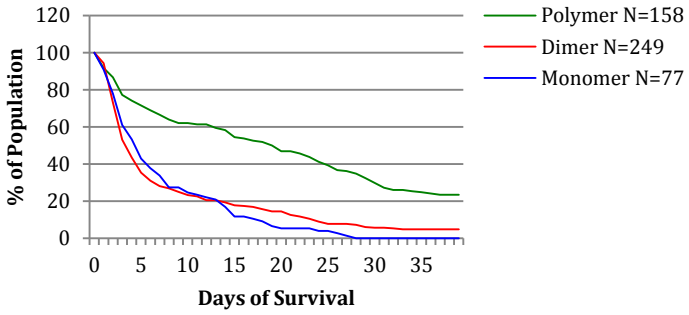

Figure 3.
Polymeric Yan rescued animals have a longer lifespan than monomeric or dimeric Yan rescued animals. The longevity of Yan rescued animals was monitored after they were aged through eclosure. Graphed here is the percentage of the rescued population remaining versus the number of days of survival.

A B

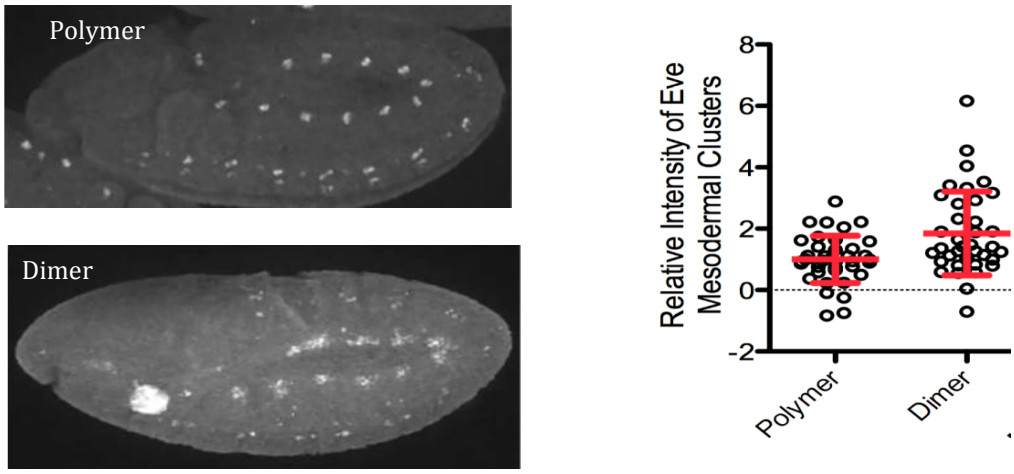

Figure 4. Yan dimers are less effective repressors of Eve transcription than polymers A, B. (A) Stage 11 drosophila embryos with 11 mesodermal clusters. (B) Relative intensity of Eve mesodermal clusters. The dimeric Yan has a higher mean intensity of Eve expression that is statistically significant.

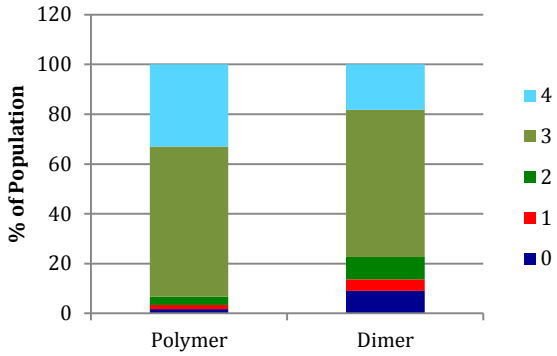

Figure 5. Dimeric Yan rescued animals exhibit a reduced flight ability.The ability of the Drosophila to fly was measured based on the flight quadrant they landed in. Graphed here is the percentage of the dimeric Yan rescued or polymeric Yan rescued population that occupied a particular flight quadrant. A greater percentage of the Dimeric Yan rescued population occupied the lower quadrant and a greater percentage of the Polymeric Yan rescued population occupied the upper quadrants.

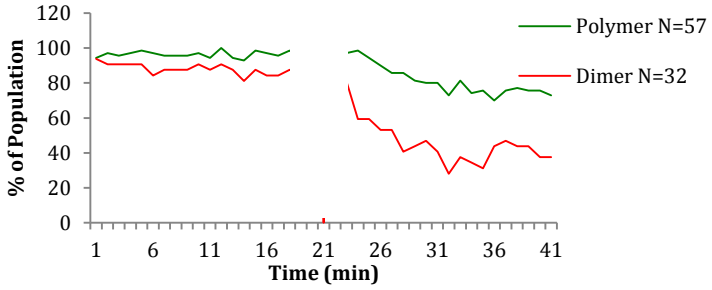

Figure 5. Dimeric Yan rescued animals display a reduced ability to exert a climbing response. The percentage of the dimeric Yan rescued population or polymeric Yan population to climb past a certain threshold is graphed here. A greater percentage of the polymeric Yan rescued population was able to climb past the threshold and the difference between the two animals after being subjected to a heat shock is even more apparent.

Conclusions/Discussion

The results from this study support the hypothesis that a higher order structure beyond a dimer, formed via SAM mediated polymerization, contributes to active repression and thus ensures precise gene regulation. In order to demonstrate the role of the higher order structure in contributing to active repression and ensuring precise gene regulation, the different constructs of Yan were studied in several contexts; including their genetic rescue ability, transcriptional repression strength, and cardiac function.

If a Yan higher order structure beyond a dimer is required for transcriptional repression then dimeric Yan would fail to fully rescue a Yan null mutant in the genetic rescue assays. The results of the genetic rescue assays are consistent with the hypothesis because the dimer displayed a decreased capacity to rescue Yan null mutants from lethality relative to the polymer. The inability of the dimer to fully rescue the Yan null mutants suggests there is a functional requirement for a higher order structure beyond a dimer to mediate proper development. The results for the embryonic genetic rescue experiement

are subtle but are further validated by the differences observed between the polymer and the dimer in the results of the survival assay.

The results for the capacity of the polymer, dimer, or monomer to support the organism through eclosure are inconsistent with the hypothesis because the dimer displays increased genetic rescue ability in comparison to both the polymer and the monomers. This discrepancy may be due to deleterious interactions with the balancer chromosome at this stage.

If my hypothesis is correct, dimeric Yan rescued animals, when compared to polymeric Yan rescued animals, would be expected to display increased expression of target gene Eve. An increase in Eve expression in dimeric Yan rescued animals would demonstrate the role of the higher order structure in precise transcriptional repression. The experimental results are also consistent with these expectations. Dimeric Yan rescued animals when compared to polymeric Yan rescued animals displayed increased variability and increased expression of target gene Eve, indicating a need for the higher order structure to confer precision in gene expression.

Finally, due to the role of Eve in regulating heart development, the cardiac functional capacities provided an indirect means to determine the biological consequences of the disrupted expression of target gene Eve. Dimeric Yan rescued animals were expected to display decreased cardiac functional capacities arising from disrupted heart development due to the decreased regulation of Eve expression. Again, the results remain consistent with initial

expectations with dimeric Yan rescued animals displaying reduced fitness levels.

The demonstrated contributions of a higher order structure beyond a dimer in activating precise transcriptional repression summons a new set of questions regarding what mechanisms the higher order structure can undertake that the dimer cannot. One consideration to take in answering this question is that transcription is not a linear process. Transcription and the dynamics of transcription depend on the three dimensional environment that surrounds the chromatin. One part of this three dimensional environment is protein concentration. In the context of the dynamics of transcriptional assembly and disassembly higher protein concentrations favor transcriptional assembly. Yan polymers, unlike Yan dimers, provide these high local Yan concentrations, and may facilitate the formation of stable active complexes. The inherent property of Yan polymers to provide higher concentrations of Yan favoring the assembly of active repression complexes is just one of the major differences between the polymer and the dimer that could account for the observed functional differences.

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