A regulatory role for the unstructured C-terminal domain of the CtBP transcriptional corepressor

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ABSTRACT

The C-terminal Binding Protein (CtBP) is a transcriptional corepressor that plays critical roles in development, tumorigenesis, and cell fate. CtBP proteins are structurally similar to alpha hydroxyacid dehydrogenases and additionally feature an unstructured C-terminal domain (CTD). The role of a possible dehydrogenase activity has been postulated for the corepressor, although *in vivo* substrates are unknown, but the functional significance of the CTD is unclear. In the mammalian system, CtBP proteins lacking the CTD are able to function as transcriptional regulators and oligomerize, putting into question the significance of the CTD for gene regulation. Yet, the presence of an unstructured CTD of ~100 residues, including some short motifs, is conserved across Bilateria, indicating the importance of this domain. To study the *in vivo* functional significance of the CTD, we turned to the *Drosophila melanogaster* system, which naturally expresses isoforms with the CTD (CtBP(L)), and isoforms lacking the CTD (CtBP(S)). We used the CRISPRi system to test dCas9-CtBP(S) and dCas9-CtBP(L) on diverse endogenous genes, to directly compare their transcriptional impacts *in vivo*. Interestingly, CtBP(S) was able to significantly repress transcription of the *E2F2* and *Mpp6* genes, while CtBP(L) had minimal impact, suggesting that the long CTD modulates CtBP's repression activity. In contrast, in cell culture, the isoforms behaved similarly on a transfected *Mpp6* reporter. Thus, we have identified context-specific effects of these two developmentally-regulated isoforms, and propose that differential expression of CtBP(S) and CtBP(L) may provide a spectrum of repression activity suitable for developmental programs.

INTRODUCTION

Eukaryotic transcription factors and cofactors are rich in unstructured domains; these proteins have a higher percentage of predicted intrinsically disordered regions (IDR) than the average protein (Uversky, 2016). Some unstructured domains have been shown to participate in specific transcriptional processes, such as the C-terminal domain (CTD) of RNA polymerase II, which is a platform for association of factors involved in capping, splicing, and polyadenylation (Harlen and Churchman, 2017). However, the roles of many IDRs present in these factors are still unknown. Transcriptional regulators can take on a diversity of roles in the cell, and unstructured domains may not necessarily play a role specific to gene regulation; yet, it has been speculated that these IDRs can assist with protein-protein interactions, or in phase separation of transcription condensates.

The C-terminal binding protein (CtBP) is a highly conserved transcriptional corepressor that contains a prominent IDR in its CTD. The CtBP CTD of about 100 amino acids is conserved across Bilateria, and despite overall lower sequence conservation than other parts of the protein, it retains certain properties, such as the predicted unstructured nature of the domain (Raicu *et al.* 2023). A few lineages, such as roundworms and flatworms, have novel, derived CTD sequences that are predicted to form structures. However, the conservation in primary sequence, length, and unstructured property of the CTD in bilaterians suggests that this IDR plays an important role, perhaps in gene regulation.

Mammalian genomes encode the CtBP1 and CtBP2 paralogs, which play overlapping and non-redundant roles in regulating expression of genes involved in apoptosis, the epithelial to mesenchymal transition, and cell differentiation (Grooteclaes *et al.* 2003; Fang *et al.* 2006; Jin *et al.* 2007; Paliwal *et al.* 2012). The CtBP1 and CtBP2 CTDs exhibit 50% sequence conservation, which is much lower than that of the central core dehydrogenase domain (Raicu *et al.* 2023). This core domain contains residues critical for oligomerization of CtBP monomers and for NADH binding, as well as *in vitro* dehydrogenase activity (Madison *et al.* 2013). CtBP can oligomerize and repress genes without the CTD, putting into question the significance of the CTD in gene regulation (Kumar *et al.* 2002; Madison *et al.* 2013). Interestingly, CtBP isoforms without the CTD exist in certain tetrapods such as birds and amphibians (Raicu *et al.* 2023). Additionally, the single *Drosophila melanogaster* CtBP locus encodes short isoforms that lack the CTD (CtBP(S)) and another which retains the long CTD (CtBP(L); Mani-Telang and Arnosti, 2007). Thus, *D. melanogaste*r is an appropriate model system to test for a possible role of the CtBP CTD in gene regulation.

Previous work using GAL4-CtBP fusions in the Drosophila embryo demonstrated that the two isoforms have similar repressive effects on *even-skipped-lacZ* reporters, and both isoforms individually rescue a *CtBP* null fly, albeit with some phenotypes (Sutrias-Grau and Arnosti, 2004; Zhang and Arnosti, 2011). Thus, the CTD does not seem to play an essential role in developmental programs.The expression pattern of the two isoforms exhibit developmentally distinct profiles; CtBP(S) is expressed throughout development, while CtBP(L) is highly expressed in the embryonic stage (Mani-Telang and Arnosti, 2007). The fact that short isoforms have been independently derived in other insects, such as Hymenoptera, and in other lineages in Bilateria suggests that expression of both isoforms is somehow important (Raicu *et al.* 2023). The conflicting evidence compelled us to test how the two CtBP isoforms regulate gene expression *in vivo*.

Here, we have made use of precise genetic tools in Drosophila to probe the function of the fly CtBP isoforms, CtBP(L) and CtBP(S), for their ability to regulate gene expression in a developing fly. Specifically, we used the CRISPRi system to assess the function of chimeric dCas9-CtBP proteins targeted to gene promoters *in vivo*. This method allowed us to compare the activity of the long and short isoforms in the same context, in both fly wing tissue and in cell culture. We found that CtBP(S) is a more potent repressor of the *E2F2/Mpp6* bidirectional promoter than CtBP(L), but that this difference in repression ability is not observed on a transiently transfected *Mpp6*-luciferase reporter. Thus, in some contexts theCTD seems to provide a regulatory function, but the difference observed between endogenous gene regulation and transient transfections raises the possibility that the effect may be chromatin-dependent. Additionally, gene promoters targeted here had differential sensitivity to CtBP recruitment, indicating a further level of regulatory specificity, in accord with recent high-throughput assays (Jacobs *et al.* 2022, bioRxiv).

RESULTS

Creation of dCas9-CtBP chimeras to regulate gene expression

To investigate differences in gene regulation by the CtBP(L) and CtBP(S) isoforms in Drosophila, we employed CRISPRi (Reviewed in Kampmann *et al.* 2018). We fused the coding sequence of each CtBP isoform to a nuclease dead Cas9 (dCas9) enzyme to recruit CtBP corepressors to target promoters using gene-specific guide RNAs (gRNA; **Figure 1A**).

dCas9-CtBP(L) and dCas9-CtBP(S) are expressed at similar levels in S2 cells, according to western blot (**Figure S1**).

We expressed the chimeric proteins in L3 wing discs using the *nubbin*-GAL4 driver. Flies homozygous for both *nub*-GAL4 and UAS:dCas9-CtBP were crossed to lines obtained from Harvard TRiP expressing two tandem gRNAs targeting a gene's proximal promoter (**Figure 1B;** Zirin *et al.* 2022). We previously tested dCas9-Rb chimeras in L3 discs, where we observed gene-specific effects after targeting ~30 different gene promoters; here, we targeted many of the same promoters (Raicu, Castanheira, Arnosti. *bioRxiv*; **Supplementary Table 1**).

The epithelial cells of the developing wing are a highly sensitive tissue that has been used to measure developmental perturbation of a number of regulatory pathways. To screen for genetic effects, we allowed the flies expressing the three transgenes to grow to adulthood, and then assessed adult wing phenotypes from targeting each promoter. We note that the *nub*-GAL4>UAS:dCas9-CtBP flies crossed to a non-targeting gRNA control fly line (QUAS) produced mild wing phenotypes, consisting chiefly of supernumerary bristles (**Figure 2A**). We presume that ectopic CtBP, even when fused to dCas9, may interact with diverse endogenous CtBP binding sites on the genome. The control gRNAs used here did not produce phenotypes with dCas9-Rb corepressor fusions tested previously, so the effect here is CtBP-specific (Raicu, Castanheira, Arnosti. *bioRxiv*).

CtBP isoforms have diverse effects on gene promoters

We recruited CtBP(L) and CtBP(S) to a number of gene promoters, with specific effects observed only on a few (**Supplementary Table 1**). Here, we detail the effects of targeting the *E2F2/Mpp6* bidirectional promoter, the insulin receptor (*InR*) promoter, and the promoter of *Acf*, a nucleosome remodeling subunit (**Figure 2**). Targeting CtBP(S) to the divergent *E2F2/Mpp6* promoter produced small wings with severe morphological defects, similar to that seen with dCas9-Rb proteins (**Figure 2B**; Raicu, Castanheira, Arnosti. *bioRxiv*). Intriguingly, CtBP(L) did not produce this phenotype, but instead produced milder effects, including wings with ectopic veins and supernumerary bristles (**Figure 2B**). dCas9 alone did not produce any phenotypic effect, indicating that the observed phenotypes are CtBP-specific. The clear difference between the long and short isoforms on this promoter suggests that the long CTD may inhibit CtBP's gene regulatory activities.

Interestingly, the strong CtBP(S) effect is only seen when using two gRNAs; recruitment using the individual gRNAs produced milder effects, including the ectopic veins seen with the CtBP(L) isoform when both gRNA were used (**Figure S2**). Interestingly, the number of wings with supernumerary bristles was less than that observed for the non-targeting control QUAS gRNA; we speculate that nonspecific CtBP overexpression effects are suppressed by targeting the chimeric protein to specific DNA locations.

Targeting the *InR* promoter produced adult wings with mild phenotypes, similar to those produced with the non-targeting QUAS gRNA control, so this effect is difficult to distinguish from a mild overexpression phenotype rather than specific *InR* targeting (**Figure 2C**). Clearly, positioning the CtBP chimeras near the transcriptional start site does not strongly affect the wing, although we know that positioning dCas9-Rb chimeras at this promoter does impact development and transcription (Raicu, Castanheira, Arnosti. *bioRxiv*). This distinct effect is consistent with CtBP promoter selectivity, a property illustrated from recent high-throughput assays (Jacobs *et al.* 2022, bioRxiv).

Recruitment to the *Acf* promoter region generated a different spectrum of phenotypes. In this case, a significant proportion of wings from the dCas9 control cross showed supernumerary bristles, evidence that dCas9 alone can disrupt gene function in certain locations. Notably, the position of one of the gRNAs used here is 3' of the initiation site for the divergently transcribed *Mccc1* gene, a position from which transcriptional inhibition is possible by dCas9 (Qi *et al.* 2013). Despite this dCas9 effect, the CtBP fusions had specific effects, with CtBP(S) causing a larger proportion of wings to be affected (80%) than CtBP(L) (60%; **Figure 2D**). Results from these targeted promoters indicate that CtBP exhibits gene-specific effects, and that in some cases, CtBP(S) has a more pronounced effect than CtBP(L).

CtBP(S) is a more potent transcriptional repressor than CtBP(L) on *E2F2/Mpp6*

Given the noticeable differences in phenotypes as a result of targeting the two CtBP isoforms to the *E2F2/Mpp6* shared promoter, we measured transcript levels of both of these genes in the wing disc using RT-qPCR. The two gRNAs used here bind at -577 and -672 relative to the *E2F2* TSS, and at -18 and +57 relative to the *Mpp6* TSS (**Figure 3A**). CtBP(S) showed specific repression of the *Mpp6* gene, whereas CtBP(L) effects were indistinguishable from those of dCas9 alone (**Figure 3C**). Effects on *E2F2* were more modest, with no apparent change for CtBP(L), and a small but significant reduction of similar magnitude for both dCas9 and CtBP(S) (**Figure 3B**). Interestingly, although CtBP(L) had a weaker effect on transcription than dCas9 alone at the time point measured (late L3 larval stage), it clearly showed more pronounced phenotypic effects in the adult stage. This may be due to gene regulatory effects later in development, where we did not measure transcriptional impacts. Taken together, these results indicate that both of the dCas9-CtBP corepressors do have specific effects, and at least CtBP(S) can be found to demonstrate classical repression effects.

Position-sensitive CtBP repression in cell culture

Many tests of CtBP function have relied on transiently transfected reporter genes; however, few studies have directly compared repression activity on the same genes in their endogenous chromosomal location. To further assess CtBP(L) and CtBP(S) function, we expressed the dCas9 chimeras in S2 cells, using an *Mpp6* reporter, which we have previously demonstrated is susceptible to repression by dCas9-Rb proteins (Raicu, Castanheira, Arnosti. *bioRxiv*). Here, we employed seven different gRNAs to test for possible position effects on this 1 kbp promoter region (**Figure 4A**). Both CtBP(S) and CtBP(L) showed strongest effects with gRNA 2 and 5; dCas9 alone did not mediate significant repression from the gRNA 2 position, but did from gRNA 5, likely due to steric effects (**Figure 4B-D**). The dCas9 control did not mediate repression from any other site, clearly different from the CtBP effects with gRNAs 1, 2, and 3. A simple distance effect, with stronger repression proximal to the transcriptional start site, was not evident. Additionally, CtBP(S) appeared to be more effective at the more distal gRNA 1 and B positions than near the TSS, at 4. Overall, it is striking that CtBP(L) performed similarly to CtBP(S) on this reporter, given the clear differences *in vivo*.

DISCUSSION

Our study of CtBP(L) and CtBP(S) isoforms using a CRISPRi approach has revealed that these repressors do exhibit different functional potential, and that CtBP itself shows promoter selectivity, consistent with the findings of the Stark laboratory (Jacobs *et al.* bioRxiv). Our data suggest that CtBP proteins are involved in selective modulation of their gene targets, consistent

with a "soft repression" form of regulation that may characterize many repressive interactions in the cell (Mitra, Raicu *et al.* 2021).

Evolutionary conservation of the CTD of CtBP indicates that this portion of the corepressor must be of importance, yet most assays employed in previous studies have not identified a difference in function at the transcriptional level (Kumar *et al.* 2002; Madison *et al.* 2013). One possible explanation is that the domain is involved in other aspects of CtBP biology, such as turnover or intracellular targeting, which may be overlooked in overexpression assays. Alternatively, its function in gene regulation may not have been identified yet, as the context in which CtBP has been assayed is limited; even the recent high throughput assessment of GAL4-CtBP was carried out with transient transfections and effects of the CTD were not assessed (Jacobs *et al.* 2022, bioRxiv).

Few studies have tested the impact of CtBP proteins with or without the conserved, long CTD on expression of endogenous genes, with the exception of genomic rescue experiments that demonstrated that viability is possible with either a CtBP(S) or CtBP(L) rescue construct (Zhang and Arnosti, 2011). However, the survivors from genomic rescues employing single isoforms showed a variety of phenotypes, including elevated embryonic lethality and aberrant wing development, indicating that limiting expression to one isoform alone does not fully satisfy developmental demands. Here, by directly testing CtBP isoforms in a CRISPRi setting on endogenous genes, we uncovered a striking difference between CtBP(L) and CtBP(S). On the *E2F2/Mpp6* bidirectional promoter, CtBP(S) was a potent repressor of gene expression and caused a severe wing phenotype, while CtBP(L) was much milder in its transcriptional and phenotypic effects. What might be the molecular action of the CTD on CtBP itself? Biochemical assays have shown that this intrinsically disordered domain is not required for NAD(H) binding or oligomerization, which are required for *in vivo* functionality (Kumar *et al.* 2002; Bellesis *et al.* 2018; Jecrois *et al.* 2021). The CTD of mammalian CtBP has been shown to be a target of post-translational modifications, which may affect conformation or protein-protein interactions of this domain. Our CRISPRi system ensures targeting to the promoter, thus the CTD regulatory impact is likely to be at the level of transcriptional action, rather than promoter binding. It is interesting that a different eukaryotic dehydrogenase-like corepressor, NPAC/GLYR1, similar to CtBP, forms tetramers and possesses an IDR that is involved in functional contacts with histone-modifying lysine demethylases (Marabelli *et al.* 2019). Our finding that the CtBP(L) isoform is less active only on the chromatinized endogenous *E2F2/Mpp6* regulatory region, but not when this element is tested in a transient reporter assay, provides support for the notion that the CTD regulation is chromatin-related, but deeper understanding will require further biochemical and molecular genetic studies.

MATERIALS AND METHODS

Plasmids used in this study

To create UAS:dCas9-CtBP constructs, the FLAG-tagged (DYKDDDDK) coding sequences for CtBP(L) and CtBP(S) were used, as described previously (Sutrias-Grau and Arnosti, 2004). These coding sequences were amplified from their parent vector using 5' PacI and 3' XbaI sites, and inserted in place of Rbf1 in the UAS:dCas9-Rbf1 plasmid described previously (Raicu, Castanheira, Arnosti. *bioRxiv*). CtBP(L) is isoform F and CtBP(S) is a combination of isoform E and J, based on Flybase nomenclature. The *Mpp6*-luciferase reporter construct uses the *Mpp6* promoter, which includes the *Mpp6* 5'UTR, to drive luciferase expression, as was described previously (Raicu, Castanheira, Arnosti. *bioRxiv*). The gRNA plasmids used in transfections were described previously, and target different sites of the *E2F2/Mpp6* bidirectional promoter (Raicu, Castanheira, Arnosti. *bioRxiv*).

Transgenic flies

Flies were fed on standard lab food (molasses, yeast, corn meal) and kept at RT in the lab, under normal dark-light conditions. The *nubbin*-GAL4 fly line was obtained from the Bloomington Drosophila Stock Center (BDSC; #25754) and was maintained as a homozygous line with a Chr 3 balancer obtained from BDSC #3704 *(w[1118]/Dp(1; Y)y[+]; CyO/Bl[1]; TM2, e/TM6B, e, Tb[1]*). Homozygous UAS:dCas9-CtBP flies were generated by using the ϕC31 integrase service at Rainbow Transgenic Flies Inc. #24749 embryos were injected with each dCas9-CtBP construct to integrate into Chr 3, landing site 86Fb. Successful transgenic flies were selected through the mini-white selectable marker expression in-house, and maintained as a homozygous line with Chr 2 balancer (from BDSC #3704). *nub*-GAL4 and UAS:dCas9-CtBP homozygous flies were crossed to generate double homozygotes (*nub*-GAL4>UAS:dCas9-CtBP), using the Chr 2 and Chr 3 balancers (from #3704). sgRNA fly lines were obtained from the BDSC (fly line numbers indicated in **Supplementary Table 1**). Single gRNA flies (-577 and -672) were previously described (Raicu, Castanheira, Arnosti. *bioRxiv*). Homozygous *nub*-GAL4>UAS:dCas9-CtBP flies were crossed to homozygous gRNA flies to generate triple heterozygotes (-/-; *nubbin*-GAL4/sgRNA; UAS:dCas9-CtBP/+) that are used for all fly experiments described here.

Genotyping flies

All flies generated in this study were genotyped at the adult stage. Flies of each genotype were homogenized (1 fly/tube) in squish buffer (1M Tris pH 8.0, 0.5M EDTA, 5M NaCl with 1µl of 10mg/mL Proteinase K for each fly). Tubes were set at 37C for 30 minutes, 95C for 2 mins, centrifuged at 14,000RPM for 7 minutes, and stored at 4C. Following PCR amplification, amplicons were cleaned using Wizard SV-Gel and PCR Clean-Up System and sent for Sanger sequencing.

Imaging adult wings

Adult wings were collected from ~50 male and female 1-3 day-old adults. They were stored in 200 proof ethanol in -20C until mounted. Wings were removed, mounted onto Asi non-charged microscope slides using Permount, and photographed with a Canon PowerShot A95 camera mounted onto a Leica DMLB microscope. Images were all taken at 10X magnification and using the same software settings.

Wing disc dissections and RT-qPCR

50 third instar wing discs were dissected from L3 larvae and placed in 200µl Trizol (ambion TRIzol Reagent) and stored in -80C until use. RNA was extracted using chloroform and the QIAGEN maXtract High Density kit, and stored in -80C. cDNA synthesis was performed using applied biosystems High Capacity cDNA Reverse Transcription Kit. RT-qPCR was performed using SYBR green (PerfeCTa SYBR Green FastMix Low ROX by Quantabio) and measured using the QuantStudio 3 machine by applied biosystems. Three control genes were averaged (Rp49, RpS13, CG8636) for all samples with control obtained from crossing dCas9 to a non-targeting gRNA (QUAS). Primers used were described previously (Raicu, Castanheira, Arnosti. *bioRxiv*). RT-qPCR was performed on 3 biological replicates with two technical duplicates. Student's t-test (two tailed, $p<0.05$) was used to measure statistical significance. Error bars indicate SEM.

Luciferase reporter assays

Reporter assays were performed as described previously, but with dCas9-CtBP(L) and dCas9-CtBP(S) effectors here (Raicu, Castanheira, Arnosti. *bioRxiv*).

Western blot

Western blot was performed as described previously for S2 cells (Raicu, Castanheira, Arnosti. *bioRxiv*).

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FIGURES

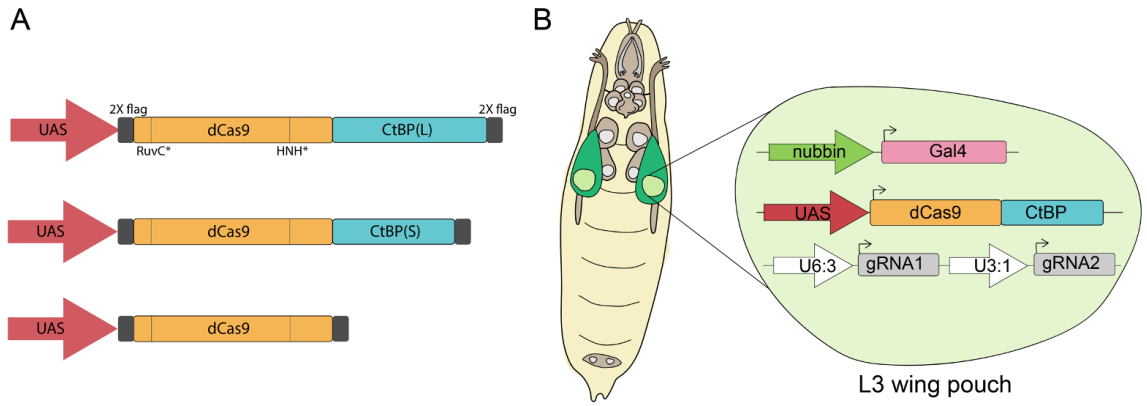

Figure 1. An *in vivo* system for targeting CtBP isoforms to gene promoters using CRISPRi. A) The fly CtBP(L) and CtBP(S) FLAG-tagged coding sequences were fused to the C-terminus of the *S. pyogenes* nuclease dead Cas9 (dCas9; D10A mutation in RuvC catalytic domain and H840A mutation in HNH catalytic domain), and placed under UAS expression. FLAG-tagged dCas9 was used as a negative control. Vertical lines in dCas9 represent the inactivating mutations. **B)** *Drosophila melanogaster* expressing three trangenes were generated for tissue-specific expression of dCas9-CtBP effectors using GAL4-UAS. Flies express dCas9-CtBP chimeras in the *nubbin* expression pattern (wing pouch of L3 wing discs), with ubiquitous expression of two tandem gRNAs designed to target a single gene's promoter. Flies used in experiments express one copy of each of the three transgenes. gRNA flies were designed by Harvard TRiP (Zirin *et al.* 2022).

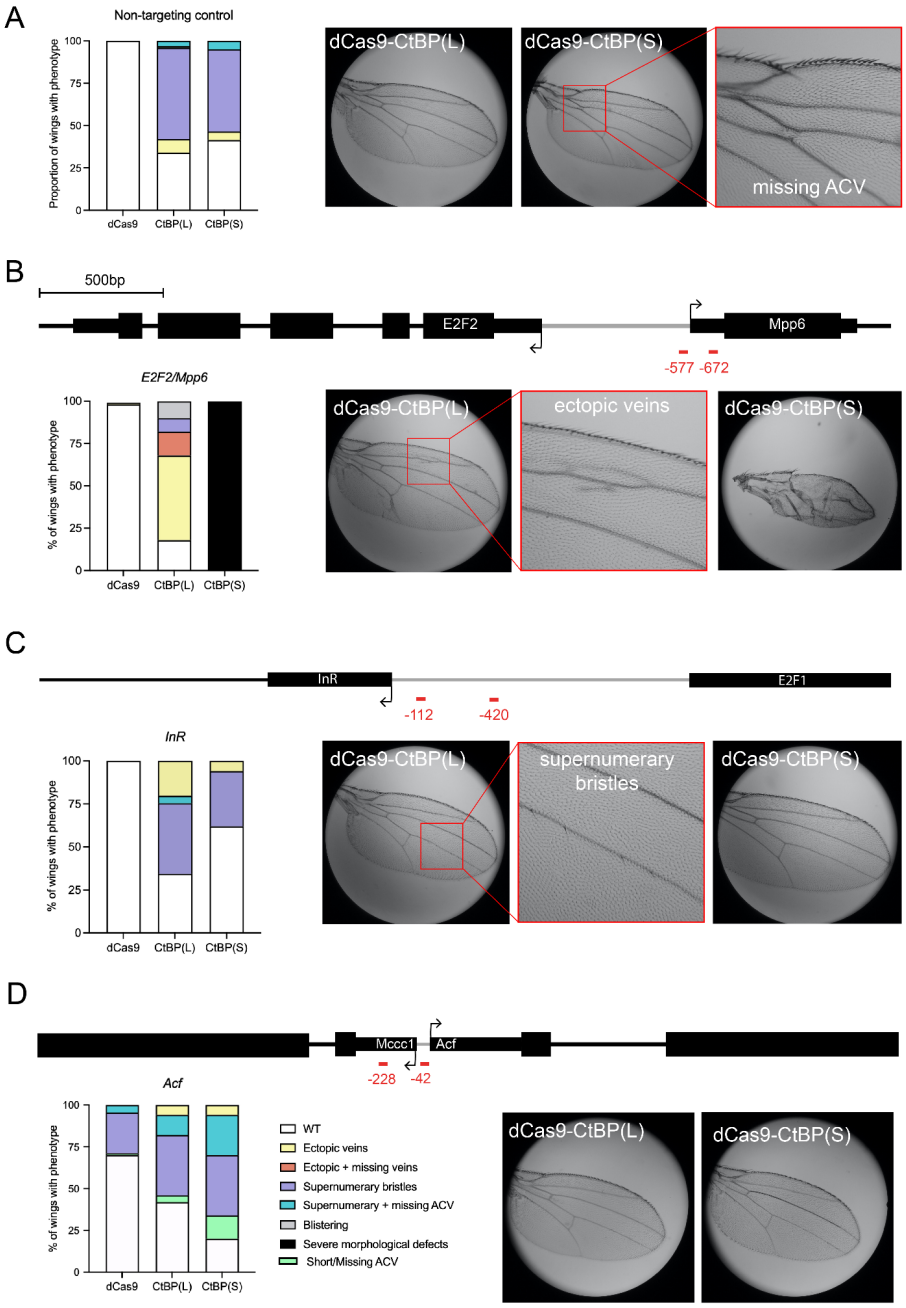

Figure 2. Targeting CtBP(S) and CtBP(L) to gene promoters leads to diverse phenotypic effects. For all crosses, ~100 wings from ~50 adults were used for analysis. Black arrows indicate the TSS, and red lines indicate gRNA binding sites relative to the target gene's TSS. **A)** Using a non-targeting control gRNA (QUAS), expression of one copy of dCas9-CtBP effectors leads to >50% of adult wings with a phenotype, such as supernumerary bristles. Legend is in panel D. **B)** Targeting the *E2F2/Mpp6* bidirectional promoter leads to severe morphological defects observed only from CtBP(S) targeting, with midler effects caused by CtBP(L). gRNA positions are relative to the *E2F2* TSS. **C)** Targeting the *InR* promoter leads to phenotypes similar to the QUAS non-targeting control, suggesting little or no specific effect on this promoter. **D)** Targeting the *Acf* promoter leads to mild phenotypes, some of which are also observed with dCas9 alone, at lower frequency. CtBP isoforms lead to a higher penetrance of phenotypes than dCas9.

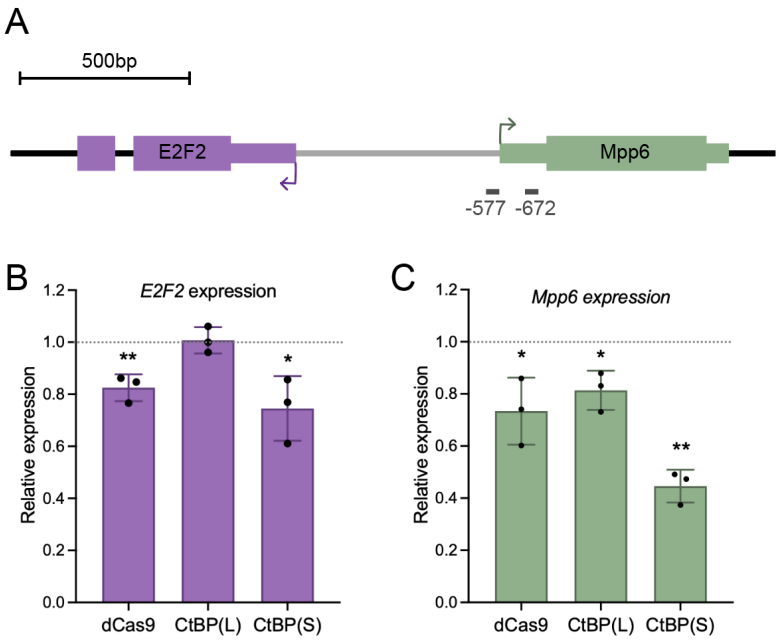

Figure 3. CtBP(S) is a more potent repressor of *Mpp6* than CtBP(L) in wing discs. A) Schematic of the *E2F2/Mpp6* bidirectional promoter, with the two tandem gRNAs indicated in gray. **B)** Targeting dCas9-CtBP(S) led to repression of *E2F2* by about 25%, similar to the effect of dCas9 alone. dCas9-CtBP(L) recruitment to the same sites did not lead to any measurable repression. **C)** Targeting dCas9-CtBP(S) led to significant repression of *Mpp6* (~50%), and this repression is greater than effects by dCas9 alone. dCas9 alone and dCas9-CtBP(L) led to about 20-25% repression. * $p<0.05$, ** $p<.01$

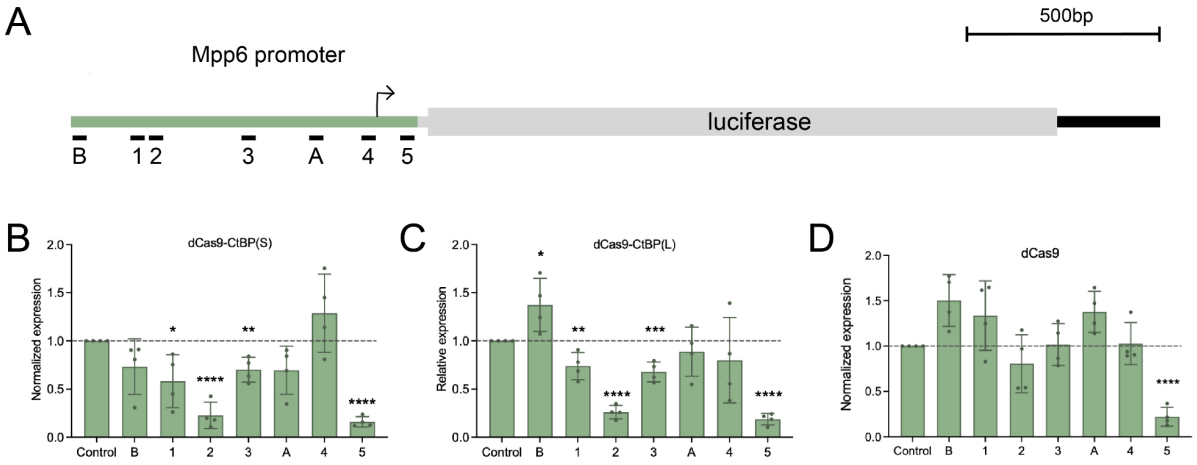

Figure 4. Testing Cas9-CtBP range of action on a luciferase reporter gene in S2 cells. S2 cells were transfected with *actin*-GAL4, the *Mpp6*-luciferase reporter, one of the dCas9 effectors, and a single gRNA. **A)** Schematic of luciferase reporter that was designed to be regulated by the *Mpp6* promoter, with gRNA positions indicated below. **B)** dCas9-CtBP(S) has position-specific effects. Position 2 led to the most severe repression. Position 5 caused the same level of repression as dCas9 alone, suggesting steric hindrance. **C)** dCas9-CtBP(L) has position-specific effects, which are similar to those of CtBP(S). **D)** The dCas9 control did not lead to significant repression, aside from position 5. The dCas9 results are the same control experiments as presented in Raicu *et al.* In prep.