ROLE OF PROTEIN OLIGOMERIZATION IN REGULATION OF WNT TARGET GENE TRANSCRIPTION

by

Chandan Bhambhani

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Doctoral Committee:

Professor Kenneth M. Cadigan, Chair
Professor Steven E. Clark
Assistant Professor Scott E. Barolo
Assistant Professor Yanzhuang Wang

“ॐ तमसो मा ज्योतिर्गमय”

“Om Tamaso Ma Jyotirgamaya”
From Darkness Unto Light
-Upanishad

To Miii, Daddyji and everyone who made this possible

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ABSTRACT

Wnt/Wg signaling is a highly conserved signaling pathway that is important for animal development and adult homeostasis. There are so many aspects of the pathway that are not well understood, and transcriptional regulation is one of them. TCF is a DNA binding mediator of signaling, and carries out transcriptional roles by associating with a variety of factors. Some of those repress Wg target genes in the absence of signaling, while some activate upon signal stimulation. One such factor is CtBP, which is both an activator and repressor of Wg targets. In this study, a mutational analysis revealed that the basis for this differential activity of CtBP is its ability to form oligomers. CtBP monomers are able to activate Wg targets, while their self-association into oligomers leads to repression of Wg targets. Furthermore, it was found that TCF can also self-associate. Some TCFs bind two different *cis*-elements termed HMG and Helper sites, in a bipartite manner. These sites have variable spacing and orientation and one attractive hypothesis is that oligmerization of TCF can overcome the requirement for a fixed spacing and orientation of these sites. Therefore, protein oligomerization plays crucial roles in regulating Wnt target gene transcription.

Chapter I

General Introduction

The 'harmony' of cell signaling

Mother nature has created several processes which are highly complex and act in accord to accomplish many different tasks. It is remarkable that a microscopic cell has a whole new world in itself that is so sophisticated. Many aspects which are a part and parcel of our daily life, exemplify the information derived from this sophistication. The sense of logic in cell cycle checkpoints, the discipline in protein folding, the engineering in proton pumps, the efficiency in utilizing the energy currency ATP, the speed in synaptic transmission – list goes on. It is amazing how the microscopic world has accomplished this level of complexity, yet manages it with so much ease.

One such complex process required for development of multicellular organisms is cell-cell communcation. Ligands serve as faithful messengers for inter-cellular communicaton and signaling pathways act as the cellular mediators of the message. Of the known signaling pathways, there are seven namely Wnt, Notch, Hedgehog, receptor tyrosine kinase, Jak/STAT, nuclear receptor and TGF-β signaling, which are used repeatedly during development (reviewed in Barolo & Posakony, 2002; Pires-daSilva & Sommer, 2003). Similar to seven notes which make up many different musical compositions, these seven pathways govern a majority of cell fate decisions. If assigned

a note to each pathway, maybe each cellular outcome can also be written down as a musical composition.

One of the outcomes of the message delivered by these diverse signaling cascades is target gene transcription. There are factors which can positively and/or negatively regulate gene expression through several mechanisms (Lodish et al, 1999). One of the main ways to achieve control over target gene expression is by regulating an event known as 'transcriptional initiation'. This initiation is a concerted effort of many factors, and involves assembly of a transcriptional complex at a region upstream of the transcription start site, known as the core promoter (reviewed in Patikoglou & Burley, 1997). An enzyme RNA polymerase, which is the core component of the transcriptional machinery, then uses DNA as a template to synthesize transcripts.

CtBP family of proteins

Historical Perspective

C-terminal Binding Protein (CtBP) is a well characterized transcriptional regulator with both activating and repressing roles in target gene regulation. In addition to being a mediator of signal induced transcription, CtBP is involved in several other transcriptional events. CtBP was identified in a screen for interaction partners of the adenoviral oncogene E1A (Boyd et al, 1993). Transformation of rats with E1A led to formation of tumors, which were greatly enhanced upon deletion of the C-terminal region of E1A, leading to a 'super transformation' phenotype (Boyd et al, 1993). Since CtBP

could bind to the C-terminus of E1A, it was implicated in suppression of E1A induced super transformation phenotype (Boyd et al, 1993).

CtBP group of proteins have been shown to play important biological roles in several transcriptional contexts, along with requirement in other cellular processes. These include synaptic transmission (Schmitz et al, 2000), golgi fission (Weigert et al, 1999) and hypoxia sensing (Fjeld et al, 2003; Zhang et al, 2002). Although the role of CtBP in golgi fission is controversial, its sensitivity to hypoxia has been implicated in tumor metastasis (Chinnadurai, 2009; Zhang et al, 2006).

Drosophila CtBP was first identified in a yeast two hybrid screen for interacting partners of an Anterior/Posterior (A/P) patterning gene Hairy (Poortinga et al, 1998). Fly embryos are patterned in repeated units called segments, which eventually define the identity of anterior through posterior tissues of the fly. During establishment of the *Drosophila* A/P body plan, each segment is the outcome of a number of molecular events controlled by the maternal coordinate genes, gap genes, pair rule genes and segment polarity genes. These genes act in a hierarchical order with gap gene mutations affecting several adjacent segments, pair rule gene mutations affecting alternating segments and segment polarity gene mutations affecting a portion of the segment or the so called para-segment.

CtBP mutant fly embroys display a severly disrupted segmentation pattern consistent with a requirement in A/P patterning (Nibu et al, 1998a; Nibu et al, 1998b; Poortinga et al, 1998). Although originally identified as an interacting partner of the pair-rule protein Hairy, it has been established through many genetic and biochemical assays

that CtBP acts at the level of the Gap genes to specify pair-rule patterns. CtBP mutant embryos show both a loss and expansion of the pair-rule stripes suggesting that it has both inductive and suppressive roles in pair-rule patterning (Nibu et al, 1998a; Poortinga et al, 1998).

Vertebrates have two CtBP genes *CtBP1* and *CtBP2*, which display structural and functional similarities (Chinnadurai, 2002; Hildebrand & Soriano, 2002). *CtBP1* has two isoforms *CtBP1 long* (*CtBP1l*) and *CtBP1 short* (*CtBP1s*) generated due to alternative spilicing. They are mostly similar in sequence except for parts of the N-Terminus and presence of a longer C-Terminus in the long isoform (Figure 1.1). The *CtBP2* gene has three isoforms *CtBP2l*, *CtBP2s* and *Ribeye*. The first two isoforms are generated by the alternative spilcing, but *Ribeye* has an alternative transcription start site and a unique N terminus, consequently leading to a distinct function when compared to other CtBP proteins (Schmitz et al, 2000).

Flies have one *CtBP* gene expressing two isoforms which code for the *CtBP short* (*CtBPs*) and *CtBP long* (CtBPl) protein (Mani-Telang & Arnosti, 2007; Nibu et al, 1998b; Poortinga et al, 1998; Sutrias-Grau & Arnosti, 2004). Similar to the vertebrate counterpart, *CtBPl* has an extended C-terminus as a result of alternative splicing of the *CtBP* transcripts. These isoforms play a redundant role when tested in different transcriptional assays (Fang et al, 2006; Sutrias-Grau & Arnosti, 2004) although there are temporal differences in their expression during larval stages of different *Drosophilids* (Mani-Telang & Arnosti, 2007).

Roles of CtBP in transcriptional regulation

CtBP acts in concert with several transcription factors in the context of target gene repression, which is the extensively explored function of CtBP. In many different cultured cell types, recruitment of CtBP to cis-elements using a heterologous DNA binding domain represses the basal activity of reporter genes (Kumar et al, 2002; Meloni et al, 2005; Nibu et al, 1998a; Phippen et al, 2000; Thio et al, 2004). However, CtBP mutants in both vertebrate and flies also show a requirement of CtBP for activation of target genes, but this function has not been extensively explored (Fang et al, 2006; Hildebrand & Soriano, 2002; Nibu et al, 1998a; Poortinga et al, 1998). For example, in flies CtBP mutants show a loss of pair-rule gene patterning or genetic interactions of CtBP with Hairy suggest that it is required for activation of a target gene fushi tarazu (Poortinga et al, 1998). Consistent with that in cultured mammalian cells CtBP has been shown to be a context dependent activator and repressor (Phippen et al, 2000). Hence CtBP has both inductive and suppressive roles in several contexts and some of the mechanisms have been discussed in the sections later on.

CtBP structure and function

CtBP group of proteins have been shown to adopt higher order quartenary structures (Kumar et al, 2002; Mani-Telang et al, 2007; Nardini et al, 2003; Shi et al, 2003; Thio et al, 2004). Since the discovery of CtBP as a suppressor of tumors induced by adenoviral gene E1A, it has been shown to bind many transcription factors and cofactors, and the interaction motifs with CtBP have been mapped for many of these

proteins (reviewed in Chinnadurai, 2007; Turner & Crossley, 2001). However, there have not been many studies which have explored the role of its quartenary structure in various functional contexts. This section provides the current understanding of the role of quartentary structure in the function on CtBP.

CtBP family of proteins is highly conserved across the animal kingdom (Chinnadurai, 2007; Turner & Crossley, 2001). They have a striking sequence and structural homology with NAD-dependent 2-hydroxy acid dehydrogenases, although this activity has no relevance in several transcriptional contexts. All CtBPs share high homology in a region spanning most of the protein which is termed the Dehydrogenase domain (e.g., fly CtBP and human CtBP1 domains are 72% identical with 84% similarity) (Figure 1.1). Information from two studies, which have reported the crystal structure for human CtBP1 (hCtBP1) and mouse CtBP2 (mCtBP2), indicates that CtBP is a dimer under conditions of crystallization (Kumar et al, 2002; Nardini et al, 2003).

The dehydrogenase domain of CtBP is divided into three regions known as the Dimerization interface, the NAD binding cleft and the Catalytic site (Figure 1.1). The available structural information is only for a truncated fragment of CtBP consisting of the Dehydrogenase domain and a part of the N and C terminus (28-353) (Figure 1.1). The structure of the full length CtBP is not yet known, hence there is a possibility of oligomer interfaces in the other regions of CtBP.

In cultured cells CtBPs exist in an equilibrium between monomers (Kim et al, 2005; Zhao et al, 2009), homo-oligomers or hetero-oligomers (Balasubramanian et al, 2003; Kumar et al, 2002; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Nardini et

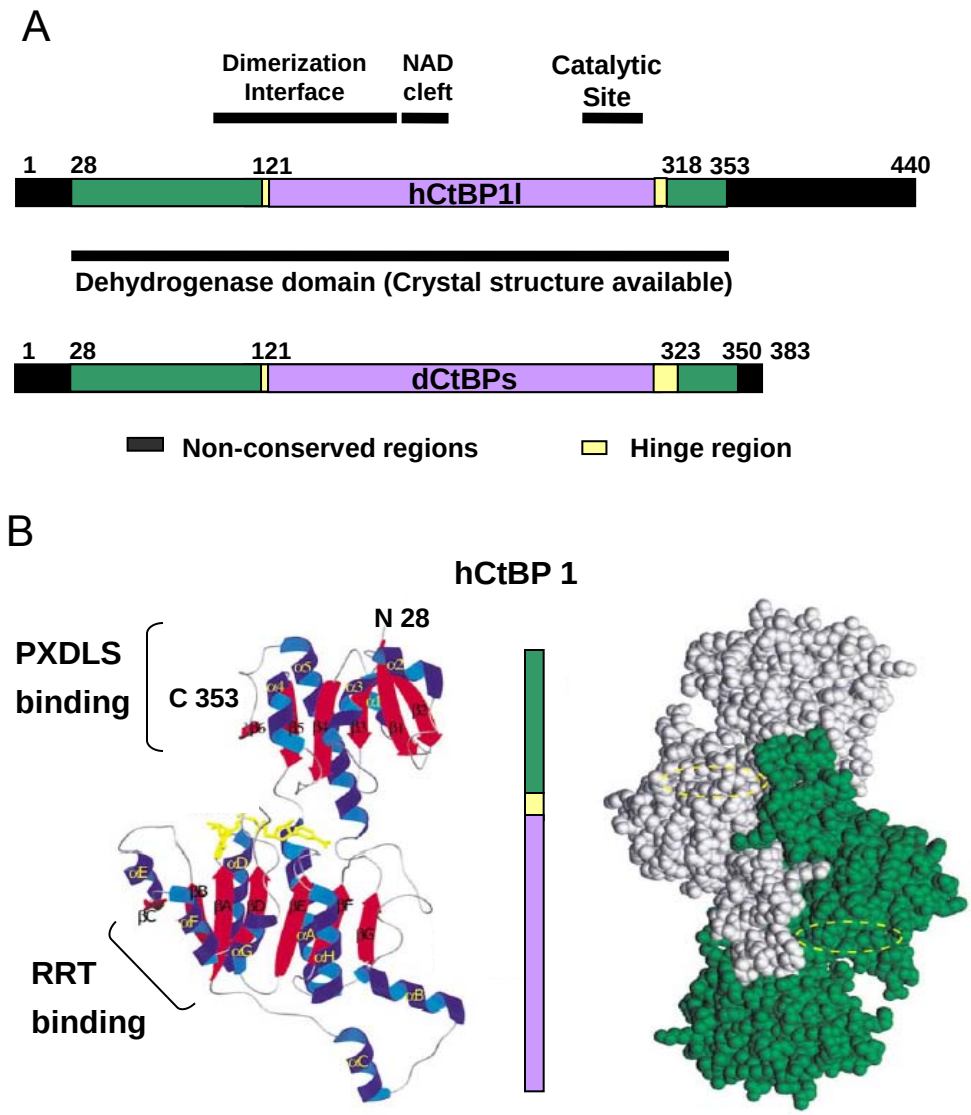


Figure 1.1. CtBP family of proteins forms higher order structures. Schematic showing the domain structure and crystal structure of CtBP family of proteins. (A) Dehydorgenase domain of CtBP has three highly conserved regions known as Dimerization interface for self-association, NAD cleft for nucleotide binding and Catalytic site for weak dehydrogenase activity. Human CtBP1 long isoform (hCtBP1l) and Drosophila CtBP short isoform (dCtBPs) are shown. (B) Crystal structure of the Dehydrogenase domain of hCtBP1l (Kumar et al., 2002) with a ribbon diagram of the monomer (left) showing the PXDLS binding and RRT binding sites and a space-fill model (right) showing the CtBP dimer

al, 2003; Shi et al, 2003; Thio et al, 2004; Zhao et al, 2009). The best supporting evidence for that was provided by treatment of cultured cells with a crosslinking reagent which covalently links primary amines of proteins (Zhao et al, 2009). It was shown that a fraction of endogenous CtBP1 and CtBP2 were not cross-linked and hence were monomers in cultured mammalian cells (Zhao et al, 2009).

In addition, higher order complexes were also found representing either CtBP oligomers, or CtBP in complex with other factors. Upon comparison of exogenously expressed wildtype and monomer mutants, it was clear that the wildtype had a pool of monomers and higher order oligomers, whereas a mutant which could not dimerize was predominantly in the monomeric size range as expected (Zhao et al, 2009). Bacterially expressed CtBP has also been purified in monomeric (Kim et al, 2005) and dimeric fractions (Balasubramanian et al, 2003; Kumar et al, 2002; Mani-Telang et al, 2007; Nardini et al, 2003) suggesting that they exist in an equilibrium between monomers and higher order oligomers.

Role of CtBP dimers in repression

The first study to report the structural information showed that hCtBP1 was a dimer based on a monomeric asymmetric unit making extensive dimer interface contacts with another subunit, in CtBP crystals (Kumar et al, 2002). Two sets of mutations were generated to biochemically test the dimerization of CtBP. Mutations of residues C134Y, L150W were used to create steric hinderance with bulky amino acids, while N138R and R141E were used to create charge repulsion between the dimeric subunits (Kumar et al,

2002). Another set of mutations R141A, R142A, R163A and R171A were used to disrupt saltbridges and hydrogen bonding across the dimer interface (Kumar et al, 2002).

Mutational analysis supported the crystal structure data that CtBP can exist as a dimer in solution. Furthermore, dimerization was shown to be important for the repression function of CtBP as mutations in the dimerization interface abolished the repressive function of CtBP (Kumar et al, 2002). CtBP monomers were unable to repress the activator E1A induced reporter activation, or when fused to a heterologous Gal4DNA binding domain, unable to repress a reporter with basal activity in cultured mammalian cells (Kumar et al, 2002). Hence the authors concluded that dimerization of CtBP was required for repression.

Consistent with this information, there are two more reports which directly link the dimerization of CtBP with its repressive activity (Kuppuswamy et al, 2008; Zhao et al, 2009). A rigorous analysis in cultured cells suggested that mutations in the dimerization interface affected the nuclear translocation of CtBP. Monomer mutants were unable to translocate to the nucleus, which might explain the loss of their repression function. This was addressed by creating a version of CtBP monomer tagged with a hetroloogous nuclear localization signal (Kuppuswamy et al, 2008). In cultured fibroblasts derived from CtBP null mice, the monomer mutant was unable to repress target genes. This was demonstrated by testing the CtBP monomer (R141A/R163L) for regulation of a *E-Cadherin* (*E-Cad*) reporter (Kuppuswamy et al, 2008), which is derived form a target repressed by CtBP (Postigo & Dean, 1999).

Although unable to repress, the CtBP monomer mutant was found to be in complex with various co-repressors which include HDACs (HDAC1 and HDAC2), CoRest and ZEB (Kuppuswamy et al, 2008; Shi et al, 2003). To explain why the monomers are unable to repress targets inspite of being able to interact with the corepressors, it was hypothesized that CtBP dimers form a bridge between different set of corepressors.

In an attempt to functionally validate the proposed model, a follow up study showed that monomers could interact with various corepressors but are impaired for repression (Zhao et al, 2009). Monomeric CtBP2 mutant was tested for interaction with the repressors ZEB and HDAC2, which are known to repress the *E-Cad* gene. While the monomer bound each of these repressors efficiently, their interaction with the monomer was mutually exclusive. Increasing levels of HDAC2 competed with ZEB for CtBP2 binding in a dose dependent manner, indicating they had overlapping interaction domains on a monomeric subunit (Zhao et al, 2009). Finally the authors showed that inspite of interacting with these repressors, the CtBP2 monomer was incompetent for repression of *E-Cad* reporter in CtBP null cells, while the wildtype was able to repress the *E-Cad* reporter significantly (Zhao et al, 2009). This study provided evidence for an interesting mode of gene repression by CtBP, wherein each monomeric subunit self-associates and forms a bridge between various repressors. Hence dimerization was necessary for the assembly of a repression complex in the context of *E-Cad*.

This dissertation is centered around the role of quartenary structure of CtBP in regulating Wnt target gene transcription as will be described in Chapter II and Chapter III. CtBP has been shown to activate and repress Wnt targets. Therefore, to provide

insightful views on the mechanisms by which it might achieve these differential roles, a description of the nuclear components of Wnt signaling is provided in the latter sections.

Wnt signaling pathway

Wnt signaling is a highly conserved signaling pathway from hydra to mammals with many distinct roles in animal development and adult homeostasis (reviewed in Cadigan & Nusse, 1997; Logan & Nusse, 2004). The pathway is activated by a family of glycoproteins called Wnts. There are a total of 19 known Wnts in humans, 7 in Drosophila and 5 in C.Elegans (Logan & Nusse, 2004). Stimulation by Wnts has several outcomes with one being stabilization of a major regulator of the pathway β-Catenin (β-Cat) and consequent transcriptional responses. This dissertation is centered around investigating how oligomer formation of two known regulators of the pathway alters β-Cat mediated transcriptional responses. Hence an overview of the current understanding of the signaling at the receptor and cytosolic level is provided, followed by a more detailed review of the nuclear components involved in regulating β-Cat mediated transcription.

Historical perspective and biology

Wingless (Wg), the first Wnt to be identified, was discovered because of the developmental phenotypes seen in flies carrying a weak allele of Wg (Babu, 1977; Sharma & Chopra, 1976). Flies homozygous for this allele were missing one or both

wings, hence the name 'Wingless'. The first mammalian Wnt was found in an investigation for the cause of large tumors in the mammary glands of mice. This led to the discovery of the *int1* locus (Nusse & Varmus, 1982) which was so named because it was a genomic 'hot spot' for the integration of Mouse Mammary Tumor Virus (MMTV) binding sites. These sites were upstream of the transcription start site of a gene, later named *int-1* (van Ooyen & Nusse, 1984). At around the same time, a screen to find regulators of the anterior-posterior body patterning of the fly embryo, led to discovery of the role of Wg in segment polarity (Nusslein-Volhard & Wieschaus, 1980). It was found later that Wg is one of the *Drosophila* Wnts involved many aspects of fly development.

The name 'Wnt' stems from the Drosophila segment polarity gene Wg (Babu, 1977; Nusslein-Volhard & Wieschaus, 1980; Sharma & Chopra, 1976) and mammalian homolog *int*, which led to mouse mammary tumors upon being constitutively activated (Nusse & Varmus, 1982). Over the next few years, as more was being understood about Wnts, their structure, biological function and diverse roles in animal development, it was discovered that misregulation of this pathway was one of the major causes of colon cancers in humans.

In 1997, it was shown that Adenomatous Polyposis Coli (APC), which was a gene linked to colon carcinoma, was associated with β-Cat, and Wnt signaling was constitutively active in APC mutants (Korinek et al, 1997; Morin et al, 1997). In today's date, there is an increasing list of biological roles that are associated with Wnt signaling and consequently diseases which stem from the abnormal functioning of the Wnt components (reviewed in Clevers, 2006; Giles et al, 2003). Hence understanding the

molecular mechanisms of the pathway is important to elucidate the basis of these diseases and find possible cures for them.

Roles of Wg signaling in fly embryonic development

The role of Wg in fly embryo patterning was uncovered in a forward genetic screen for embryonic lethals, (Nusslein-Volhard & Wieschaus, 1980) as were many other negative and positive components of the pathway in genetic screens later (reviewed in Bejsovec, 2006). Establishment of the A/P body plan of embryos is a co-ordinated effort of the genes which act in a segmentation hierarchy. Mutations in gap genes affect several adjacent segments, while pair rule gene mutations affect alternating segments and segment polarity gene mutations affect a parasegment. *Wg* is a segment polarity gene and is required for specifying cell fates in the porterior portion of each segment.

Wg is secreted from a single stripe of cells in each segment and is required for maintainance of a homeodomain gene *Engrailed* (*En*) (Cadigan & Nusse, 1997). En is also expressed in a single stripe of cells adjacent to the Wg expressing cells and marks the posterior region of the segment (Parker et al, 2002). Wg is a secreted protein, and so cells to the anterior also receive Wg, but *En* is repressed in those cells through another gene called *Sloppy-Paired* (Cadigan et al, 1994). Induction of *En* is in turn required for maintainance of Wg through the Hedgehog signaling pathway and this auto-regulation is crucial for establishing the segment polarity (Cadigan & Nusse, 1997).

At the ventral epidermis of the embryo, the anterior region of each segment is marked by rows of cells expressing hook like structures called the denticles, which help

the larvae latch on to surfaces post-hatching. The rows of cells in the posterior region of the segment have no denticles and are specified by Wg (Nusslein-Volhard & Wieschaus, 1980). Loss of Wg signaling results in the classic 'Lawn of Denticles' while hyper active signaling results in 'naked cuticle' (Figure 1.2). These phenotypes have been extensively used to identify regulators of the Wg pathway in the genetic screens (Bejsovec, 2006).

Role of Wg signaling in the fly wing primordium

The first mutant phenotype reported for Wg was in the adult wing. Flies homozygous for a weak allele of *Wg* (Wg^1) were missing one or both wings at a low penetrance (Sharma & Chopra, 1976) and showed wing to notum transformations (Figure 1.2). Although the mechanisms of the adult wing development in the context of Wg are not clearly understood, its role has been extensively studied in the wing primoridum, a tissue comprised of columnar epithelial cells that give rise to the adult wing.

At the early third instar larval stage, Wg is turned on in a stripe of cells at the dorsal/ventral (D/V) boundary of the wing imaginal disc (Couso et al, 1994; Phillips & Whittle, 1993) (Figure 1.3). Wg is secreted and acts in a cell non-autonomous manner and acts in a gradient decreasing away from the D/V boundary (reviewed in Cadigan, 2002). Wg turns on short range targets like Sensless (Sens), which are activated a few cell diameters aways from the site of Wg secretion (Figure 1.3) (Nolo et al, 2000; Parker et al, 2002). Sens is a transcription factor with a role in specification of mechanosensory bristles at edge of the wing blade (Nolo et al, 2000). Wg also turns on long-range targets like Distal-less (Dll), which are activated in cells several diameters away from the site of

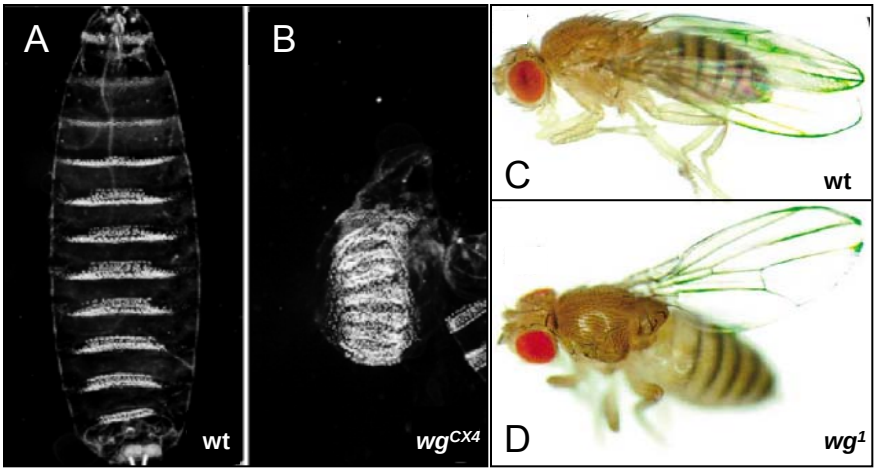


Figure 1.2. Wg signaling is important for many aspects of *Drosophila* development. Different alleles of *wg* showing disruption of segment polarity in embryos and wing to notum transformation in adults. Micrograph showing cuticles of embryos which are (A) wildtpye and show denticle belts and naked cuticle or (B) zygotic mutants for a strong allele of *wg* and display denticle fusions known as the classic 'lawn of denticles' (taken from Parker et al., 2002). Adult flies which are (C) wildtype with normal wings and (D) mutant for a weak allele of *wg* showing loss of a wing and a duplicated notum (taken from Bejsovec A., 2006).

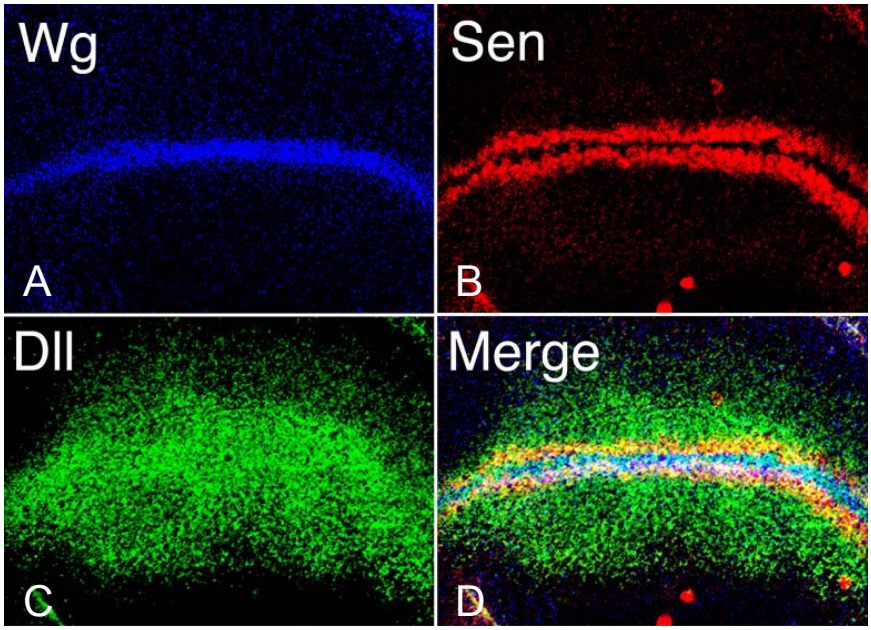


Figure 1.3. Wg activates short range and long range targets in the wing primordium. (A-D) Confocal images of wildtype third instar discs showing (A) Wg expression (blue) at the Dorsal/Ventral boundary. Wg turns on short range targets like (B) Sensless (Sen) (Red) and long range targets like (C) Distal-less (Dll)(green)

Wg secretion (Figure 1.3) (Neumann & Cohen, 1997; Zecca et al, 1996). The role of Dll in adult wing formation is not yet known. A gradient of Wg across the D/V boundary leads to differential activation of these targets, consistent with Wg acting as a morphogen. These targets have been used as readouts for Wg signaling, in validating newly identified components of the pathway.

Roles of Wnt signaling in worm development

Wnt signaling in worms has been known for the establishment of the A/P 'asymmetry' starting at the four cell stage during embryogenesis. The blastomere destined to give rise to the Endoderm and Mesoderm (EMS) receives a polarizing signal from the adjacent P2 (Posterior) blastomere after the second cell division event (Figure 1.4). The two daughter cells adopt different fates with the E cell giving rise to the Endoderm and the MS giving rise to the Mesoderm, and these decisions are regulated by Wnt signaling (Cadigan & Nusse, 1997; Lin et al, 1995; Phillips & Kimble, 2009; Rocheleau et al, 1997; Thorpe et al, 1997).

The E cells specify the formation of the intestine while the MS cells divide to form the body wall and the pharynx. Wnt signaling has instructive roles in the endoderm formation through targets like *endoderm-1* (*end-1*) (Shetty et al, 2005; Zhu et al, 1997). These endodermal cell fates are suppressed in cells destined to become the mesoderm in the absence of signaling. Loss of Wnt signaling in the early embryo leads to more mesoderm (mom) due to the loss of endodermal fates (Thorpe et al, 1997). Hyper active

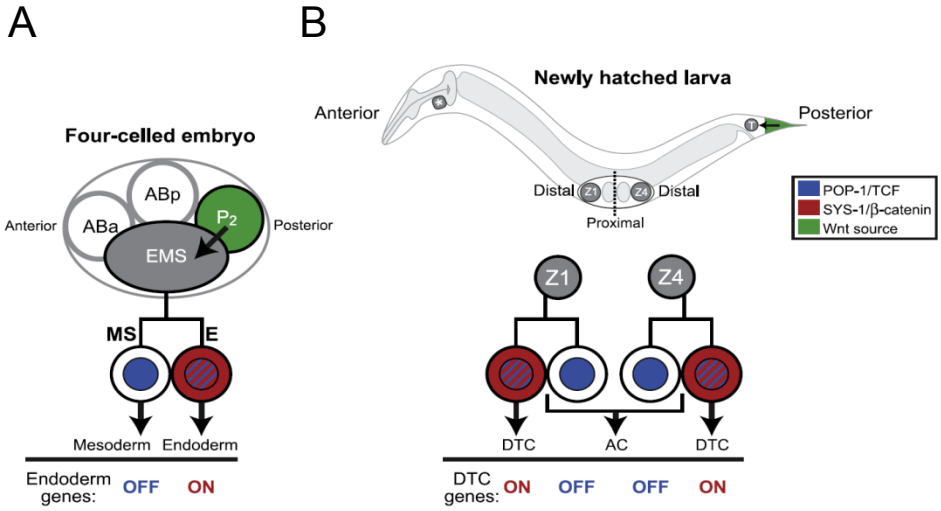


Figure 1.4. Wnt signaling induces asymmetric cell divisions during *C. elegans* development (taken from Phillips and Kimble, 2009). (A) Depiction of the four-cell stage of the worm embryo showing that the P2 cell (green) sends a polarizing signal to the EMS blastomere (grey). Asymmetric division of POP1 (blue) distinguishes between the cells adopting Mesodermal (MS) fate and Endodermal (E) fate. More nuclear POP1 antagonizes Wnt signaling keeping endoderm genes off in MS cells. β-cat and low levels of nuclear POP1 are required for Wnt stimulated activation of Endoderm genes in E cells. (B) Newly hatched larva showing the position of somatic gonadal precursors Z1 and Z4 (grey). Z1 and Z4 receive a polarizing signal which stimulates POP1 asymmetry. The distal tip cells (DTCs) have active Wnt signaling and low levels of nuclear POP1 and the anchor cells (AC) have high levels of nuclear POP1 which represses Wnt targets.

signaling due to loss of suppressive components has defects in mesoderm derived pharyngeal tissue, hence named posterior pharynx (pop) defect (Lin et al, 1995).

One of the wide spread roles of Wnt signaling in establishing the A/P cell fates is achieved by tritrating the nuclear levels of a key component of the pathway POP1 and establishing a POP1 asymmetry. In addition, Wnt signaling plays roles in T-Cell specification through a known Wnt target *phsamid socket absent-3* (*psa-3*)(Arata et al, 2006) and specification of the gonadal precursors known as Distal Tip Cells (DTCs) during larval stages, through another target called *c. elegans homeobox-22* (*ceh-22*) (Lam et al, 2006) (Figure 1.4).

Molecular mechanism of the Wnt/Wg signaling pathway

Wnt signal transduction is dependent on the turnover of one of the key effectors of the pathway called β-Cat (reviewed in Cadigan & Peifer, 2009). The curent working model for the β-Cat mediated signaling is that in the absence of ligand, a cytosolic pool of β-Cat gets phosphorylated, polyubiquitinated and targeted for degradation by the proteasome (Figure 1.5). The phosphorylation is dependent on the procurement of β-Cat by a 'destruction complex' comprising of axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1) (reviewed in Kennell & Cadigan, 2009; Kikuchi et al, 2006). This leads to low cytosolic and nuclear levels of β-Cat. In the nucleus, there is another level of regulation which keeps a check on the aberrant activation of Wnt targets in the absence of signaling. The DNA bound mediator

of signaling T-Cell Factor (TCF) recruits corepressors like Gro and HDACs to keep the signaling in an 'off' state.

Release of β-Cat from the destruction complex is stimulated by Wnts, and this release leads to the corresponding downstream events of the cascade. Wnts are a family of secreted glycolipoproteins, which bind to the Frizzled (Fz) family of serpentine receptors and single-pass membrane co-receptor LRP 5/6 to turn on the signaling. At the receptor level, there is formation of a 'signalosome' as Fz and LRP come together upon Wnt stimulation (reviewed in Cadigan & Peifer, 2009). Activation of the pathway is also correlated with the recruitment of the destruction complex to the receptors, with LRP interacting with GSK3, which recruits Axin to the membrane. This recruitment releases the 'captured' β-Cat pool, with the aid of another protein called Dishevelled (Dvl). Stimulation by Wnts leads to the recruitment of Dvl to the intracellular C-terminal domain of the Frizzled receptors.Dvl stimulates Phosphotidylinositol (PI) Kinases which leads to increased Phosphotidylinositol 4, 5 bisphosphate levels. This in turn stimulates the phosphorylation and oligomer formation of LRP leading to subsequent events (reviewed in Cadigan & Peifer, 2009).

Release of β-Cat from the degradation complex is followed by its nuclear translocation where the levels of β-Cat are fine tuned to reach the thresholds for activation. In the absence of signaling there are β-Cat 'sponges' in the nucleus which 'soak up' the β-Cat that escapes the degradation complex. Stimulation by Wnts increases the β-Cat levels to an extent that overcomes sequestration by these 'sponges'. Eventually β-Cat interacts with DNA binding mediators called T-Cell Factors (TCFs) and other cofactors like Pygopus (Pygo), Legless (Lgs) and CBP/p300, to activate target genes.

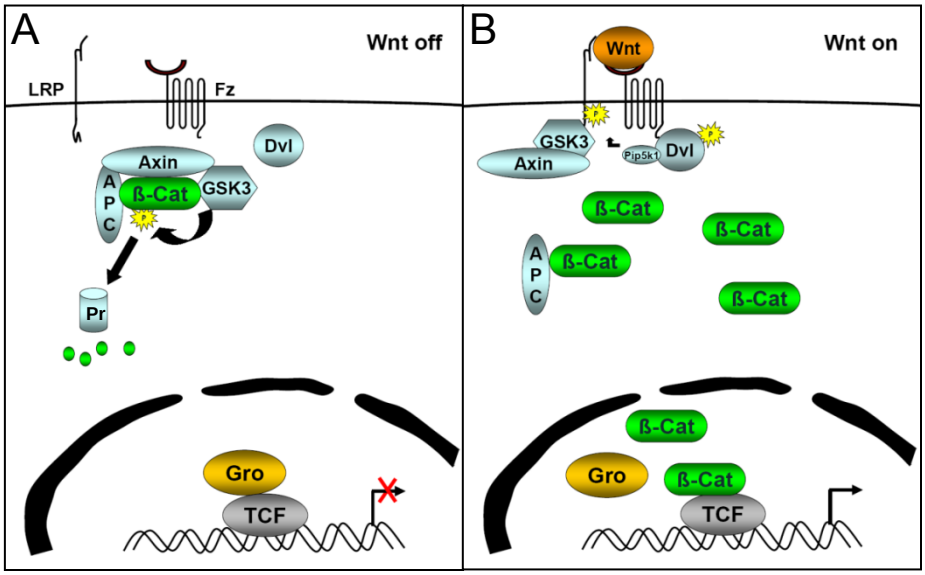


Figure 1.5. Wnt signaling leads to stabilization and nuclear translocation of β-cat for target gene regulation. Current working model of Wnt signaling. (A) In the absence of signaling (Wnt off) β-cat gets phosphorylated in complex with Axin, APC and GSK3 (destruction complex) consequently getting degraded by the proteasome (Pr). In the nucleus, TCF represses Wnt targets along with corepressors like Gro. (B) Stimulation by Wnts (Wnt on) leads to disruption of the degradation complex. β-cat gets stabilized, translocates to the nucleus and causes a nuclear switch of TCF from a repressor to an activator by replacing Gro, consequently activating Wnt targets.

The result of stimulation by Wnts is a β-Cat mediated switch of TCF from a repressor to an activator of Wnt target genes (Barolo & Posakony, 2002).

Roles of CtBP in Wnt/Wg signaling

CtBP has been shown to contribute to the nuclear switch of TCF from a repressor to an activator, and hence has dual roles in regulating Wnt/Wg targets (Figure 1.6). There are three working models based on the reported data, which suggest that CtBP has gene-specific positive and negative roles in regulating Wg targets (Figure 1.6). In the absence of signaling CtBP is recruited by unkown factors to keep some Wg targets off (Figure 1.6)(Fang et al, 2006). Upon stimulation by Wg, CtBP is recruited by the N-terminus of Armadillo (Arm; fly β-Cat) to activate some targets (Figure 1.6)(Fang et al, 2006). Once the targets are activated CtBP has been implicated in sequestering Arm away from WREs, as a mechanism to shut off signaling post stimulation (Figure 1.6) (Hamada & Bienz, 2004; Sierra et al, 2006). Since regulation of Wg signaling by CtBP comprises the major portion of this dissertation, discussed below are some of the details from available studies in this context, which form the basis for these models.

Activation of Wnt/Wg targets by CtBP

The activation function of CtBP in Wg signaling is a very recent finding from the identification of CtBP in a modifier screen, for components that regulate the Wg pathway (Fang et al, 2006). Although CtBP was known to have antagonistic roles in Wg signaling, a previously unexplored physiological role of CtBP in activation of Wg targets was found, upon analysis in flies and cultured fly cells (Fang et al, 2006). In a fly

epithelial tissue called the wing imaginal disc, CtBP was shown to be required for activation of Wg targets *Sens* and *Dll*. Mosaic clones of cells homozygous for a hypomorphic allele of CtBP showed loss of Sens and Dll, suggesting it is required for activation of these targets (Fang et al, 2006). Misexpression of CtBP enhanced the expression of Dll and a Wg responsive reporter Dll-lacZ, consistent with a positive role of CtBP on regulation of Wg targets *in vivo*.

The mechanism of a novel role of CtBP in Wg signaling was elucidated using Wg readouts in cultured fly cells. CtBP was required for maximal activation of another Wg target *CG6234* (Fang et al, 2006). CtBP was recruited to a Wg responsive cis-element (WRE) of *CG6234* in the presence of signaling (Fang et al, 2006). In order to explore if the activation function of CtBP was mediated through Arm, a chimeric Arm construct containing the hetrologous Gal4DNA binding domain, was used to activate a *UAS* reporter (Hecht et al, 1999). A truncated fragment of Arm containing the N-Terminus was able to recruit CtBP to this reporter and was functionally dependent on CtBP for acticvation (Fang et al, 2006). Therefore CtBP was implicated to be a part of the Arm activation complex for targets like *CG6234* (Figure 1.6). However, whether CtBP interacts directly with Arm, or there are other factors which bridge the interaction of CtBP with Arm, is not yet known. As this is a central question for mechanism of Wg target gene activation by CtBP, it has been discussed in Chapter V of this dissertation.

Consistent with a physiological role of CtBP in activation of Wnt targets, CtBP null mice display phenotypes seen due to loss of Wnt signaling (Hildebrand & Soriano, 2002). One of these includes stage-specific loss of a direct Wnt target *Brachury* (*T*) (Galceran et al, 2001). Although the mechanism of regulation of *T* by CtBP needs to be

elucidated, loss of function suggests that gene specific role of CtBP in activating Wnt targets is conserved from flies to mammals.

Repression of Wnt/Wg targets by CtBP

The role of CtBP in regulating Wnt signaling was first investigated, when it was found as an interacting partner of *Xenopus* Tcf-3(xTcf-3) in a yeast two hybrid screen (Brannon et al, 1999). CtBP was implicated to act as a corepressor with TCF in the absence of signaling. Since then, many labs have tried to explore the TCF dependent role in repression, but have been unable to find an interaction between CtBP and TCF (Fang et al, 2006; Hamada & Bienz, 2004; Valenta et al, 2006). Therefore, this mode of regulation by CtBP is least supported by experimental evidence.

Consistent with a previously known antagonistic role of CtBP in Wg signaling, a study in the Cadigan lab found that CtBP could supress a hyperactive Wg signaling phenotype in flies (Fang et al, 2006). In cultured fly cells, CtBP was required for the repression of a Wg target *naked cuticle* (*nkd*) in the absence of signaling (Fang et al, 2006). CtBP was recruited to the Wingless Response Element (WRE) of *nkd*, and this recruitment was shown to be independent of TCF (Fang et al, 2006). Therefore CtBP was repressing *nkd* in the absence of signaling, independent of TCF. This is the only study till date, which provides evidence for a TCF independent mode of repression by CtBP, which is physiologically relevant. However, the factors which recruit CtBP to the *nkd* WRE are not yet known.

As a mechanism to reset active signaling to an 'off' state, CtBP has been implicated to sequester β-Cat from the DNA bound TCF complex of active targets. This is by binding to a cytosolic and nuclear regulator of Wg signaling called APC (Figure 1.6) (Hamada & Bienz, 2004). The authors in this study convincingly showed that CtBP interacts directly with APC, but could not detect an interaction of CtBP with β-Cat . However, the functional validation of this model was not as strongly supported as the biochemical interaction.

The functional analysis was based on misexpression of APC variants, without many direct correlations of the function of CtBP in regulating Wnt signaling. For example, mutant versions of APC that could not bind CtBP, were shown to be impaired in their ability to antagonize signaling . It was hypothesized that APC's interaction with CtBP was important to sequester β-Cat away from Wnt targets. In order to test the hypothesis, levels of β-Cat in complex with TCF were compared in wildtype versus *CtBP* null cells (Hamada & Bienz, 2004; Hildebrand & Soriano, 2002). In these *CtBP* mutant cell lines, more β-Cat was immunoprecipitated with exogenously expressed TCF when compared to wildtype cells. A Wnt responsive reporter activation was significantly higher in the *CtBP* mutants compared to wildtype cells suggesting that CtBP was required to antagonize signaling. However, the authors did not address if this effect was rescued by misexpresion of exogenous CtBP. Finally, in no context was it shown that CtBP directly regulates Wnt signaling.

One of the missing pieces for making the conclusion that CtBP was sequestering β-Cat from Wnt targets was provided by another report, where CtBP was shown to be recruited to a Wnt target *c-Myc*, by Chromatin – Immmuno Precipitation (ChIP) (Sierra et

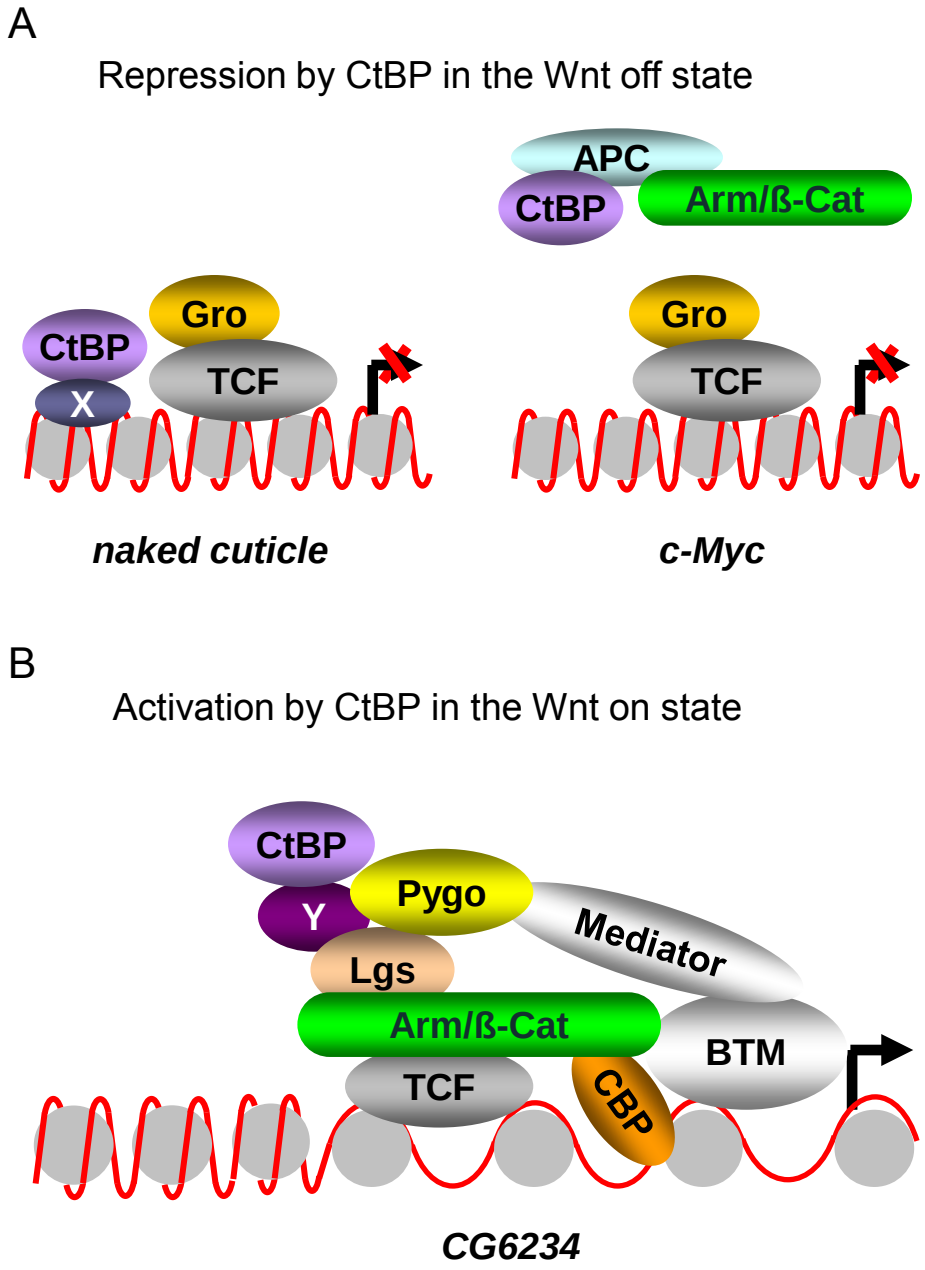


Figure 1.6. Models for activation and repression of Wg targets by CtBP. (A) Cartoon showing the models implicated for repression of Wnt targets by CtBP in the absence of signaling. TCF independent mode of repression by CtBP (left) where it is recruited to the WRE by unknown factor(s). CtBP interacts with APC to sequester β-cat away from TCF (right). (B) Cartoon showing the model implicated for activation of Wnt targets where CtBP is recruited to the N-terminus of Arm through unknown factor(s).

al, 2006). Although not supported by any functional evidence for CtBP, the authors did a time course to show the recruitment of various components of the Wnt pathway to the WRE of a Wnt target *c-Myc* (Sierra et al, 2006). In cultured cells, under active signaling conditions, APC followed by CtBP were recruited to the WRE of *c-Myc*. The recruitment of APC overlapped with a reduction in the transcript levels of *c-Myc* (Sierra et al, 2006).

Following the recruitment of APC and CtBP, there was a decrease in the ChIP signal of B-Cat and other positive regulators of *c-Myc* (Sierra et al, 2006). The time course on the *c-Myc* WRE strongly suggested a 'guilt by association model' where recruitment of full length APC along with CtBP led to a loss of β-Cat and other coactivators, possibly by sequestering B-Cat away from the WRE. However, there was no functional analysis to show that loss of CtBP was important in maintaining *c-Myc* transcriptionally active. Although biochemically well supported, there is not much functional evidence for this hypothesis. Thus it makes it an interesting but weakly supported model in the context of CtBP regulated Wnt signaling.

General mechanisms of repression by CtBP in contexts other than Wnt/Wg signaling

In the screens performed to find interacting partners of CtBP, many factors have been identified (Quinlan et al, 2006b; Shi et al, 2003). A study which used tandem affinity purification (TAP) to immunoprecipitate hCtBP1 from cultured mammalian cells reported many interacting partners of CtBP (Shi et al, 2003). Those included known

interacting partners like a transcription factor ZEB (Postigo & Dean, 1999) and a nuclear receptor RIP140 (Vo et al, 2001). In addition, they also identified chromatin modifying enzymes as novel components of the CtBP repression complex, which include class I histone deacetylases (HDACs) and histone methyl transferases (HMTs).

The Zincfinger/homeodomain containing factor ZEB (Postigo & Dean, 1999) is required for the repression of a target *E-Cad* along with CtBP . CtBP is recruited to *cis-regulatory* elements of *E-Cad* and repression by CtBP is dependent on DNA binding sites of ZEB called *E-Boxes* (Zhang et al, 2006). Repression of *E-Cad* by CtBP has been implicated in epithelial to mesenchymal transition, leading to tumor metastasis (Grooteclaes et al, 2003; Zhang et al, 2006). Due to its role in metastasis, *E-Cad* has been a widely studied and commonly used as a readout to explore the repressive function of CtBP.

In flies, CtBP has been shown to play key roles in patterning of the embryo as CtBP mutant embryos show disrupted segmentation. Some of the defects have been attributed to its function as a corepressor with the Gap genes Knirps, Kruppel and Giant (Keller et al, 2000; Nibu et al, 1998a; Strunk et al, 2001). CtBP has also been shown to interact both genetically and biochemically with Knirps and Kruppel and the interaction motifs on those proteins have been mapped to a five amino acid stretch called PLDLS motif (Keller et al, 2000; Nibu et al, 1998a; Nibu et al, 1998b).

Knirps and Kruppel co-ordinate to specify the pattern of a pair-rule gene *even-skipped* (*eve*) in the fly embryo. CtBP is required for repression of some known *eve* stripe enhancers as loss of CtBP leads to an expansion of the enhancer patterns in the fly

embryo (Nibu et al, 1998a). This is due to interaction of CtBP with the gap genes. For example, ectopic expression of Knirps in a wildtype embryo represses *eve stripe3*, but this repression is dependent on the motif required for its interaction with CtBP. Loss of Knirps or CtBP leads to derepression of some synthetic enhancers containing Knirps binding sites. Similarly, CtBP interaction motifs are required for repression of synthetic reporters regulated by *Kruppel* (Nibu et al, 1998a). Hence, CtBP coordinates with Gap genes for the A/P patterning of the fly embryo.

Apart from roles in A/P patterning, CtBP is also required for other processes during fly development. For Dorsal/Ventral (D/V) patterning of the embryo CtBP has been shown to co-ordinate with a ventrally expressed repressor Snail (Nibu et al, 1998a; Nibu et al, 1998b). Loss of CtBP or Snail leads to an expansion of targets like *Rhomboid* and *Single Minded*, which are repressed by Snail in the ventral mesoderm. In addition, CtBP interacts directly with Hairless (Barolo et al, 2002), which is an antagonist of the Notch pathway. Interaction of Hairless with CtBP and another corepressor Gro is required for Suppressor of Hairless mediated silencing of notch targets in the absence of signaling, during adult mechanosensory bristle development (Barolo et al, 2002). Hence CtBP has context specific roles in different aspects of fly development.

Coordianted role of CtBP with Chromatin modifying enzymes

Modification of the chromatin is one of the most widely known mechanisms in regulating gene transcription (Lodish et al, 1999). In general, acetylation of histones at is associated with transcriptional activity due to a more accessible chromatin and

deacetylation is correlated with transcriptional repression (reviewed in Grunstein, 1997). Effects of methylation are specific to the residue being modified, and can be context dependent for activation or repression (reviewd in Fischle et al, 2003). CtBP has been implicated to work with two classes of chromatin modifying enzymes, namely HDACs and HMTs, and inhibit histone acetyl transferase (HAT) activity to repress genes.

A group which used tandem affinity purification to identify interacting partners of CtBP, found Class I HDACs (HDAC1 & 2) in complex with CtBP (Shi et al, 2003). The immunoprecipitated complex showed HDAC activity on acetylated peptides, specifically Histone3 Lysine 9 (H3K9), although it hasn't been shown if CtBP is required for this activity *in vitro* (Shi et al, 2003). There have been other reports confirming interaction of CtBP with HDACs, albeit indirectly (Kuppuswamy et al, 2008; Subramanian & Chinnadurai, 2003; Zhang et al, 2001; Zhao et al, 2009). Most of these interactions have been tested in the context of mammalian proteins, however an interaction between fly class I HDACs (Rpd3 and HDAC3) and CtBP has not been reported.

There is some evidence that the HDAC activity of immunoprecipitated Knirps and RpD3 (fly HDAC1) is dependent on the CtBP binding site in Knirps (Struffi & Arnosti, 2005), suggesting that this mechanism of repression by CtBP may be conserved. Functionally, a Class I and Class II HDAC inhibitor TrichostatinA has been shown to derepress genes regulated by CtBP in mammalian cells (Shi et al, 2003; Subramanian & Chinnadurai, 2003; Zhao et al, 2009), but not in context of targets regulated by fly CtBP (Ryu & Arnosti, 2003). However the interaction domains of Class I HDACs with CtBP have not yet been mapped. Hence there could be novel motifs on these HDACs which are required for a direct interaction with CtBP.

Alternatively, CtBP might only be stable with HDACs when in a complex with cofactors like ZEB (Kuppuswamy et al, 2008; Shi et al, 2003; Zhao et al, 2009) and CoREST (Kuppuswamy et al, 2008; Shi et al, 2003; You et al, 2001), which have been found in CtBP immunoprecipitates. Hence CtBP has been shown to repress through class I HDACs, albeit indirectly through HDAC inhibitors, in many different contexts. However, these conclusions needed to be treated with caution as there is no convincing evidence yet that recruitment of HDACs occurs through factors like ZEB and CoREST, and that their activity is dependent on CtBP.

CtBP has also been shown to interact with HMTs like EuHMT and G9a (Kuppuswamy et al, 2008; Shi et al, 2003). Immunoprecipitates from CtBP show demethylase activity on core histones and mononucleosomes in vitro at H3K9 and H3K27 (Shi et al, 2003). A H3 peptide acetylated at K9 can be deacetylated and methylated by the CtBP complex *in vitro*. H3K9 monomethylation has been shown to be a CtBP dependent modification, relevant to repression of *E-Cad* gene in cultured mammalian cells (Shi et al, 2003). Depletion of CtBP, EuHMT and G9a leads to significantly greater activation of a *E-Cad* reporter (Shi et al, 2003). Although supported by good biochemical evidence, CtBPs co-ordinated role with HMTs has not been explored functionally in as many contexts as the role of CtBP with HDACs.

CtBP has been shown to interact with HATs p300, GCN5 and P/CAF and inhibit their enzymatic activity *in vitro* (Kim et al, 2005; Meloni et al, 2005; Senyuk et al, 2005), although this mechanism is not functionally supported. One of the studies proposed that CtBP monomers compete for binding to Histones, and showed that a dimerization defective mutant interacts with the Histone binding Bromodomain of p300. However

whether the mutant used in that study is monomeric, is questionable. Hence CtBP might interact with HATs to repress their function, although the exact mechanisms are unclear. Since the functional relevance of the interaction of p300 and CtBP monomers has not been elucidated, it is possible that CtBP monomers function as activators in different contexts, as activation is a more global funciton of HATs.

Role of NAD binding and Catalytic site in CtBP function

Structurally, the high homology to NAD+ dependent dehydrogenases led to many different groups exploring this unique property of CtBP in its transcriptional function. CtBP displays a weak NAD+ dependent dehydrogenase activity *in vitro*, although the biological relevance of this activity is unknown. CtBP has been implicated in sensing cellular redox levels, a property found in very few transcriptional regulators e.g., Sir2 (Imai et al, 2000) and NPAS2 (Rutter et al, 2001). However, Sir2 and NPAS2 use NAD as a substrate while CtBP uses it as a cofactor, and the physiological substrate for the weak dehydrogenase activity of CtBP is not yet known. With several substrates for other dehydrogenases tested so far, CtBP does not show an equivalent catalytic efficiency which suggests that catalysis may not have any biological role in the activity of CtBP (Achouri et al, 2007; Balasubramanian et al, 2003; Kumar et al, 2002).

The role of NAD binding (Zhao et al, 2009) and catalytic activity in the repression function of CtBP is controversial. There are two predicted modes, thought to be prominent in these domains playing any role in the function of CtBP. Firstly, NAD binding has been shown to induce dimerization of CtBP in many biochemical analyses

(Balasubramanian et al, 2003; Kim et al, 2005; Kumar et al, 2002; Thio et al, 2004) although it is not absolutely required (Mani-Telang et al, 2007; Thio et al, 2004). Therefore, NAD binding might affect the oligomeric state and repression by CtBP in some contexts.

Secondly, changes in the NAD+/NADH ratios in the cell is thought to influence the repression function of CtBP, considering that it has ~200 fold higher binding affinity for NADH compared to NAD+ (Fjeld et al, 2003; Zhang et al, 2002). Upon changes in the cellular redox state, more NADH bound CtBP can form dimers, and catalysis to NAD+ can influence the activity of CtBP, possibly due to conformational changes and release of NAD+ (Fjeld et al, 2003).

In several different contexts, mutations in key residues involving NAD binding or catalytic activity do not affect the repressive function of CtBP, suggesting it might not be important (Fang et al, 2006; Grooteclaes et al, 2003; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Phippen et al, 2000; Sutrias-Grau & Arnosti, 2004). In one case, mutating four residues required for catalytic activity abolished the repression function, although the authors fail to test if the mutant had proper nuclear localization or if the mutations were too severe for the native structure of the protein(Kumar et al, 2002). Hence most of the available data point toward these domains not playing a role in the transcriptional activity of CtBP in several contexts.

Interaction motifs found in factors which interact with CtBP

A number of cofactors, which interact with CtBP and are thought to recruit it to target loci, have been shown to have two ‘signature’ CtBP interaction motifs

(Chinnadurai, 2007; Quinlan et al, 2006a; Quinlan et al, 2006b; Turner & Crossley, 2001). The first one is called PXDLS motif (X= any amino acid), and was originally identified in E1A, the first interacting partner of CtBP (Boyd et al, 1993). Most of the CtBP interactions have been shown to be dependent on this motif in one to three copies, eg., ZEB, p300, APC, Kruppel etc. Based on structural information with the PLDLS peptide, mCtBP2 residues interacting with these different motifs have been identified. The PLDLS motif interacts with the N-terminus of CtBP (Figure 1.1) and specifically residues L18, A20, M31, T39, A41, F42, C43, H52 and V55 (Nardini et al, 2003). Consistent with the structural findings, the N-terminus of CtBP has also been shown to be important for repression in some contexts (Meloni et al, 2005; Nardini et al, 2003; Quinlan et al, 2006b; Thio et al, 2004).

A second CtBP binding motif identified in other proteins is termed the RRT motif (consensus: RRTGXPPXL). Crystal structure of mCtBP2 with the RRT peptide shows that two salt bridges R1-D220 and R2-E164 are a key feature of this interaction (Figure 1.1) (Quinlan et al, 2006a). Additionally, side chains of multiple residues make contacts with the RRT peptide and those are Y129, A150, H218, R245, Q246, G247, A248, F249 and R274. Surprisingly, the salt bridge mutations reduce the interaction of CtBP with RRT motif but functionally do not affect its activity in all contexts.

Some proteins which contain the RRT motif also contain additional PXDLS motifs e.g., a Zinc finger protein ZNF217 , where the RRT motif was originally identified (Quinlan et al, 2006a), suggesting that this may play a role in stabilizing protein interactions. In this case, both the PXDLS and RRT motif were shown to be important for CtBP mediated repression of ZNF217. Secondly, it is also possible that each

monomeric subunit of CtBP may intereact with its cofactors, either through the RRT or PXDLS motif, hence recruiting a different set of cofactors through two different binding motifs. There are also proteins which interact directly with CtBP and are independent of the PXDLS motifs eg., Tramtrak (Wen et al, 2000). Therefore, a systematic deletion analysis might lead to mapping of novel interaction domains for known interacting partners with CtBP.

Currently the mechanisms by which CtBP carries out the dual roles in Wnt/Wg signaling are unknown. What factors recruit CtBP to repress targets in the absence of signaling? What factors bridge the interaction of CtBP with the N-terminus of Arm to activate Wg targets? A detailed description of the nuclear effectors of Wnt/Wg signaling is provided to explain the rationale for designing experiments, which can elucidate these mechanisms, as described in Chapter V of this dissertation.

TCF family of proteins and molecular switches

Historical perspective

TCFs get their name from a role in activation of T-Cell specific receptors like CD3ε, which serves as one of the early markers for T-Cell lineage commitment (Travis et al, 1991; van de Wetering et al, 1991; Waterman et al, 1991). TCFs were originally identified in screens for T-Cell specific transcription factors eg., a gel-retardation screen with an enhancer element essential for activation of the CD3ε gene (van de Wetering et al, 1991). Association of TCFs with Wnt signaling came from a yeast two hybrid screen and its interaction with β-Cat, which was the most downstream component of Wnt

signaling known at that time (Behrens et al, 1996; Huber et al, 1996; Molenaar et al, 1996). β-Cat was initially discovered as a cell-adhesion molecule linking the cytoskeleton to cadherins (Nagafuchi & Takeichi, 1989; Ozawa et al, 1989). After its discovery as a component of the Wnt pathway, the molecular mechanisms of signal transduction by β-Cat were not clear. An interaction with TCF laid the foundation for the first connection to a DNA bound mediator, which would complete the circuit of β-Cat translocating to the nucleus to turn on signaling.

Many groups after that showed a functional requirement of TCFs in regulating Wnt/Wg signaling. The first piece of evidence came when misexpression of a TCF family member, Lymphoid Ehancer Factor-1 (LEF-1) in Xenopus embryos led to a secondary axis induction- a classical hyperactive Wnt signaling phenotype (Behrens et al, 1996; Huber et al, 1996; Molenaar et al, 1996). β-Cat binding domains of TCF family members were mapped by two independent studies, which showed that mutants missing these interaction domains had an antimorphic effect on the axis duplication (Behrens et al, 1996; Molenaar et al, 1996). However, a more physiological relevance for the role of TCF in Wg signaling came from studies with the fly TCF.

Fly embryos homozygous for hypomorphic alleles of TCF showed a weak segment polarity phenotype, consistent with its requirement in Wg signaling (van de Wetering et al, 1997). In contrast hyperactive Wg signaling induced by a gain of function mutation in Armadillo (Arm; fly B-Cat), was dramatically suppressed in a TCF mutant background, suggesting that TCF was a downstream component of the pathway (van de Wetering et al, 1997). Supported by more rigorous analyses, TCF was shown to be an essential nuclear mediator of the Wnt/Wg pathway.

TCF family of proteins: conserved domains and structure

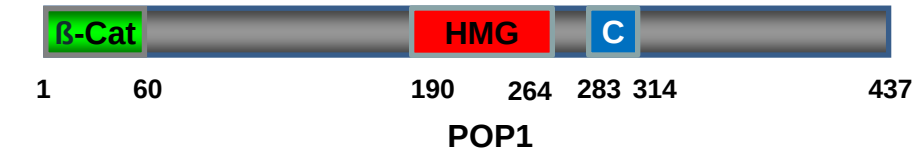
The TCF family of proteins is conserved from hydra to humans and share regions of high homology (reviewed in Arce et al, 2006). Mammals have four TCFs while flies and worms have one. TCF family members share at least two or three conserved domains. These include two DNA binding domains, with one being isoform specific, and a N-terminal β-Cat binding domain (Figure 1.7).

The first DNA binding domain is more centrally located in most TCFs and termed the High Mobility Group (HMG) box. The second one is known as the C-Clamp and is found in a region of the C-terminus termed the E-Tail. There are several different isoforms of TCFs expressed in different cell types (Arce et al, 2006; Hovanes et al, 2000; Van de Wetering et al, 1996; Weise et al) and one of the key difference between various vertebrate isoforms is the presence of the E-tail (Fig). One of the TCF family members LEF-1 (Lymphoid Enhancer Factor-1) does not code for an E-Tail. However a majority of the invertebrate TCF isoforms code for an E-tail and express the C-Clamp. It has been shown for some TCFs that they bind DNA in a bipartite fashion through the HMG box and the C-Clamp (Atcha et al, 2007; Chang et al, 2008b).

HMG Box: the DNA binding domain common to all TCFs

HMG box containing proteins are well known for their DNA binding properties, with TCF family forming one class of HMG containing proteins. The HMG domain is highly conserved amongst TCFs (~95% similarity) (Figure 1.7) (Arce et al, 2006). *In vitro* studies of LEF-1 with double stranded oligo nucleotides have shown that this basic

A



B

Figure 1.7. Domain structure and sequence of TCFs. (A) Schematic of the *C. elegans* TCF homolog POP1 showing the conserved β-cat binding domain, and two DNA binding domains - HMG Box and C-Clamp. (B) Amino acid sequence of the *human* TCF1E, *Drosophila* TCF and POP1 with the three conserved domains boxed.

HMG domain makes contacts with the minor groove of the consensus CCTTTGAA (Giese et al, 1991).

TCFs were originally discovered as T-Cell specific regulators of targets like CD3ε and the cis-element for binding of the HMG box in the enhancer of CD3ε was found to be AACAAAG (inverse of CTTTGTT) (van de Wetering et al, 1991). With functional evidence that TCFs play a role in Wnt signaling, a study used an *in vitro* selex based methodology and found that HMG box of fly TCF preferentially bound the sequence CCTTTGAT (van de Wetering et al, 1997). Thereafter many studies have functionally and biochemically validated this as a 'preferred' and high affinity TCF binding site (Atcha et al, 2007; Chang et al, 2008a; Chang et al, 2008b; Hallikas et al, 2006; Hatzis et al, 2008; Parker et al, 2007).

Synthetic reporters containing this concatemerized site respond potently to Wnt signaling in vertebrate systems (reviewed in Barolo, 2006; Parker et al, 2007). Intriguingly such synthetic reporters containting only HMG sites do not respond well in flies (Barolo, 2006; Chang et al, 2008b), suggesting more information is required for TCFs to activate these 'simple' reporters. Furthermore, genome wide binding studies for TCF suggest that this might not be the only consensus for TCF binding. There are variations to the consensus, important for functioning of *cis-elements* which respond to Wnt signaling termed Wnt/Wg Response Elements (WREs).

A genome wide study, found that in cultured colon carcinoma cells, 30% of about 6800 hTCF4 occupied 1kb regions did not have the consensus HMG motif (Hatzis et al, 2008), although these need to be functionally validated for conclusions. Another study

looking at the genome wide occupancy of β-Cat in another cultured carcinoma cell line, found that 16% of ~400 β-Cat occupied regions did not have the consensus HMG binding motif (Yochum et al, 2007). While this could be explained by other factors recruiting β-Cat to chromatin, considering that a vast majority of Wnt targets have TCF as the DNA binding mediator of β-Cat, it seems likely that there are other sites which TCF might bind to.

It has also been demonstrated that in the context of repression of some targets by Wg signaling, there is a different consenus AGAWAW, required for repression of the WRE reporters (Blauwkamp et al, 2008). These sites are required for repression of the Wnt responsive genes examined and remarkably swapping the AGAWAW sites with CCTTTGAT leads to activation of these WREs (Blauwkamp et al, 2008). This strongly suggests that allosteric regulation of TCFs based on the *cis*-binding element might be one of the key mechanisms differentiating activated targets from repressed targets.

The second property of this conserved domain, which is characteristic of other HMG DNA binding domains is to bend DNA in solution (Behrens et al, 1996; Giese et al, 1992; Love et al, 1995). This bending is shown to be enhanced when Lef-1 is in complex with β-Cat (Behrens et al, 1996). *In vitro* studies suggest that when bound to Lef-1, the consensus oligo CCTTTGAA in a probe shows a bend of about 130º (Giese et al, 1992). DNA bending is one of the mechanisms correlated to bringing transcriptional cofactors in vicinity of the TCF, β-Cat complex to regulate target genes.

C-Clamp motif: the second DNA binding domain found in TCFs expressing the E-Tail

The C-Clamp is also a highly conserved DNA domain amongst the isoforms which express the E-Tail (Hovanes et al, 2000). C-Clamp is so named due to the presence of four cysteines, which are important for DNA binding. Although there is no crystal structure available for this motif with DNA oligos, one interesting hypothesis is that the four cysteines might adopt a Zinc finger like conformation, which is a DNA binding motif found in numerous transcription factors (Atcha et al, 2007).

The fly TCF C-Clamp binds to a consensus sequence GCCGCCA/G termed the Helper site, which is required for activating Wg targets and provides another level of specificity for identification of targets by TCF (Chang et al, 2008b). It has been demonstrated for the fly targets, that only putative WREs containing the Helper sites and HMG sites, in a particular distribution in the genome, are Wg responsive (Chang et al, 2008b). A similar distribution of only HMG consensus sequences alone was not responsive to Wg signaling (Chang et al, 2008b). Hence the biochemical and functional data indicate a bipartitie model of TCF binding to these sites, where Helper sites contribute significantly to this binding and consequently function (Chang et al, 2008b). In an *in vitro* binding assay, a similar extended binding site for the C-clamp of human TCF-1E was identified (A/GCCG) suggesting that the bipartite binding is a conserved phenomenon (Atcha et al, 2007). However, functional validation of this consensus in mammalian WREs is required to show that is is important for Wnt target gene activation.

Fucntionally, the C-Clamp has been shown to be important for activation of fly and mammalian WREs in cultured cells (Chang et al, 2008b). Requirement of C-Clamp for the function of TCF *in vivo* is yet to be determined. Considering that a majority of the mammalian TCF isoforms do not express the E-Tail, it is intriguing what mechanisms are used to acquire specificity in finding targets. An interesting hypothesis is that these isoforms may form hetero-oligomers with ones expressing E-Tails, and use 'Helper' like sequences to locate targets.

β-Cat binding domain

TCF has been shown to interact with β-Cat and the interaction domains have been mapped to 50 N-Terminal amino acids which are conserved (~60% similarity) amongst the various TCFs (Arce et al, 2006). The crystal structures of truncated versions of β-Cat and N-terminus of mammalian TCFs are consistent with at least two conserved alpha helix modules and a beta-hairpin module (for eg., beta hairipin module residues 7-15 and alpha helix module residues 16-29 and 40-52 in xTcF3) which make extensive contacts with central regions of B-Cat (Graham et al, 2000; Poy et al, 2001).

There is significant biochemical and genetic evidence to support that this domain is required for TCF to function in the Wnt/Wg pathway (reviewed in Arce et al, 2006; Parker et al, 2007). The first few studies which mapped the B-Cat interaction domain showed that misexpression of a N-terminal truncated form of TCF was antimorphic and antagonized signaling (Behrens et al, 1996; Hsu et al, 1998; Molenaar et al, 1996). Based on expression analysis, some TCF isoforms missing the B-Cat binding domain are

endogenously expressed. These serve as natural antimorphs of Wnt signaling in those cell types, suggesting that this might be a natural means of keeping the pathway in check (Parker et al, 2007).

TCF as a transcriptional switch

In the absence of signaling, at least two different modes of regulation are utilized to keep the Wnt/Wg signaling off. One is by phosphorylation and consequent proteasome mediated degradation of cytosolic B-Cat aided by the destruction complex. The second is by silencing of targets in some of the known contexts by TCF, in conjunction with other corepressors which keep a check on aberrant expression of the targets. Activation of the pathway causes the 'transcriptional switch' of TCF from a repressor to an activator for positively regulated genes and the intriguing question is how the switch is achieved (Figure 1.8 & 1.9).

Transcriptional switch of DNA binding effectors is a common theme in other conserved developmental signaling pathways also (reviewed in Barolo & Posakony, 2002). In the notch signaling pathway, CSL family of DNA binding proteins are known to repress targets in the absence of signaling while they are required for activation upon signal stimulation. The hedgehog signaling pathway uses Gli/CI proteolytic modifications as a mode of regulation between active and inactive states of signaling to differentially regulate target genes . In the case of Wnt signaling many factors have been reported to aid the TCF mediated repression in the absence of signaling and activate through TCF upon signal stimulation as described in the next few sections.

Repression by TCF in the absence of signaling

Evidence for repression of targets by TCFs in the absence of signaling comes from mutational analysis of HMG binding cis-elements, or mutating TCF proteins, which leads to activation of targets in the absence of signaling. Chronologically the first piece of physiological evidence came with the discovery of POP1, the only TCF gene in C. Elegans (Lin et al, 1995). At that time, POP1 was shown to be required for specifying the Mesoderm fate in the early embryo, but the role of Wnt signaling in C.Elegans was not known. The first *C. elegans* Wnt ligand Mom-4 was discovered after POP1 but it was shown to be required for endodermal fate instead (Thorpe et al, 1997). Therefore, in the early embryo, POP1 was antagonizing the Wnt induced E fate and promoting the MS fate suggesting that POP1 was playing a repressive role in Wnt signaling.

This was inconsistent with the known positive roles of mammalian and fly TCFs in Wnt/Wg pathway. At around the same time, the fly *Ultrabithorax* (*Ubx*) gene was shown to be a target of Wg. Mutation of three TCF sites in the WRE of *Ubx* led to a mild derepression of Ubx in the fly midgut suggesting that TCF was repressing *Ubx* in some tissues (Riese et al, 1997). There was a loss in expression of *Ubx* in tissues where it is activated by Wg signaling (Riese et al, 1997). Consistent with a role in repression of Wg targets, many targets since then have been shown to be repressed by TCF in the absence of signaling both in vertebrates and invertebrates eg., *Siamois* (Brannon et al, 1999) and *naked cuticle* (Fang et al, 2006).

Role of *cis-elements* in the switch

There are many WREs contain multiple HMG sites which are important for target gene activity. One intriguing question which stems from that is if these sites promote both the activation or repression function, or are there dedicated sites for either of the two functions. The only known target which requires one HMG consensus for both activation and repression is a worm endoderm determining gene *end-1*. Mutation of the HMG site leads to depression of a *end*-1 reporter in the MS cells, while loss of Wnt mediated activation in the E cells (Shetty et al, 2005). A similar reduction is seen in a POP1 mutant background, which can be rescued by wildtype POP1, but not a HMG box mutant (Shetty et al, 2005) suggesting that in this case, the same HMG site mediates the dual roles POP1.

However, considering that the fly TCF has been shown to require additional cis-elements called Helpers, it is possible that different putative helpers in the *end-1* WRE mediate formation of different POP1 activation and repression complexes via a common HMG site. Presence of Helpers in another worm target *ceh-22* has been explored in Chapter IV of this dissertation.

Mechanisms of Wnt/Wg target gene repression in the absence of signaling

There are many factors known to co-ordinate with TCF in order to repress targets in the absence of signaling (Figure 1.8). They have been categorized into TCF dependent and TCF independent factors and discussed in the following section.

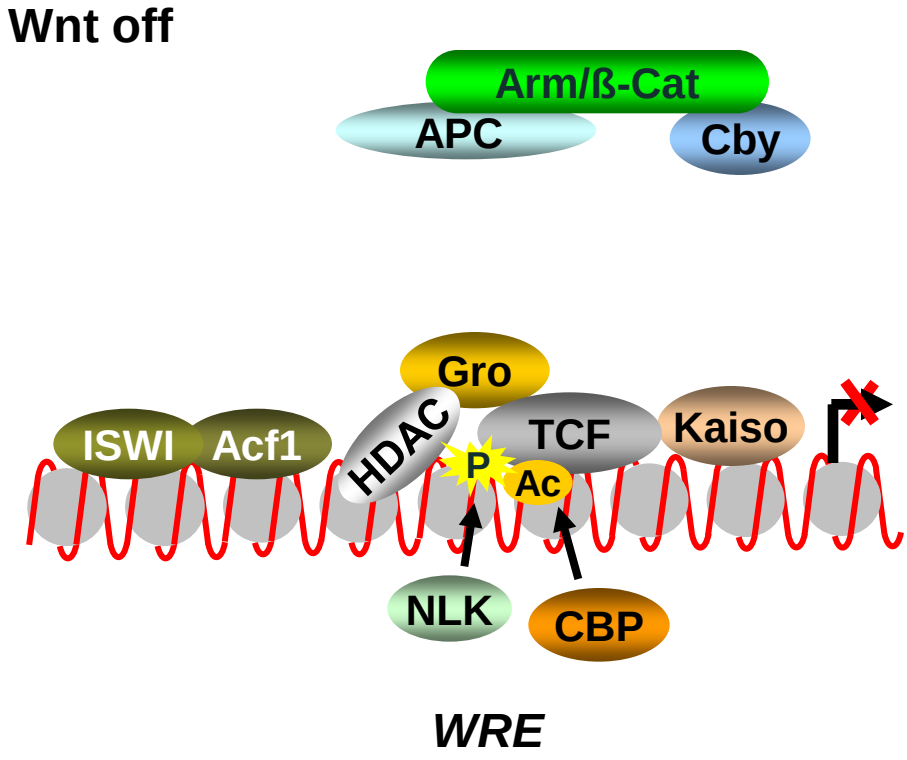


Figure 1.8. TCF represses target genes in concert with corepressors during the Wnt off state. Cartoon showing the different cofactors recruited to Wingless Response Elements (WREs) for repression of targets in the absence of signaling. Recruitment of Gro is TCF dependent while Kaiso is TCF independent, although it interacts with TCF. Gro recruits chromatin modifying HDACs and function. Chromatin remodelling factors ISWI/Acf1 are recruited independent of TCF. TCF gets phosphorylated by NLK and acetylated by CBP which reduces its affinity for β-cat. In the absence of signaling, β-cat which escapes the degradation complex is sequestered in the nucleus by 'spunges' like APC and Cby.

TCF dependent factors

Groucho

A more direct genetic evidence for TCF being a repressor came when loss of function alleles of *TCF* suppressed Wg mutant phenotypes in the fly embryonic cuticle (Cavallo et al, 1998). TCF was originally discovered as a positive regulator of the pathway, so the reduced severity of Wg and TCF double mutants compared to Wg single mutants came as a surprise. In the same screen the authors found a corepressor Gro, which also suppressed the Wg mutant phenotypes consistent with an antagonistic role of Gro, similar to TCF (Cavallo et al, 1998).

In parallel, another group found Gro in the same two hybrid screen in which β-Cat was found to be an interacting partner of TCF (Roose et al, 1998). Misexpression of Gro could anatagonize Wg signaling in Xenoups embryos, by inhibiting dorsal cell fates (Roose et al, 1998). Gro misexpression was also shown to reduce endogenous transcripts of two Wnt targets known to be important for axis specification (Roose et al, 1998). Gro was shown to interact TCF, and the interaction domain mapped to region overlapping the N-terminal β-Cat binding domain. Mammalian Gro homologs have been shown to interact genetically with the different TCFs, which share a common Gro interaction domain for repression (Brantjes et al, 2001). Gro has also been shown to be transiently recruited to the WRE of a Wnt target *c-Myc* in cultured mammalian cells, in the absence of signaling (Sierra et al, 2006), or with other negative regulators to shut off signaling.

Therefore, Gro is till date the most well characterized corepressor of the Wnt/Wg pathway in the absence of signaling.

HDACs

Gro family members have been shown to exercise their repressive function by through HDACs, the enzymatic activity of which leads to a less accessible chromatin. The fly HDAC1 homolog Rpd3 has been shown to bichemically interact with Gro (Chen et al, 1999). Genetically double mutants of Rpd3 and Gro show more severe cuticular defects compared to Gro or Rpd3 alone (Chen et al, 1999). Mammalian HDAC-1 has also been shown to interact with Gro and antagonize Wnt reporter activation (Brantjes et al, 2001). Similar to recruitment of Gro, HDAC-1 is also transiently recruited to the WRE of a Wnt target *c-Myc* (Sierra et al, 2006).

Another convincing demonstration for repression by TCF in concert with Gro and HDAC-1 comes from loss of function analysis in *C. elegans*, suggesting that this mechanism of gene silencing in the absence of signaling is highly conserved. As was demonstrated in one study, depletion of the worm homologs of Gro, HDAC-1 and TCF led to derepression of *end-1*, an endoderm fate determining gene at early embryonic stage. Using an endodermal marker, the authors show that the MS cells were transformed into the E fate upon depletion of these components, which is indicative of hyperactive Wnt signaling (Calvo et al, 2001).

Furthermore, in cultured mammalian cells it was shown Gal4POP1 was able to repress the basal activity of a *UAS*-reporter (Calvo et al, 2001). POP1 mediated

repression of the *end-1* reporter was greatly enhanced by misexpression of HDAC and Gro, and the HMG domain of POP1 was required for this repression (Calvo et al, 2001). Therefore, a co-ordinated role of these factors is required for repression of endogenous Wnt targets in the absence of signaling.

TCF independent factors

ISWI/Acf1

Apart from the chromatin modifying HDACs, ISWI and Acf1 which are ATP dependent chromatin remodeling factors of the ISWI family, contribute to repression of Wg targets in the absence of signaling. ISWI and Acf1 are required for repression of several Wg targets in the developing wing (Liu et al, 2008). ChIP analysis suggests that they are recruited to broad regions on target gene loci and their depletion in cultured fly cells leads to derepression of several Wg targets in the absence of signaling (Liu et al, 2008). This derepresison is not due to defects in a more global role of the remodeling complex like post-mitotic chromatin assembly, rather is more specific to Wg targets. Furthermore, stimulation by Wg reduces the levels of widespread localization of Acf1 (Liu et al, 2008) demonstrating how Wg signaling may relieve the repression complexes along the gene loci for activation of targets .

Kaiso

Another TCF independent mode of repression is by a DNA binding protein known as Kaiso. Depletion of Kaiso or mutation of a Kaiso binding *cis-element* was

shown derepress some Wnt targets in *Xenopus* embryos (Park et al, 2005). Furthermore, derepression of a Wnt target *Siamois* reporter was rescued upon misexpression of *xenopus* TCF3 suggesting that TCF3 and Kaiso synergize to repress *Siamois* (Park et al, 2005). Misexpression of Kaiso suppresses the β-Cat induced secondary axis duplication of *Xenopus* embryos (Park et al, 2005), consistent with an antagonistic role in Wnt signaling.

However, the mechanism of repression of Kaiso may not be totally independent of TCF as they interact with each other, although it is not known if Kaiso recruitment to WREs is dependent on TCF. Apart from a role in repressing Wnt targets, Kaiso is also required for Wnt stimulated activation of the *Siamois* reporter in the dorsal marginal zone, a region where Wnt signaling turns on *Siamois* in *Xenopus* embryos (Iioka et al, 2009). Hence in this context Kaiso contributes to the switch of TCF from a repressor to an activator upon signal stimulation.

Chibby

A different mechanism of keeping signaling off before ligand binding or post stimulation, is through proteins called β-Cat/Arm 'buffers' like I-Cat, Chibby (Cby) and the APC-CtBP complex. Current evidence based on loss of function analysis of Cby suggests that in the absence of signaling, some of the Arm is in the nucleus, presumably escaping the degradation complex (Parker et al, 2007). A dramatic example comes from the analysis of the *Ubx* reporter in the fly embryonic midgut, which is ectopically activated upon depletion of *Cby* in a Wg mutant background (Takemaru et al, 2003).

However this activation is Arm dependent, suggesting that Cby antagonizes Arm, intriguingly in the absence of stimulation by Wg (Takemaru et al, 2003).

In mammalian cells, Cby has been shown to antagonize β-Cat mediated reporter activation. Although this was not at the level of β-Cat stability, Cby was shown to interact with β-Cat and compete with Lef-1 for binding to β-Cat (Takemaru et al, 2003). Evidence from more recent reports unravels a mechanism where Cby exports β-Cat from the nucleus in conjunciton with a protein 14-3-3 (Li et al, 2008). Hence these data suggest that there is a pool of Arm which escapes degradation in cells unstimulated by Wg. Therefore, proteins like Cby act as 'sponges' to soak up the nuclear β-Cat/Arm which 'erroneously' escapes the destruction complex in the absence of signaling.

Mechanisms of activation of Wnt/Wg targets

The nuclear switch of TCF from a repressor to an activator or vice versa is dependent on nuclear β-Cat levels as has been demonstrated by several studies (reviewed in Mosimann et al, 2009; Parker et al, 2007). How is this switch achieved? While there are many possibilities e.g., β-Cat sequestering the repressors or separate sites of TCF which are bound by repression and activation complexes, the experimental evidence argues that β-Cat replaces the repressors associated with TCF to activate target genes (Figures 1.5 & 1.9).

This was demonstrated biochemically for Gro, which is the most well-characterized corepressor in the absence of signaling (Daniels & Weis, 2005). β-Cat and Gro were shown to compete for an overlapping binding site on TCF *in vitro* (Daniels &

Weis, 2005). Consistent with a displacement model, ChIP analysis of the WRE of a Wnt target *c-Myc* suggested that the recruitment of corepressors like Gro and coactivators like Arm was non-overlapping (Sierra et al, 2006). In that study, the authors did a time course to show the recruitment of various components of the Wnt pathway to the *c-Myc* WRE in cultured cells. Gro was shown to be present at the *c-Myc* WRE before signal stimulation, however was absent upon recruitment of the coactivators β-Cat, Lgs, Pygo and p300 (Sierra et al, 2006). TCF/Lef was present at this WRE throughout the time course (Sierra et al, 2006), supporting a switch model of TCF/Lef from a repressor to activator upon signal induction.

These data indicate that recruitment of β-Cat is not only essential to activate targets, but also to relieve the repressive effect. This is also evident by derepression of some targets e.g. *nkd*, in the absence of corepressors like Gro (Fang et al, 2006). The transcriptional switch of TCF to displace the repression complex and recruit the activation complex seems to be the mode of activation of Wnt targets. Discussed below are the known components of the activation complex.

β-Catenin

The term 'β-Catenin' gets its name from the latin word 'Catena' which means chains. Catenin proteins were so named because of their function as linkers of cytoskeleton to the cell-adhesion molecules. Originally discovered as an interacting partner of E-Cadherin (E-Cad), this 94KDa protein was shown to be missing from lysates of mouse fibroblast expressing C-terminal deletion mutants of the E-Cad in

Wnt on

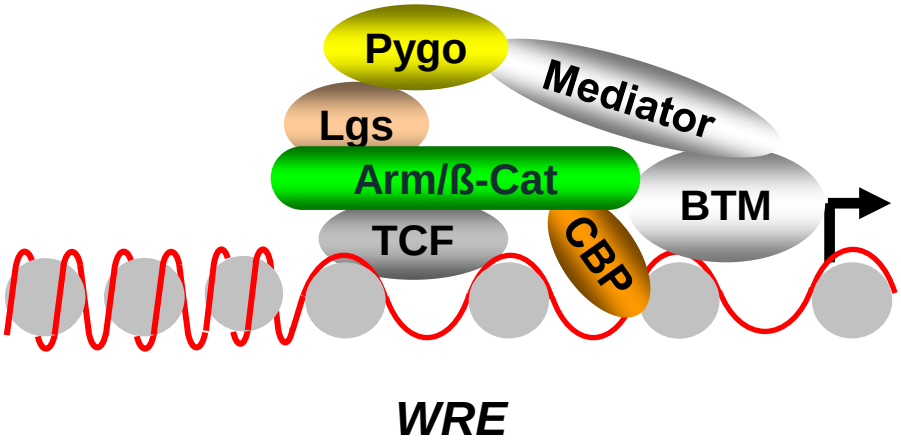


Figure 1.9. Nuclear switch of TCF from a repressor to an activator by β-cat in the Wnt on state. Cartoon showing the different cofactors recruited to Wingless Response Elements (WREs) for activation of targets upon stimulation by Wnts. β-cat is recrutied by TCF. Lgs is recruited by the N-terminus of β-cat and Pygo is recriuted by Lgs. CBP is recruited by the C-terminus of β-cat. Pygo associates with the mediator complex and C-terminus of β-cat associates with TATA-binding protein, a component of basal transcriptional machinery (BTM).

immunoprecipitation assays (Kemler & Ozawa, 1989). A subsequent report identified three proteins associated with E-Cad in immunoprecipitates and direct interaction assays thus naming them α-Catenin, β-Catenin and γ-Catenin for their role in linking the cytoskeleton to E-Cad (Ozawa et al, 1989).

The fly β-Cat homolog Armadillo was discovered as a gene required for establishing segment-polarity of fly embryos (Nusslein-Volhard & Wieschaus, 1980). However, Arm and β-Cat were categorized in the same family in the pre-genome era (Peifer & Wieschaus, 1990), and functionally connected to mammalian Wnt signaling, based on an antibody directed against Arm which could detect β-Cat (Peifer et al, 1992). Injection of antibodies against Arm into *xenopus* embryos led to axis duplications, phenocopying hyperactive Wnt signaling (McCrea et al, 1993). In flies, *Arm* mutant embryos had segment polarity phenotypes similar to that of Wg, suggesting they might be acting in a similar manner during development (Noordermeer et al, 1994; Nusslein-Volhard & Wieschaus, 1980; Siegfried et al, 1994). Hence based on the mutant phenotypes, it was deduced that β-Cat /Arm might be a part of Wnt/Wg signal transdsduction.

Considering that β-Cat had a key role in cytoskeltal contact at cell membrane with E-Cads and was implicated in Wnt/Wg signaling, its role as a nuclear effector of Wnt/Wg signaling was surprising. In flies it was known that Arm was regulated post-transcriptionally by Wg (Riggleman et al, 1990), and subject to phosphorylation (Peifer et al, 1994). β-Cat was implicated to have transactivation potential narrowed down to the C-terminus (Peifer & Wieschaus, 1990) and later on shown to interact with components of the basal transcriptional machinery (Hecht et al, 1999).

Although misexrpression of the C-terminus of Arm/ β-Cat had hyperactive Wnt phenotypes in some assays eg., secondary axis induction in *xenopus* embryos (Vleminckx et al, 1999), it was not sufficient to rescue the loss of Arm in flies (Cox et al, 1999). Futhermore excess β-Cat in the nucleus could antagonize Wnt signaling (Prieve & Waterman, 1999). This suggested that other cofactors aid the activation of Wnt target by β-Cat, which might be squelched upon misexpression. Thereafter, studies with β-Cat truncation fusions in yeast were shown to activate a reporter, and activation potential of β-Cat narrowed down to two potent transactivation domains – one at the N-terminus and the other at the C-terminus (Hsu et al, 1998). Only the C-terminal transactivation domain was shown to interact with components of the basal transcriptional machinery (Hecht et al, 1999). Hence it was deduced that there are other cofactors required for activation through the N-terminus.

Structural analysis suggests that Arm defines a class of its own. Named after Armadillo, the protein has twelve of the so called 'Arm repeats' (Riggleman et al, 1989), with each repeat being a superhelical segment comprising of three alpha helices (Huber et al, 1997). Arm repeats in B-Cat form a highly positively charged surface groove, which have shown to make surface contacts with various factors (reviewed in Mosimann et al, 2009; Parker et al, 2007). Consistent with that, the N-terminus and C-terminus have been shown to interact with several cofactors which aid in the transcriptional functions of β-Cat.

The N-Terminal transactivation domain

The discovery that the N-terminus of Arm contains a transactivation domain came from a systematic analysis of β-Cat deletion fragments in cultured mammalian cells (Hsu et al, 1998). However, with the concern that overexpression of the truncated fragment was stabilizing endogenous Arm, the analysis was carried out in yeast, which lack β-Cat and almost all components for the Wnt pathway (Hecht et al, 1999). Truncated N-terminal Arm/ β-Cat fragments upto Arm repeat 6 were shown to activate reporters by a chimeric β-Cat recruited to DNA (Hecht et al, 1999).

Consistent with that, other reports have shown that N-terminus of β-Cat activates Wnt/Wg responsive reporters in various cell culture assays (Fang et al, 2006; Stadeli & Basler, 2005). A further systematic deletion analysis narrowed down the minimal domain to be the Arm repeat regions 1-4, which were sufficient in potently activating Wg responsive reporters in fly cell culture (Stadeli & Basler, 2005).

Two cofactors have been shown to interact with the N-terminal region of Arm named Legless (Lgs) and Pygopus (Pygo) (Kramps et al, 2002). Lgs and Pygo are required for the transactivation by N-terminal domain of Arm. This was evident when a minimal fragment with the Arm repeat 1-4 fused to the transactivation domain of Pygo could potently activate reporters in the absence of endogenous Lgs and Pygo (Stadeli & Basler, 2005). The assembly of the transcriptional complex has been termed ‘chain of adaptors’ (Stadeli & Basler, 2005), considering that TCF, Arm, Lgs and Pygo act as adaptors for the complex assembly.

The C-terminal transactivation domain

The activation function of Arm was mapped to the C-terminus based on alleles of Arm, which displayed more severe mutant phenotypes with longer C-terminal deletions (Cox et al, 1999). In *xenopus*, misexpression of the C-terminus of β-Cat was shown to induce hyperactive Wnt phenotypes (Vleminckx et al, 1999). A chimera with Gal4 containing only the C-terminal region downstream of Arm repeat 12 was shown to have transactivation function (Hecht et al, 1999). Many studies have shown that this construct is an activator in cell culture (Fang et al, 2006; Hsu et al, 1998; Stadeli & Basler, 2005).

The C-terminal transactivation domain has been shown to interact with a different set of cofactors compared to the N-terminus. Those are the chromatin modifiying HAT p300/CBP, chromatin remodeling Brahma complex, a protein interacting with the basal transcriptional machinery known as Hyrax and TATA Binding Protein, a component of the basal transcriptional machinery (reviewd in Mosimann et al, 2009; Parker et al, 2007). Hence, based on *in vitro* interaction assays, the C-terminus of β-Cat activates by interaction with the components of the basal transcriptional machinery, while the N-terminus through Pygo and the mediator complex (Carrera et al, 2008). Hence multiple interactions of the full length B-Cat with other cofactors are required for maximal activation of Wnt/Wg targets.

Arm domains involved in gene-specific repression

Wnt/Wg signaling also leads to β-Cat/Arm mediated repression of many targets (reviewed in Parker et al, 2007). Arm mediated repression has a different mechanism

that activation, as was demonstrated for repressed targets in fly cell culture (Blauwkamp et al, 2008). A mutant version of Arm termed as 'Disarmed' has shown to be required for repression of Wg targets, though not competent for activation (Blauwkamp et al, 2008).

Disarmed carries a point mutation that abolishes Lgs binding and has a C-Terminal truncation removing the region downstream of the Arm repeats (Blauwkamp et al, 2008). This suggests that components that interact with N-terminus and C-terminus of Arm for activation, are not required for repression by Arm. Intriguingly, TCF still follows the switch model for repressed targets, as these targets have basal activity in the absence of signaling (Blauwkamp et al, 2008). Hence Arm mediated repression may be a result of displacement of unknown coactivators from these targets or recruitment of a novel set of cofactors to repress actively transcribed genes.

Legless

The N-terminal transactivation of Arm has been attributed to association with 'adaptors' Lgs and Pygo. Lgs was originally identified in a modifier screen for genes which would dominantly suppress a ectopic Wg phenotype (Kramps et al, 2002). Consistent with a being a key component of the Wnt pathway, it was shown to be essential for many Wg directed developmental events downstream of Arm stabilization (Kramps et al, 2002).

Lgs has three highly conserved domains termed the homology domains (HD) HD1, HD2 and HD3. While HD3 is dispensable for the function of Lgs in Wg signaling, HD2 interacts with Arm while HD1 interacts with Pygo (Kramps et al, 2002).

Remarkably, a Pygo-HD2 fusion fragment completely rescues severe mutant phenotypes of both Lgs and Pygo in flies (Kramps et al, 2002). An Arm-PygoNHD fusion construct can rescue activation of a Wg responsive reporter upon loss of Pygo and Lgs in cultured fly cells (Stadeli & Basler, 2005). Therefore, Lgs is a nuclear adaptor linking Pygo to the N-terminus of Arm to activate Wg targets.

Pygopus

Pygo was discovered independently in different screens and has an essential role in many Wg directed developmental contexts (Belenkaya et al, 2002; Kramps et al, 2002; Parker et al, 2002; Thompson et al, 2002). Further investigation of this novel member in the Wg/Wnt pathway showed that the loss of function phenocopied loss of Wg signaling in several different tissues. Epistasis analysis for Pygo in fly embryos and wing primordium indicated that it was downstream of Arm stabilization (Parker et al, 2002).

Pygo has two conserved domains known as the plant homeodomain (PHD) and N-terminal homology domain (NHD) (Kramps et al, 2002). The PHD domain of Pygo interacts with Lgs and is required for recruitment of Pygo to the Arm activation complex (Kramps et al, 2002; Stadeli & Basler, 2005). The NHD domain is required for activation function of Pygo in the arm compelx (Hoffmans et al, 2005; Stadeli & Basler, 2005). The NHD contains a conserved nuclear localization signal and fly pygo has been shown to predominantly nuclear (Parker et al, 2002).

In one of the intial studies it was shown that Lgs nuclear localization was affected in Pygo mutant clones hence suggesting that it may be recruiting Lgs and Arm to the

nucleus (Townsley et al, 2004). However another group provided convincing evidence for the fact that Pygo recruitment through Lgs is essential for transcriptional activity of Arm/ β-Cat, and not just its nuclear localization (Hoffmans et al, 2005). In fly embryos, a point mutant of *Lgs* could not rescue the Wg signaling defects, even with nuclear localized constitutive form of Arm (Hoffmans et al, 2005). Furthermore, a point mutant of Pygo NHD which could bind Lgs and is localized to the nucleus, could not rescue Wg signaling defects in a Pygo mutant background (Hoffmans et al, 2005). These data provided strong evidence for the fact that Pygo's NHD domain plays a crucial role in the transcriptional activity of Arm/ β-Cat.

Consistent with a requirement for transcriptional activity, Pygo was shown to be one of the key components for the Arm activation complex. Pygo was shown to interact directly with Lgs, and co-immunoprecipitate Arm/ β-Cat in various mammalian cell lines (Kramps et al, 2002). Misexpression of Pygo also antagonized Wg signaling in some contexts, probably due to antimorphic effects on the activation compelx (Parker et al, 2002).

Pygo is recruited by Lgs to the N-terminal of Arm through the PHD domain binding to the HD1 of Lgs (Kramps et al, 2002), and has a transctivational function in the NHD (Belenkaya et al, 2002; de la Roche & Bienz, 2007; Stadeli & Basler, 2005). It contains a conserved NPF motif, one of the signature motifs in some transactivators (Stadeli & Basler, 2005)(Basler). The NPF tripeptide is essential for the transactivity of Pygo (Stadeli & Basler, 2005), but the Lgs binding PHD domain is dispensable (Kramps et al, 2002; Stadeli & Basler, 2005).

Mechanistically, NHD domain of Pygo interacts with components of the mediator complex Med 12 and Med 13, which is the nexus to the basal transcriptional machinery (Carrera et al, 2008). These mediator subunits are essential for activation of some Wg targets in the wing primordium and interact with the Arm activation complex in cultured cells (Carrera et al, 2008). Hence the N-terminal transactivity of Arm is through interaction with Pygo and the mediator complex in many contexts, although it is not yet known if Pygo interacts directly with the mediator proteins.

Pygo is associated some Wg targets in the absence of signaling. ChIP assays have shown that this recruitment is TCF dependent (de la Roche & Bienz, 2007). What does association of Pygo with target genes in the absence of signaling imply? Immunostains of Pygo and TCF on *Drosophila* polytene chromosomes, which are devoid of Wg signaling, showed that they have an overlapping localization pattern (de la Roche & Bienz, 2007). This pattern was shown to be dependent on the NHD domain of Pygo but not on the Lgs binding PHD domain (de la Roche & Bienz, 2007). Therefore, it was hypothesized that in the absence of signaling, Pygo might serve as a cognate protein for TCF for recruitment of Arm to target loci, although not functionally validated.

Another mode for Pygo to function as a positive regulator of signaling has been suggested, where it serves as an anti-repressor, implicated to relieve the repression of Gro on target genes (Mieszczanek et al, 2008). In fly embryos, Pygo is required for activation of a Wg target *En* and for Wg dependent A/P segment polarity (Parker et al, 2002). In Gro mutant embryos, the requirement of Pygo is not completely required for these Wg dependent events (Mieszczanek et al, 2008).

However, most of the Gro dependent phenotypes have been tested in tissues where Wg signaling is active, which makes the anti-repressor model potentially weak. One possibility is that removal of Gro in the presence of Arm may bypass the need for Pygo, as Arm has atleast two potent transactivation domains. It is possible that in a Gro mutant background, the C-terminal transactivation is enough for target gene activation. Also considering that Gro has many roles in embryonic segmentation, the anti-repressor Pygo effect may be indirect, as Wg is required only for maintainance of *En* in the embryo, and not the activation. Therefore, testing this anti-repressor effect in cultured cells may be better to decipher the mechanisms underlying the effect. Association of Pygo with the chromatin in the absence of signaling to recruit Arm, seems to be the more attractive model compared to the anti-repressor model.

CBP

One of the key regulatory events for recruiting the basal transcriptional machinery to proximal promoters is making the chromatin more accessible. Histone Acetyl Transferases (HATs) are one family of enzymes which reversibly acetylate histone tails in order to loosen the DNA-histone complexes (Grunstein, 1997). The C-terminal region of β-Cat has been shown to interact with two closely related HATs known as CBP (CREB- Binding Protein) and p300 (Hecht et al, 2000; Li et al, 2007). These HATs have been shown to play a positive role in enhancement of β-Cat mediated transcriptional activation, as judged by Wnt transcriptional readouts in cultured cells (Parker et al, 2007).

As HATs are enzymes which globally induce histone acetylation, loss of function analysis has been extremely hard to demonstrate their physiological roles in specific contexts. However, in cultured fly cells, CBP was shown to be required for Wg stimulated activation of several targets (Li et al, 2007; Parker et al, 2008). Functionally CBP was shown to enhance the transactivation function of the C-terminus of Arm (Li et al, 2007). Consistent with a requirement for activation of Wg targets under physiological conditions, CBP mutant mosaic cells show a loss of Wg readouts in fly wing imaginal discs (Li et al, 2007).

CBP has also been shown to be recruited to several Wnt/Wg targets during active signaling (Li et al, 2007; Sierra et al, 2006). Furthermore, this recruitment has been shown to induce widespread chromatin acetylation for several Wg targets in cultured fly cells and fly embryos (Parker et al, 2008). This is consistent with a model where CBP nucleates essential chromatin modification events. These might relieve the inhibitory roles of other chromatin modifying and remodeling enzymes in order to activate target genes.

Modifications of TCF

Acetylation

In addition to a positive role in Wnt signaling, CBP antagonizes signaling through mechanisms different from its role in activation of Wnt targets. *In vitro*, CBP interacts with one of the mammalian TCFs (Li et al, 2007) and acetylates the fly TCF on a Lysine 25 which lowers its affinity for Arm (Waltzer & Bienz, 1998). CBP antagonizes Wg

signaling in some contexts, consistent with acetylation of TCF by CBP affecting Arm and TCF interaction (Waltzer & Bienz, 1998). In cultured mammalian cells, depletion of CBP and p300 enhances the response of a Wnt stimulated reporter (Li et al, 2007). Therefore, CBP/p300 are bimodal regulators of Wnt/Wg signaling.

Phosphorylation

Another mode of Wnt target gene regulation is by phosphorylation of TCFs, first observed in worms where members of the Map Kinase (MAPK) group of proteins Lit-1 and Mom-4 were found to have Wnt like phenotypes (Meneghini et al, 1999; Shin et al, 1999). In worms, one of the major roles of Wnt signaling is to specify anterior cell fates and this is achieved by asymmetrically distributing the levels of worm TCF homolog POP1, in EMS blastomere. Lit-1 and Mom-4 mutants do not show an asymmetric nuclear division of POP1 suggesting Lit-1 might be regulating POP1 levels (Meneghini et al, 1999; Shin et al, 1999). RNAi depletion assays have shown that POP1 is downstream of Lit-1(Rocheleau et al, 1999). Similarly the MAPKKK Mom-4 regulates POP1 asssymetry in the anterior posterior cell divisions (Shin et al, 1999).

When initially discovered, a surprising aspect of POP1 mutant was that its embryonic phenotypes were completely opposite of the Wnt or β-Cat mutant phenotypes, suggesting that it was an antagonist of signaling (Lin et al, 1995; Thorpe et al, 1997). However, one key difference in the molecular mechanism of Wnt signaling in worms is that the only worm β-Cat known at that time, WRM1, did not interact with POP1, but was shown to interact with the MAPK Lit-1 (Rocheleau et al, 1999). *In vitro*

phoshporylation assays showed that POP1 could directly be phosphorylated by Lit-1 and this was WRM1 dependent (Rocheleau et al, 1999). The most striking result was that in Lit-1 mutants POP1 was predominantly nuclear (Meneghini et al, 1999), suggesting that titrating the levels of POP1 from the nucleus as a result of phosphorylation was the key to activate Wnt targets. Consistent with that, a nuclear export protein Par5 was shown to be required for the asymmetry of POP1 and phosphorylation by Lit-1 was important for the asymmetry (Lo et al, 2004). This formed the basis of a novel mechanism of Wnt signaling in worms where WRM1 dependent phosphorylation of POP1 by Lit-1 was required to activate Wnt signaling

This mechanism may be conserved for regulation of TCFs in mammals too, although the physiological relevance of this mechanism has not been extensively explored. The TGF-β Activated Kinase1 (TAK1) and Nemo Like Kinase (NLK), which are homologs of the MAP3K and MAPK respectively, antagonize signaling in cultured mammalian cells and Xenopus embryos (Ishitani et al, 1999). NLK can directly phosphorylate some TCFs and antagonize B-Cat complex formation, suggesting that phosphorylation may be weakening the affinity of TCF for B-Cat (Ishitani et al, 1999).

The phosphorylation sites have been mapped to Serine/Threonine residues in LEF-1and TCF-4 between the B-Cat binding domain and the HMG box, which are also conserved in POP1 (Ishitani et al, 2003). These residues have been shown to be important for NLK mediated antagonism of Wnt activated reporters in cultured cells (Ishitani et al, 2003). However, mutation of these residues does not completely abolish the NLK mediated phosphorylation suggesting that there are additional phosphorylation sites (Ishitani et al, 2003).

A more rigorous analysis of this mechanism carried out in worms led to the identification of five additional Serine/Threonine residues, important for nuclear export of POP1 (Lo et al, 2004). Out of the five, two serines were identified as substrates for NLK and phosphoryaltion at these sites was shown to be important for the interaction of POP1 with a nuclear export protein Par-5 in cultured mammalian cells (Lo et al, 2004). However mutation of these two sites rescued the POP1 mutant phenotypes completely (Lo et al, 2004), suggesting that the additional serines were functionally required for nuclear export of POP1. Although there is increasing evidence for a direct role of POP1 in activating Wnt targets in worms (Phillips & Kimble, 2009), relieving the repression of POP1 by nuclear export is a major mode of activating Wnt signaling in worms. However, the physiological relevance of phosphorylation of TCFs in flies and mammals is yet to be determined.

Summary of the results presented in this dissertation:

Currently there is no insight into the mechanisms which govern the differential role of CtBP in regulating Wg targets. What provides it the ability to be recruited to activation and repression complexes? Is it a gene-specific switch forming a docking site for activation and repression complexes? I have tested the hypothesis that quartenary structure of CtBP determines its role in activation and repression of Wg targets. CtBP monomers are regulating activated Wg targets while CtBP oligomers are regulating repressing Wg targets. Hence dimerization provides a differential activity to CtBP.

Does CtBP interact with known regulators of the pathway, which include Gro, Kaiso, TCF, HDACs and/or ISWI/Acf1 (Figure 1.8) to repress Wg targets in the absence of signaling? Are there previously unidentified transcription factors which recruit CtBP to WREs for repression? Based on a previous study and unpublished data from the Cadigan lab, we know that CtBP has a mode of repression independent of TCF and Gro, and based on unpublished data it is independent of ISWI/Acf1. So other known candidates are being currently tested.

For activation of Wg targets, CtBP is recruited by the N-terminal transactivation domain of Arm, but a direct interaction with Arm has not been detected so far. What factors might be bridging the interaction of CtBP with Arm? The only known interacting partners for the N-terminal domain are Pygo and Lgs (Figure 1.9). Therefore, the possibility of CtBP interacting with Pygo or Lgs is being explored.

Apart from trans-regulatory factors, another central component of target gene regulation by transcription factors is *cis-elements* which help transcription factors locate their targets. These DNA sequences act as a platform for formation of different complexes and hence demand a level of specificity which makes them stand out from the other loci, and recruit the transcription factors. How do the WREs 'array' themselves to 'attract' transcription factors and provide the desired transcriptional output for homeostasis?

Recently, a study from the Cadigan lab identified novel *cis-elements* called the 'Helper' sites, which along with the known HMG sequences are required for bipartite binding of TCF. Helper sites provide another level of specificity for identification of Wg

targets by TCF in fly TCF and some vertebrate TCFs. I have extended those studies to a worm WRE in order to explore if the bipartite binding of TCF is a conserved mechanism. Helper-like sequences are required for the binding of worm TCF to a worm WRE. However the distribution of HMG and Helper sites in WREs does not show a stringent requirement of spacing and orientation. So a novel finding from my analysis is that POP1 self-associates and form oligomers. Hence the orientation and spacing requirements are less stringent as TCF might form oligomers on these WREs. Although a more rigorous analysis is required to address this, *in vitro* and *in vivo* studies are currently underway to test the hypothesis that TCF oligomerization is required for activation of Wnt targets. Provided below is a summary of the work reported in this dissertation.

Chapter II: The oligomeric state of CtBP determines its role as a transcriptional coactivator and corepressor of Wingless targets.

The requirement of dimerization of CtBP for regulation of Wg targets has never been explored. In this study, I found that mutations in the dimerization interface separate the activator and repressor functions of CtBP. Function of CtBP monomers has never been reported and I found that CtBP monomers positively regulate Wg targets. Using a strategy of complementary mutations in the dimerization interface, I explored the reconstitution of CtBP dimers from monomers and found that dimers repress Wg targets.

Chapter III: Mutational analysis to investigate the role of CtBP in Wg signaling.

In Chapter II, it is reported that CtBP monomers can activate Wg targets but cannot repress Wg target and CtBP self-association was required for repression. One of the unanswered questions is can CtBP dimers activate Wg targets? In this study, I used strategies like engineering a more hydrophobic dimerization interface, or covalently linking two CtBP monomers, to force dimerization of CtBP. What I found was that the forced dimer was non-functional and the concatemer was degraded, hence no conclusive evidence could be deduced in the context of function of CtBP dimers. A requirement of the NAD dependent catalytic activity was also tested for activation of a Wg target, and found that it was not required. Also to further explore the role of activation of Wg targets by CtBP monomers *in vivo*, I looked at activation of Wg target Sens by CtBP monomer mutant and found that it could not activate Sens.

Chapter IV: Exploring POP1 oligomerization and investigating the role of Helper-like *cis-elements* in binding of POP1.

Most invertebrate TCF isoforms and some vertebrate isoforms have at least two DNA binding domains known as the HMG box and the C-Clamp. HMG box binds to HMG sites and C-Clamp binds to Helper sites in WREs. *In vitro* analysis in this study shows that Helper sites are required for binding of worm TCF homolog POP1 to Helper-like sequences in the WRE of *ceh22*. Also a previously unexplored self-association of POP1 is reported in this study.

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Chapter II

The oligomeric state of CtBP determines its role as a transcriptional coactivator and corepressor of Wingless Targets

Abstract

C-terminal binding protein (CtBP) is a well-characterized transcriptional co-repressor. Homo-dimerization of CtBP is required for this activity. CtBP is known to inhibit the Wnt/ß-catenin pathway in flies and vertebrates, but we have previously reported that CtBP is also required for activation of some Wingless nuclear targets in *Drosophila*. Here, we examine the role of dimerization of CtBP in these opposing processes. CtBP mutants that cannot dimerize are able to promote Wingless signaling, but are defective in repressing Wingless targets. To further test the role of dimerization in repression, basic and acidic residues which form inter-molecular salt-bridges in the CtBP dimerization interface were swapped. These mutants cannot homo-dimerize and are compromised for repression. However, their co-expression leads to hetero-dimerization and consequent repression of Wingless targets. Our results support a model where CtBP is a gene-specific regulator of Wingless signaling, with some targets requiring CtBP dimers for inhibition while other targets utilize CtBP monomers for activation of their expression.

Introduction

Wnt/ß-catenin signaling plays crucial roles in many aspects of embryonic development and adult homeostasis (Cadigan, 2008; Cadigan & Nusse, 1997; Clevers, 2006; Logan & Nusse, 2004). Misregulation of this pathway is causal for several different cancers and other diseases (Giles et al, 2003; MacDonald et al, 2009). This pathway is activated by a highly conserved group of secreted glycolipoproteins called Wnts, which promote the stabilization and nuclear translocation of cytosolic ß-catenin (Cadigan & Peifer, 2009; Kikuchi et al, 2006; MacDonald et al, 2009). Members of the T-Cell Factor/Lymphoid Enhancer Factor-1 (TCF/LEF-1) family of transcription factors are major nuclear binding partners of ß-catenin. Given the widespread importance of Wnt/ß-catenin signaling in normal and pathological states, elucidating how the pathway regulates target gene expression through TCFs remains an important goal in furthering our understanding of Wnt biology.

In the absence of Wnt signaling, several different modes of regulation operate to repress target gene expression. Although ß-catenin is constantly synthesized, it is also constitutively subjected to phosphorylation by a protein complex which includes Axin, Adenomatous Polyposis Coli (APC), Casein Kinase I and Glycogen Synthase Kinase 3. Phosphorylated ß-catenin is then ubiquitinated and subjected to proteosome mediated degradation (Kennell & Cadigan, 2009; Kikuchi et al, 2006). ß-catenin that escapes this destruction is prevented from binding to TCFs by several factors, which bind to either TCF or ß-catenin and/or promote nuclear efflux of ß-catenin (Hamada & Bienz, 2004; Parker et al, 2007; Takemaru et al, 2003). These factors serve to set the threshold of nuclear ß-catenin needed to affect gene regulation. Finally, many Wnt transcriptional

targets are repressed in the absence of signaling by TCFs in conjunction with corepressors. TCF mediated recruitment of ß-catenin to Wnt Regulated Elements (WREs) causes a 'transcriptional switch' of TCF from a repressor to an activator hence turning on Wnt target gene expression (Mosimann et al, 2009; Parker et al, 2007).

Many factors have been reported to contribute to TCF-mediated repression of WREs in the absence of signaling and TCF-mediated activation of WREs upon stimulation of the pathway. Negative regulators include the co-repressor TLE/Groucho, the transcriptional repressor Kaiso, and the Brahma and ACF chromatin remodeling complexes (Cavallo et al, 1998; Collins & Treisman, 2000; Liu et al, 2008; Park et al, 2005; Roose et al, 1998). These factors are either physically displaced or somehow counteracted upon ß-catenin binding to TCFs (Daniels & Weis, 2005; Liu et al, 2008; Parker et al, 2007). ß-catenin then recruits many co-activators to WREs, e.g., the Legless (Lgs)-Pygopus (Pygo) complex to the N-terminal transactivation domain of ß-catenin, and CBP/p300 and Paraformbin/Hyrax to the ß-catenin's C-terminal transactivation domain (Hecht et al, 2000; Li et al, 2007; Mosimann et al, 2006; Stadeli & Basler, 2005).

C-Terminal Binding Protein (CtBP) is another factor that has been shown to play important roles in modulating the Wnt/β-catenin pathway. Overexpression of CtBP can inhibit Wnt signaling (Brannon et al, 1999; Fang et al, 2006; Hamada & Bienz, 2004; Valenta et al, 2003). Consistent with CtBP acting as a transcriptional co-repressor in many contexts (Chinnadurai, 2007; Turner & Crossley, 2001), CtBP has been reported to bind directly to TCFs (Brannon et al, 1999; Valenta et al, 2003). However, more recent reports have been unable to find a detectable interaction (Hamada & Bienz, 2004; Valenta et al, 2006). Instead, a CtBP-APC complex was shown to bind to β-catenin, and

prevent its interaction with TCF4, thus blocking Wnt target gene activation (Hamada & Bienz, 2004; Sierra et al, 2006).

Our laboratory has previously shown that in *Drosophila* cells, CtBP is required for repression of several Wingless (Wg, a fly Wnt) targets. CtBP is enriched at the WREs of these targets, in a similar pattern as TCF (Fang et al., 2006). However, CtBP recruitment to WREs is not dependent on TCF (Fang et al, 2006). In addition, several Wg targets were not repressed by CtBP, but instead required CtBP for maximal activation by the pathway. CtBP was recruited to the WREs of these genes in a TCF and Armadillo (Arm, the fly β-catenin) dependent manner (Fang et al, 2006). Thus, CtBP contributes to both aspects of the TCF transcriptional switch, in a gene-specific manner.

The CtBP family of proteins all contains a conserved central domain with high homology to NAD^+/NADH dependent dehydrogenases (Kumar et al, 2002; Nardini et al, 2003). Dehydrogenase activity has been detected in recombinant human CtBP1 (hCtBP1) (Achouri et al, 2007; Balasubramanian et al, 2003; Kumar et al, 2002) but the role of catalytic function in the transcriptional activity of CtBP is controversial. Mutations in the catalytic site compromise co-repressor activity (Kumar et al, 2002), although not in all contexts (Grooteclaes et al, 2003; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Phippen et al, 2000; Sutrias-Grau & Arnosti, 2004). In addition, the role of CtBP in potentiating Wg signaling in fly cells does not require dehydrogenase activity (Fang et al, 2006).

Another important factor that can affect the transcriptional activity of the CtBP family of proteins is their quaternary structure. In cells, CtBP is thought to exist in an equilibrium between monomers (Kim et al, 2005; Zhao et al, 2009), homodimers and

possible higher order structures (Balasubramanian et al, 2003; Kim et al, 2005; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Shi et al, 2003; Thio et al, 2004; Zhao et al, 2009). Dimerization is stimulated by NAD^+/NADH binding (Balasubramanian et al, 2003; Kim et al, 2005; Kumar et al, 2002; Kuppuswamy et al, 2008; Nardini et al, 2009) but mutations in NAD^+ binding domain do not abolish dimerization in all cases (Mani-Telang et al, 2007; Thio et al, 2004) . When crystallized, mammalian CtBP proteins exist as dimers, and the dimerization interface has been well defined (Kumar et al, 2002; Nardini et al, 2003). Mutations in the dimerization interface have been shown to reduce the function of CtBP as a corepressor in several contexts (Kumar et al, 2002; Kuppuswamy et al, 2008; Zhao et al, 2009).

In this report, we examine whether dimerization of CtBP plays a role in mediating the Wg/Wnt transcriptional switch in fly cells. Mutant forms of CtBP that cannot dimerize are still able to activate Wg targets, but are no longer capable of repression. However, co-expression of different monomeric forms of CtBP that can heterodimerize restores the repression activity. We conclude that CtBP dimers act in repression of Wg targets while CtBP monomers function in transcriptional activation of Wg targets. In addition, the tools developed in this study to uncouple CtBP activation and repression in Wg signaling can be utilized to explore the requirement of CtBP oligomerization in other contexts where CtBP plays important biological roles.

Results

Monomeric CtBP activates Wg signaling in flies

CtBP is thought to exist in an equilibrium between monomeric (Kim et al, 2005; Zhao et al, 2009), homodimeric and possibly higher ordered homo-oligomeric complexes (Balasubramanian et al, 2003; Kim et al, 2005; Kumar et al, 2002; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Nardini et al, 2003; Shi et al, 2003; Thio et al, 2004; Zhao et al, 2009) . While the native oligomeric state has mostly been determined for mammalian CtBP proteins, the entire dehydrogenase domain of fly CtBP is highly conserved (e.g., fly CtBP and hCtBP1 domains are 72% identical with 84% similarity). Nearly all of the residues making intermolecular contact in the hCtBP1 homodimers are identical in fly CtBP (Kumar et al, 2002). This information was utilized to construct a fly CtBP protein that should not be able to dimerize, and thus remain monomeric.

There are several different isoforms of fly CtBP which express proteins containing 383, 386, 476 and 479 residues (Nibu et al, 1998b; Poortinga et al, 1998; Sutrias-Grau & Arnosti, 2004). The short and long isoforms differ in their C-termini, downstream of the dehydrogenase domain. They are thought to play a redundant role in transcriptional regulation in some developmental contexts (Sutrias-Grau & Arnosti, 2004). Consistent with this, both isoforms can activate Wg/Arm-dependent transcription (Fang et al, 2006). Hence the short isoform(383) was used for all subsequent experiments in this report.

To generate a monomeric CtBP, four conserved residues, previously shown to be important for hCtBP1 self-association (Kumar et al, 2002), were mutated in fly CtBP. The resulting CtBP variant is referred to as $CtBP^{Mono}$. The C134Y and N138R substitutions should result in steric and electrostatic hindrance, hence preventing homo-

dimerization and the R141A and R142A mutations should disrupt inter-molecular salt bridges and hydrogen bonds as predicted for hCtBP1 (Kumar et al, 2002). The normal equilibrium between monomers and dimers in wild-type CtBP ($CtBP^{WT}$) should be dramatically shifted to the monomeric state for $CtBP^{Mono}$ (Figure 1A).

To test if the mutations in $CtBP^{Mono}$ abolished its ability to self-associate, differentially tagged CtBP forms were co-transfected in the *Drosophila* hemocyte-derived cell line Kc167 (Kc) and assayed for binding using co-Immunoprecipitation (co-IP). While $CtBP^{WT}$-Flag could co-IP $CtBP^{WT}$-HA, it was not able to pull down $CtBP^{Mono}$-HA (Figure 1B). Mutations also disrupted the ability of $CtBP^{Mono}$ to homo-oligomerize as judged by this assay (Figure 1B). These results demonstrate that $CtBP^{Mono}$ cannot dimerize, resulting in a "forced monomer" version of CtBP.

To test the role of dimerization in regulating Wg signaling, transgenic lines were constructed containing $CtBP^{WT}$ or $CtBP^{Mono}$ under the control of the Gal4/Upstream Activating Sequence (UAS) inducible promoter. These CtBPs can then be expressed in any fly tissue for which a Gal4 driver line is available (Phelps & Brand, 1998). We previously reported that overexpression of CtBP, via nearby insertion of a P[GSV] element (Toba et al, 1999) in the developing eye could suppress a small eye phenotype caused by overstimulation of Wg signaling (Fang et al, 2006). Consistent with these results, several P[UAS-$CtBP^{WT}$] lines were able to suppress the small eye phenotype caused by GMR-Gal4 dependent expression of a stabilized form of Arm (Arm*) (Figure 2A, B). Thus CtBP antagonizes Wg signaling downstream of Arm stabilization in this assay. In stark contrast, misexpression of $CtBP^{Mono}$ caused a significant enhancement of

the *GMR-Gal4::UAS-arm** small eye phenotype (Figure 2A, C), suggesting that CtBP monomers promote Wg signaling in this context.

Wg signaling also plays a significant role in defining the wing margin that originates from the Dorsal/Ventral (D/V) boundary of the wing imaginal disc. Antagonism of Wg signaling in this tissue leads to a loss of the wing margin, causing notches in the adult wing (Couso et al, 1994; Phillips & Whittle, 1993). To assay the role of $CtBP^{Mono}$ in Wg directed wing margin formation, a sensitized genetic background was created by misexpression of Pygo at the anterior/posterior (A/P) boundary of the wing disc using *Patched-Gal4* (*Ptc-Gal4*) (Figure 2D-F). Although Pygo is known to positively regulate Wg signaling (Belenkaya et al, 2002; Kramps et al, 2002; Parker et al, 2002; Thompson et al, 2002), misexpression of Pygo antagonizes Wg signaling, possibly due to disruption of the stoichiometry of a protein complex (Parker et al, 2002).

In the *Ptc-Gal4*::*UAS-Pygo* background employed in this assay, over 80% of the adult wings displayed notches. These notches were categorized into two groups based on their size. Small notches had loss of wing margin only between the L3 and L4 veins (Figure 2D, E). Big notches extended beyond these veins (e.g., Figure 2F). Coexpression of $CtBP^{Mono}$ significantly suppressed the loss of wing margin caused by Pygo, with a dramatic reduction in the frequency of big notches (Figure 2G). These data provide another line of evidence supporting a positive role for $CtBP^{Mono}$ in regulation of Wg signaling.

To further test the role of $CtBP^{Mono}$ in Wg signaling, expression of a Distalless enhancer trap line (*Dll-lacZ*) was monitored. In larval third instar wing imaginal discs, *Dll-lacZ* is activated by Wg in a broad domain centered on the D/V boundary of the

presumptive wing blade (Neumann & Cohen, 1997; Zecca et al, 1996) (Figure 3A). Transgenic flies carrying UAS-CtBP^{WT} or UAS-CtBP^{Mono} transgenes were crossed to a *Engrailed-Gal4 (En-Gal4)* driver, leading to expression of transgenes in the posterior half of the disc (Figures 3E, H). Lines expressing CtBP^{WT} and CtBP^{Mono} at similar levels resulted in an enhancement in the *Dll-lacZ* expression (Figure 3D, F, G, I). These results provide additional support for positive regulation of the Wg pathway by CtBP^{Mono}.

CtBP Monomers Promote Activation of Wg targets in Kc cells

We have previously shown that the expression of the genes *CG6234* and *naked cuticle (nkd)* is activated by Wg signaling in Kc cells (Fang et al, 2006). In the absence of signaling, CtBP and TCF act in parallel to repress *nkd* expression, but CtBP is not required for activation of *nkd* expression by Wg signaling (Fang et al, 2006). In contrast, CtBP repression of *CG6234* in the absence of signaling is minimal, but CtBP is required for maximal activation of *CG6234* upon Wg stimulation (Fang et al, 2006).

To test whether the positive regulation of *CG6234* by CtBP is occurring at the transcriptional level, a reporter gene containing a minimal WRE from this target gene was examined. Figure 4A shows the location of a minimal WRE (539 bp) derived from a previously reported 2.2 kb WRE (Fang et al., 2006; see Materials and Methods) which is comparatively more responsive to Wg signaling (data not shown).

The *CG6234* WRE reporter was highly activated by expression of Arm* in a TCF-dependent manner (Figure 4B). RNAi mediated depletion of *CtBP* also caused a dramatic reduction in activation of the *CG6234* WRE reporter (Figure 4B). Consistent with the data for *CG6234* transcripts (Fang et al, 2006), there was still residual activation

of the *CG6234* WRE reporter by Arm* in *CtBP* RNAi treated cells (Figure 4B). This data suggests that CtBP is acting in parallel with other co-activators recruited by Arm for activation of *CG6234* WRE reporter.

To test the role of CtBP dimerization in promoting Wg-mediated transcriptional activation of the *CG6234* WRE reporter, a *CtBP* gene replacement strategy was employed. Endogenous *CtBP* was depleted using dsRNA corresponding to the 5'UTR of *CtBP*. These cells were then transfected with the reporter, plus transgenes expressing $CtBP^{WT}$ or $CtBP^{Mono}$. These *CtBP* transgenes contained a heterologous 5' UTR, so they were not targeted by the *CtBP* RNAi. Activation of the *CG6234* WRE reporter by Wg conditioned media was then assayed. Transfection of $CtBP^{Mono}$ rescued the *CtBP* RNAi defect to a similar level as seen with $CtBP^{WT}$ transfection (Figure 4C; compare the second and third groups). A similar rescue of *CG6234* WRE reporter activation by $CtBP^{Mono}$ was observed when the Wg pathway was stimulated by expression of Arm* (Figure 4D). $CtBP^{Mono}$ was expressed at similar levels as $CtBP^{WT}$ in these experiments (Figure 4D and data not shown). These results demonstrate that $CtBP^{Mono}$ is capable of substituting for endogenous CtBP to promote activation of the *CG6234* WRE reporter.

CtBP has previously been shown to be required for activation Gal4-Arm* dependent activation of a *UAS-luc* reporter (Fang et al, 2006). Both $CtBP^{WT}$ and $CtBP^{Mono}$ had no effect on *UAS-luc* when co-expressed with Gal4DBD (Gal4 DNA binding domain). However, both CtBP forms dramatically enhanced the ability of Gal4-Arm* to activate *UAS-luc* (Figure 4E). Taken together with the data from Figure 4D, these results indicate that like $CtBP^{WT}$, $CtBP^{Mono}$ is functioning downstream of Arm stabilization to activate Wg transcriptional targets.

CtBP dimerization is required for its antagonistic role in Wg signaling in Kc cells

We have previously identified three *WREs* in the *nkd* locus, two upstream of the *nkd* transcriptional start site (*nkd-UpE1* and *nkd-UpE2*) and one in the first intron of *nkd* (*nkd-IntE*) (Chang et al, 2008). While reporters for all three WREs were derepressed upon depletion of *CtBP*, the *nkd-UpE1* reporter consistently exhibited the largest response (Figure S1). In addition, *TCF* knockdown also caused derepression of *nkd-UpE1*, while having no effect on *nkd-UpE2* or *nkd-IntE* (Figure S1). Therefore, *nkd-UpE1* (Figure 5A) was used for all subsequent experiments to assay the role of CtBP oligomers in regulating this *nkd* WRE. Similar to *nkd* mRNA (Fang et al, 2006), simultaneous knockdown of *CtBP* and *TCF* resulted in a far greater derepression of *nkd-UpE1* reporter activity than with either factor alone (Figure 5B). TCF has already been shown to be enriched at the *UpE1* WRE (Chang et al, 2008). As judged by chromatin IP (ChIP), CtBP was also enriched at the *nkd-UpE1* (Figure S2) supporting a direct role for CtBP in repression of this WRE.

To test if dimerization of CtBP was required for inhibition of Wg targets in the absence of signaling, the ability of $CtBP^{Mono}$ to repress the *nkd-UpE1* reporter was assayed. This was done using a similar gene replacement strategy as described in Figure 4. As expected, transiently expressed $CtBP^{WT}$ was able to repress the *nkd-UPE1* in the absence of signaling. Strikingly, $CtBP^{Mono}$ was unable to perform this function (Figure 5C). Mutations which abolish self-association of CtBP, while having no affect on its ability to promote Wg signaling, severely disrupted its ability to repress Wg target gene expression in the absence of signaling.

The approach described above is similar to that used in several other studies to provide evidence that CtBP dimerization is required for transcriptional repression (Kumar et al, 2002; Kuppuswamy et al, 2008; Zhao et al, 2009), i.e., correlating loss of dimerization with loss of CtBP activity. However, this approach cannot rule out that the mutations disrupting homo-dimerization also affect other aspects of CtBP function. To provide a more convincing demonstration of the importance of CtBP self-association in antagonizing Wg signaling, monomeric versions of CtBP were created that cannot homo-dimerize, yet possess the ability to hetero-dimerize with each other. If dimerization is essential for repression by CtBP, then the monomeric forms should not be able to repress Wg targets but co-expression of these complementary monomeric forms should reconstitute dimerization and hence the repressive function of CtBP.

The strategy for engineering complementary monomeric forms of CtBP required identifying the salt-bridges in the CtBP dimer and then switching the positions of the acidic and basic residues forming the salt bridge. Such intermolecular salt bridge swaps have been previously used to show interaction or self-association of various proteins (Venkatachalan & Czajkowski, 2008; Watt et al, 2001; Xiao et al, 1999). Using the structural information of the highly conserved hCtBP1, two salt bridges (E126 – R173 and E127 – R171) formed by conserved residues were targeted for a swap. $CtBP^{Basic}$ contains E126R and E127R substitutions, while $CtBP^{Acidic}$ has R173E and R171E alterations. When expressed on their own, $CtBP^{Basic}$ and $CtBP^{Acidic}$ should be monomeric but have the ability to form $CtBP^{Basic}$/$CtBP^{Acidic}$ heterodimers when co-expressed (Figure 6A).

As expected, coexpression of CtBP^{Basic} and CtBP^{Acidic} led to association of these molecules at levels similar to those seen with CtBP^{WT} (Figure S3 and Figure 6B, lane 2). Coexpression of differentially tagged versions of CtBP^{Acidic} did not result in an appreciable co-IP (Figure 6B, lane 1). Assaying self-association of CtBP^{Basic} was complicated by the fact that the V5-tagged version of this protein was highly unstable when expressed with a Flag tagged CtBP^{Basic} (Figure 6C, lane 1). Stability was greatly increased by coexpression with CtBP^{Acidic} (Figure 6C, lane 2). Although V5 tagged CtBP^{Acidic} was more readily expressed, it also appeared to be more stable in the presence of the complementary CtBP^{Basic} (Figure 6C, lanes 3 & 4). In contrast to the V5-tagged proteins, the Flag tagged versions were relatively stable when expressed under all conditions (Figure 6C). Taken together, these data demonstrate that the CtBP^{Acidic} and CtBP^{Basic} mutants function as predicted, being unable to homo-dimerize but capable of efficient hetero-dimerization.

When tested for their ability to rescue the derepression of the *nkd-UpE1* reporter in cells depleted of endogenous CtBP, neither CtBP^{Acidic} nor CtBP^{Basic} were able to provide significant repressive activity, similar to the original CtBP^{Mono} mutant (Figure 6D). Remarkably, coexpression of CtBP^{Basic} and CtBP^{Acidic} restored the inhibition of *nkd-UPE1* in the absence of signaling, to a similar extent as observed with CtBP^{WT}. These data provide compelling evidence that self-association is required for the function of CtBP as a corepressor of a Wg transcriptional target.

CtBP dimerization is required for its ability to inhibit wing margin formation

To extend the findings summarized in Figures 5 and 6 to fly tissues, the ability of $CtBP^{WT}$ and $CtBP^{Mono}$ to affect development of the wing was examined. Expression of $CtBP^{WT}$ at the A/P boundary of the wing disc using the *Decapentaplegic-Gal4* (*Dpp-Gal4*) driver gave rise to a high frequency of wing notches (Table I). Expression of CtBP had no effect on the expression of Wg (Figure S4). This phenotype is consistent with a reduction in Wg signaling at the presumptive wing margin. However expression of $CtBP^{Mono}$ never resulted in wing notching (Table 1). These data provide further support for a model where CtBP self-association is required for the ability of CtBP to antagonize events downstream of Wg expression in the wing primordium.

To confirm that CtBP dimerization was required for inhibition of wing margin formation, $CtBP^{Basic}$ and $CtBP^{Acidic}$ transgenes were also tested for a wing phenotype when misexpressed using *Dpp-Gal4* (Table 1). Flies containing two UAS transgenes each were generated in the following combinations: Acidic/Acidic, Basic/Basic or Acidic/Basic. While expression of both complementary combinations ($Acidic^1$/$Basic^1$ or $Acidic^2$/$Basic^2$) resulted in significant wing notching, $Acidic^1$/$Acidic^2$ or $Basic^1$/$Basic^2$ combinations did not (Table I). Immunostaining with CtBP antisera was performed to ensure that comparisons were made with CtBP variant proteins expressed at similar levels (Figure S4). The CtBP $Acidic^1$/$Basic^1$ and $Acidic^1$/$Acidic^2$ backgrounds were expressed at similar levels, while the $Basic^1$/$Basic^2$ and $Acidic^2$/$Basic^2$ combinations were expressed at slightly lower levels (Figure S4). As with $CtBP^{WT}$ and $CtBP^{Mono}$, Wg expression at the presumptive margin was not affected by any of the Acidic/Basic combinations (Figure

S4). These results indicate that dimerization of CtBP is required for antagonism of Wg signaling during wing margin formation.

Discussion

Oligomeric state of CtBP determines its effect on Wg signaling

CtBP is well known for its role as a co-repressor for many transcription factors (Chinnadurai, 2007; Kuppuswamy et al, 2008; Turner & Crossley, 2001). It is also known to antagonize Wnt/β-cat signaling, possibly by binding to some TCFs (Brannon et al, 1999; Valenta et al, 2003) or by acting with APC to divert β-catenin away from TCF (Hamada & Bienz, 2004; Sierra et al, 2006). In *Drosophila* Kc cells, we have previously shown that CtBP works in parallel with TCF to repress expression of the Wg target *nkd* in the absence of signaling (Fang et al, 2006). In addition, we found that CtBP was required for activation of several Wg targets in cultured cells and fly tissues (Fang et al, 2006). Our data indicate that CtBP can both repress and activate the Wg pathway in a gene-specific manner.

In this report, we provide a dramatic example of this differential regulation of Wg-mediated transcription by CtBP using *WRE* reporter constructs. While CtBP is required for silencing the *nkd-UpE1* reporter in the absence of signaling (Figures 5 & 6), depletion of CtBP results in a significant reduction of the *CG6234* WRE reporter activation upon Wg stimulation (Figure 4). Since both these *WREs* are directly activated by TCF-Arm (Chang et al, 2008; Fang et al, 2006), these results indicate that additional sequence information must exist in these elements that influence CtBP's relationship with TCF and

Arm. Further dissection of these WREs will be needed to identify the cis-acting motifs responsible for their differential regulation by CtBP.

How can CtBP both promote and repress transcription of Wg targets? Our data demonstrate that the quaternary state of the CtBP protein determines its role as an activator and repressor. CtBP mutants which cannot homo-dimerize are unable to repress *nkd-UpE1* expression (Figures 5C, 6D) or inhibit wing margin formation (Table I). However, coexpression of complimentary monomeric CtBP mutants which can hetero-dimerize restores CtBP repression activity in both these readouts (Figure 6D and Table I). This provides a compelling argument that self-association of CtBP is required for its ability to antagonize Wg transcriptional targets.

In contrast to targets where CtBP inhibits Wg signaling, monomeric CtBP can rescue the loss of Wg activation of the *CG6234* WRE reporter in Kc cells depleted of endogenous CtBP (Figures 4C and 4D). In addition, CtBP monomers enhance an Arm-induced small eye phenotype (Figure 2C), can rescue a weak loss of Wg signaling defect in the fly wing (Figure 2G) and activate the Wg target *Dll-lacZ* (Figure 3). Taken together, our data strongly support a model where CtBP monomers activate and CtBP dimers repress the Wg pathway in a gene-specific manner.

Mechanism of CtBP action on Wg signaling

Why is dimerization required for repression by CtBP? CtBPs have been shown to bind to many transcriptional repressors and some interactions require the dimerization of CtBP (Balasubramanian et al, 2003; Chinnadurai, 2007; Kumar et al, 2002; Kuppuswamy et al, 2008; Turner & Crossley, 2001). Although homodimerization is dispensable for

interaction of CtBP with some factors, it is clearly required for the function of CtBP as a potent corepressor in complex with those factors (Kuppuswamy et al, 2008; Zhao et al, 2009). CtBPs have also been shown to associate with several chromatin modifying enzymes which play crucial roles in transcriptional repression (Kuppuswamy et al, 2008; Shi et al, 2003; Zhao et al, 2009). A recent report provides good evidence that in the case of repression of *E-Cadherin* by the repressor ZEB, human CtBP2 dimers act as adaptors between ZEB and histone deacetylase 2 (HDAC2) (Zhao et al, 2009). Binding of ZEB and HDAC2 to CtBP monomers was mutually exclusive, necessitating the need for CtBP dimerization to form the ZEB-CtBP-HDAC2 complex (Zhao et al, 2009). It is possible that this mechanism is also operating in the silencing of *nkd* expression in Kc cells, though further work is required to identify the binding partners of CtBP in this system.

In the case of activation we have previously shown that CtBP functionally interacts with the N-terminal transactivation domain of Arm (Fang et al, 2006). This domain of Arm is bound by Lgs, which is in a complex with Pygo to promote transcriptional activation (Kramps et al, 2002; Li et al, 2007; Stadeli & Basler, 2005). This suggests that CtBP may interact with Pygo or Lgs, a possibility that we are currently exploring. Given the fact that the Lgs-Pygo complex is generally required for Wg signaling throughout fly development (Belenkaya et al, 2002; Parker et al, 2002; Thompson et al, 2002), there would have to be other factors involved to explain why only a subset of Wg targets require CtBP for activation. In addition, Pygo has also been demonstrated to regulate some Wg targets in the absence of signaling, suggesting a possible link with CtBP in this context (de la Roche & Bienz, 2007; Mieszczanek et al, 2008).

Regulation of the oligomeric state of CtBP

CtBPs are highly homologous to NAD^+/NADH dependent dehydrogenases and can bind NADH with high affinity (Fjeld et al, 2003). However the role of cofactor based differential regulation of CtBP oligomerization is controversial. An increase in the NAD^+/NADH levels stimulates dimerization of mammalian CtBPs (Balasubramanian et al, 2003; Kim et al, 2005; Kumar et al, 2002; Thio et al, 2004). Mutations in the NAD^+/NADH binding site of CtBPs abolishes or reduces oligomerization, (Kumar et al, 2002; Kuppuswamy et al, 2008; Nardini et al, 2009; Thio et al, 2004) although NAD^+ binding is not always essential for dimerization of CtBP (Mani-Telang et al, 2007). In the case of the short isoform of fly CtBP, mutations in the NAD binding cleft (G181V, G183V) make the protein highly unstable in Kc cells (Bhambhani and Cadigan, unpublished). This precludes any functional studies to test if the NAD^+/NADH ratio might affect its role in regulating Wg targets.

Does stimulation by Wg influence the oligomeric state of CtBP? In the absence of Wnt signaling, TCF acts with many other co-repressors to silence target gene expression. This repression is then counteracted by Arm/β-cat binding to TCF (Mosimann et al, 2009; Parker et al, 2007). Given the fact that CtBP dimers repress some Wg targets and CtBP monomers promote the Wg-dependent activation of some targets, it is tempting to speculate that Wg signaling causes a conversion of CtBP dimers to monomers. However we have been unable to detect any difference in CtBP self-association in our co-IP assay (data not shown). Perhaps a more sensitive assay is required to detect changes in the oligomeric state upon Wg stimulation.

An alternative to the Wg pathway influencing the oligomeric state of CtBP is a model where CtBP monomers and dimers exist in the cell in equilibrium, and selective protein-protein interactions recruit either form to Wg targets. In the case of CtBP monomers, this recruitment to WRE is predicted to require Arm. Although Wg signaling does not appear to influence the overall CtBP concentration in some fly tissues (Fang et al, 2006), protein-protein interactions may cause changes in the monomer-dimer ratio on the WRE chromatin.

The role of CtBP oligomerization in other systems

The reagents and methodology described in this report can be applied to other systems where CtBP plays important roles in regulating gene expression. For example, loss of CtBP1 and CtBP2 in the mouse results in loss of posterior structures in the embryo, a phenotype that has many similarities to Wnt3a mutants (Hildebrand & Soriano, 2002). This suggests that mammalian CtBPs also play a positive role in Wnt signaling. However, it is also possible that the phenotype is indirect, i.e., CtBP represses a negative regulator of the Wnt pathway. Similar to fly CtBP, if murine CtBP monomers also play a positive role in regulating Wg targets, then a gene knockin of monomeric mCtBP1 or mCtBP2 should rescue the defect in posterior structures of CtBP knockouts.

In fly embryogenesis, loss of CtBP results in dramatic disruption of segmentation, due to defects in the striped pattern of the primary pair-rule genes (Nibu et al, 1998a; Poortinga et al, 1998; Strunk et al, 2001). Many of these defects can be explained by requirement of CtBP to bind to gap gene transcription factors (e.g., Kr) and promote repression (Keller et al, 2000; Nibu & Levine, 2001; Nibu et al, 1998a; Struffi et al, 2004;

Strunk et al, 2001). However, there are aspects of the CtBP mutant phenotype (e.g., loss of pair rule stripes; (Nibu et al, 1998a; Poortinga et al, 1998)**)** and genetic interactions (Phippen et al, 2000; Poortinga et al, 1998) that suggest that CtBP may play a positive role in regulating transcription. Testing whether $CtBP^{Mono}$ can rescue aspects of the CtBP segmentation phenotype may help determine whether CtBP plays a direct role in activating transcription in regulatory hierarchies beyond the Wg pathway.

Materials and Methods

Drosophila cell culture

Kc cell culture and RNAi mediated knockdown were performed as reported previously (Fang et al, 2006). Cells (10^6/ml) were soaked in 10 µg dsRNA for 4 days, before seeding for transfections. Primers for dsRNA synthesis have been described elsewhere (Fang et al, 2006). Transient transfections were performed using Fugene 6 (Roche Applied Science) as per the manufacturer's instructions.

Plasmids and reporter assays

$pAcCtBP^{short}$ with 2x Flag tags at the C-terminus (kindly provided by Dr. D. Arnosti) was used for all rescue assays. Site directed mutagenesis of $pAcCtBP^{short}$ (hereafter referred to as $CtBP^{WT}$) was used to introduce mutations in the dimerization interface to generate $CtBP^{Mono}$ (C134Y, N138R, R141A, R142A), $CtBP^{Basic}$ (E126R, E127R) and $CtBP^{Acidic}$ (R171E, R173E). The C-terminal HA tagged versions were generated by replacing the 2x Flag tags of $pAcCtBP^{short}$ by 4x HA tags. The C-terminal V5 tagged versions were created by cloning the $CtBP^{WT}$ and mutant cDNAs into the

KpnI and NotI sites of *pAC 5.1 V5-His* (Invitrogen). *pGL3nkd-UpE1*, *pAcArm**, *pAcGal4DBD, pAcGal4Arm*, pUAS-luc* and *pActinlacZ* constructs have been described elsewhere (Chang et al, 2008; Fang et al, 2006). *pGL3CG6234* minimal WRE (*CG6234* WRE), a 539 bp fragment, was generated using PCR based subcloning of a 617 bp region (-3220 to -2603 relative to the *CG6234* transcription start site) from the previously described *pCG6234* (Fang et al, 2006). Deletion of the region (-2603 to -1465) and an internal 80 bp deletion (-2860 to -2781) led to ~4.5 fold increase in the activation of the WRE by Arm* in cell culture assays, and hence this reporter was used thereafter. For transgenic lines, cDNAs for the $CtBP^{WT}$ and mutants with two C-Terminal flag tags were subcloned into *pUAST* vector using the KpnI and XbaI sites.

CG6234 WRE and *nkd-UpE1* reporter assays were performed by transiently transfecting 10 to 50 ng of the reporter and 150 ng to 500 ng CtBP expression plasmids in 2.5 x 10^5 cells/well. The *CG6234* WRE was activated using 100 µl of Wg-CM (derived from stable pTubWg S2 cells kindly provided by Dr. R. Nusse) or 50-100 ng of Arm*. For assays with *pUAS-luc*, 10ng of the reporter and 5 ng of Gal4Arm* was used with 500ng to 1µg of the CtBP constructs. $CtBP^{Mono}$ protein was less stable compared to $CtBP^{WT}$ and to achieve equal expression levels, two times more of the $CtBP^{Mono}$ plasmid was transfected compared to $CtBP^{WT}$ in all assays. For all reporter assays 5 ng of *pAcLacZ* was transfected for normalization and pAC5.1 (Invitrogen) or Gal4DBD to control for DNA amounts. Luciferase and LacZ assays were performed as described (Fang et al, 2006).

Drosophila genetics

Transgenic *UAS-CtBP* lines were generated using the injection facility at BestGene Inc. (Chino Hills, CA). w^{1118}, *GMR-Gal4*, *Ptc-Gal4*, *Dpp-Gal4*, *En-Gal4* and *Dll-lacZ* were obtained from Bloomington Stock Center. CtBP transgenes were analyzed for their effect on the small eye phenotype of P[*GMR-Gal4*] P[*GMR-Arm**] flies as described previously (Cadigan et al, 2002; Parker et al, 2002). Experiments with *Ptc-Gal4*, *GMR-Gal4* and *En-Gal4* were carried out at 25ºC and *Dpp-Gal4* at 27ºC or 29 ºC.

Immunoblots and immunostains

For western blot analysis, anti-Flag (1:2500, Sigma), anti-V5 (1:5000, Invitrogen), anti-HA (1:1000, Roche) and anti-Tubulin (1:4000, Sigma) were used followed by HRP conjugated anti-Mouse or anti-Rat IgG (Jackson Immunochemicals). Signal was detected using ECL kit (Amersham). Immunostaining of wing imaginal discs was performed as described previously (Fang et al, 2006) using anti-LacZ (1:1000, Abcam), anti-Wg (1:100) and anti-CtBP (1:1000). Alexa 488- and Cy3- conjugated secondary antibodies were obtained from Molecular Probes and Jackson Immunochemicals. Samples were examined using a Leica triple channel confocal microscope DM6000B-CS and processed using Adobe Photoshop 8.0.

Co-IP and ChIP

For co-IPs, 6-10 x10^6 Kc cells were seeded with 1µg pAcCtBP/10^6 cells for 3 days before harvesting. Cells were resuspended in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.9, 1% CHAPS, 10% glycerol, 0.1 mM EDTA with Complete mini-EDTA

free protease inhibitor cocktail, (Roche)) and sonicated thrice on ice in pulses of 6 seconds. Lysates were pre-cleared using Protein A/G sepharose beads. Total protein concentration was measured using *DC* protein assay (Bio-Rad). Lysates corresponding to 3mg total protein was used for each IP. 15 % of this lysate was saved as input. The remainder was incubated with 5µg primary antibody for 2 hours at 4ºC followed by incubation with Protein A/G sepharose beads for 30 minutes at 4ºC. The antibody-antigen complexes were washed 4 times with lysis buffer and eluted in 60µl of Laemmli sample buffer for western blot analysis. Results shown are representative of three independent experiments.

ChIP analysis was performed as described previously (Fang et al, 2006). Briefly 3 $x10^6$ cells and 10 µl of anti-CtBP antisera were used for every pulldown and precipitated DNA subject to Quantitative RT-PCR. Data are expressed as a percent of the input DNA. Specific primer pairs for the *UPE* and *ORF* correspond to N#1 and N#0 in the *nkd* locus as reported elsewhere (Chang et al, 2008; Fang et al, 2006).

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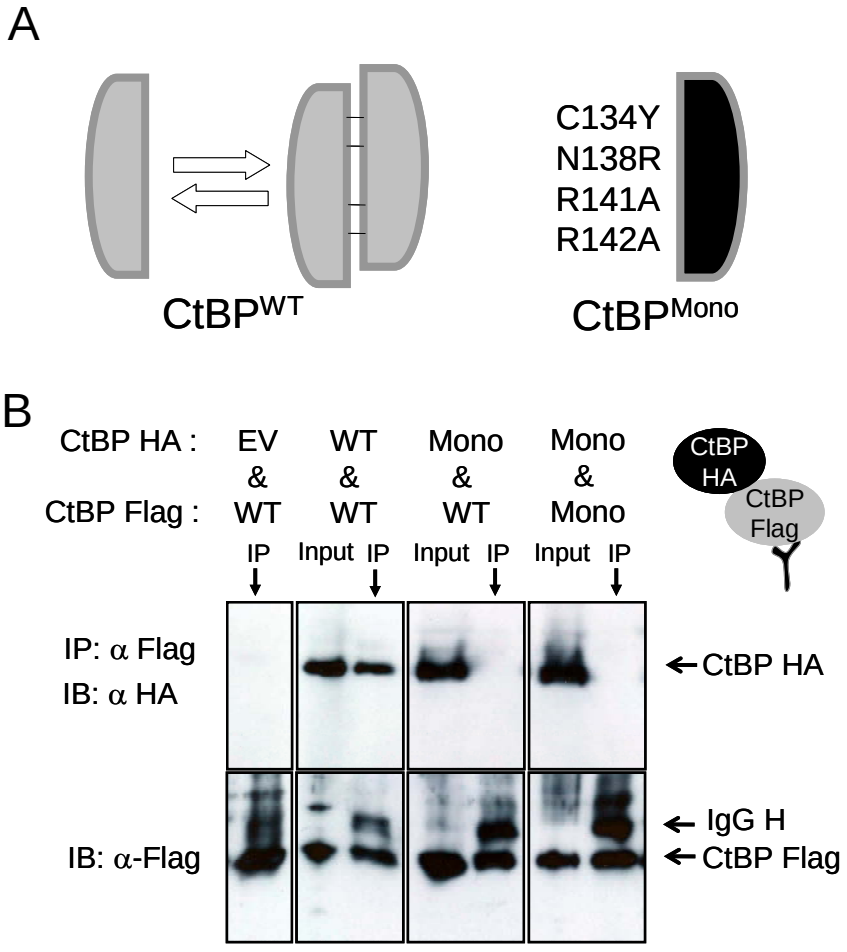

Figure 2.1 Conserved residues in the dimerization interface are required for self-association of CtBP. (**A**) Cartoon illustrating the equilibrium between monomers and dimers of $CtBP^{WT}$ and how four mutations in the dimerization interface in $CtBP^{Mono}$ shift the equilibrium to monomers. (**B**) $CtBP^{Mono}$ cannot associate with itself or $CtBP^{WT}$. A Flag tagged version of $CtBP^{WT}$ can co-IP $CtBP^{WT}$-HA (top panel, lane 3). No signal was observed if $CtBP^{WT}$-HA was left out of the transfection (lane 1). In contrast to $CtBP^{WT}$, $CtBP^{Mono}$-HA did not co-IP with $CtBP^{WT}$-Flag (lane 5) or $CtBP^{Mono}$-Flag (lane 7). The Flag tagged forms of $CtBP^{WT}$ or $CtBP^{Mono}$ were pulled down with a similar efficiency (lanes 1, 3, 5 & 7, bottom panel). Inputs (15% of total) for each co-IP are shown in lanes 2, 4 & 6.

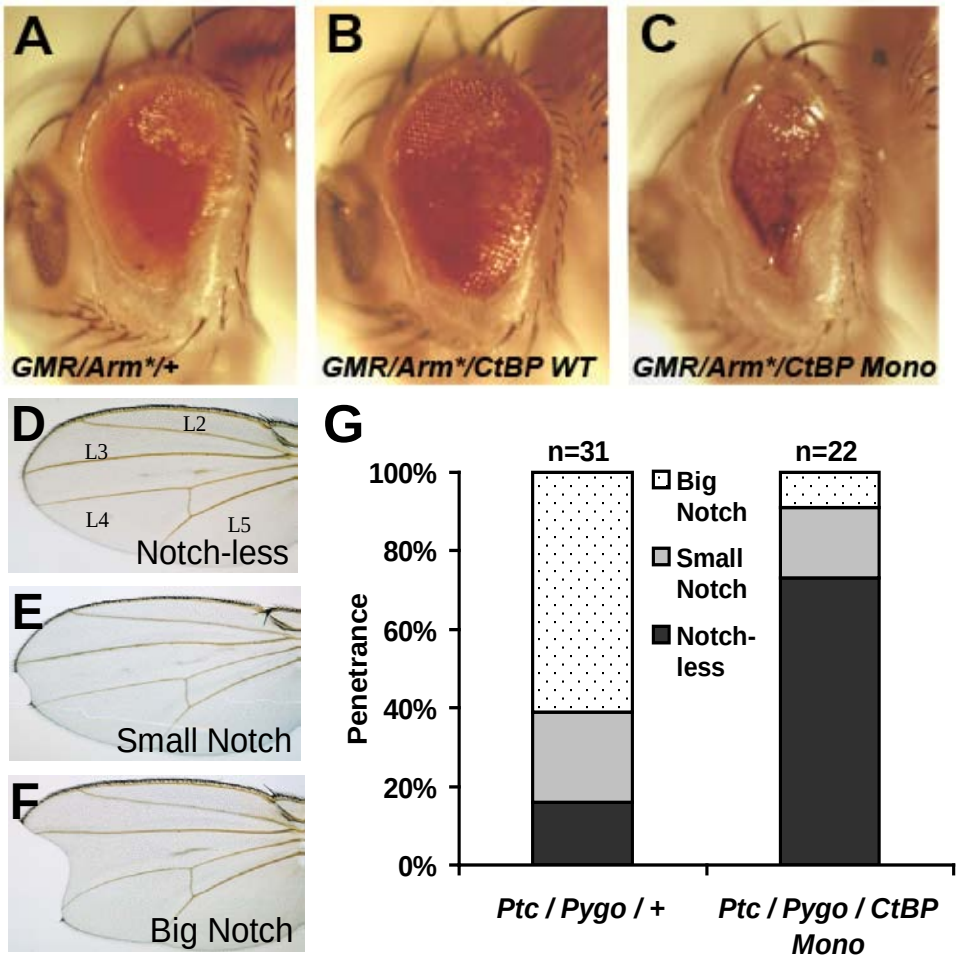

Figure 2.2 CtBP^{Mono} positively regulates Wg signaling in fly tissues. (**A-C**) Adult eyes of *GMR-Gal4::UAS-Arm** flies containing no transgene (A), *UAS-CtBP^{WT}* (B) or *UAS-CtBP^{Mono}* (C). The reduction in the fly eye size caused by Arm* expression is suppressed by co-expression CtBP^{WT} and is enhanced by CtBP^{Mono}. (**D-F**) Representative adult wings from *Ptc-Gal4::UAS-Pygo* flies that either lack a notch, contain a small notch (between the L3 and L4 vein) or contain a big notch (between the L2 and L5 vein) due to antagonism of Wg signaling. (**G**) The effect of CtBP^{Mono} on the frequency of the *Ptc-Gal4::UAS-Pygo* dependent notches. CtBP^{Mono} causes a marked reduction in the frequency and size of the wing notches.

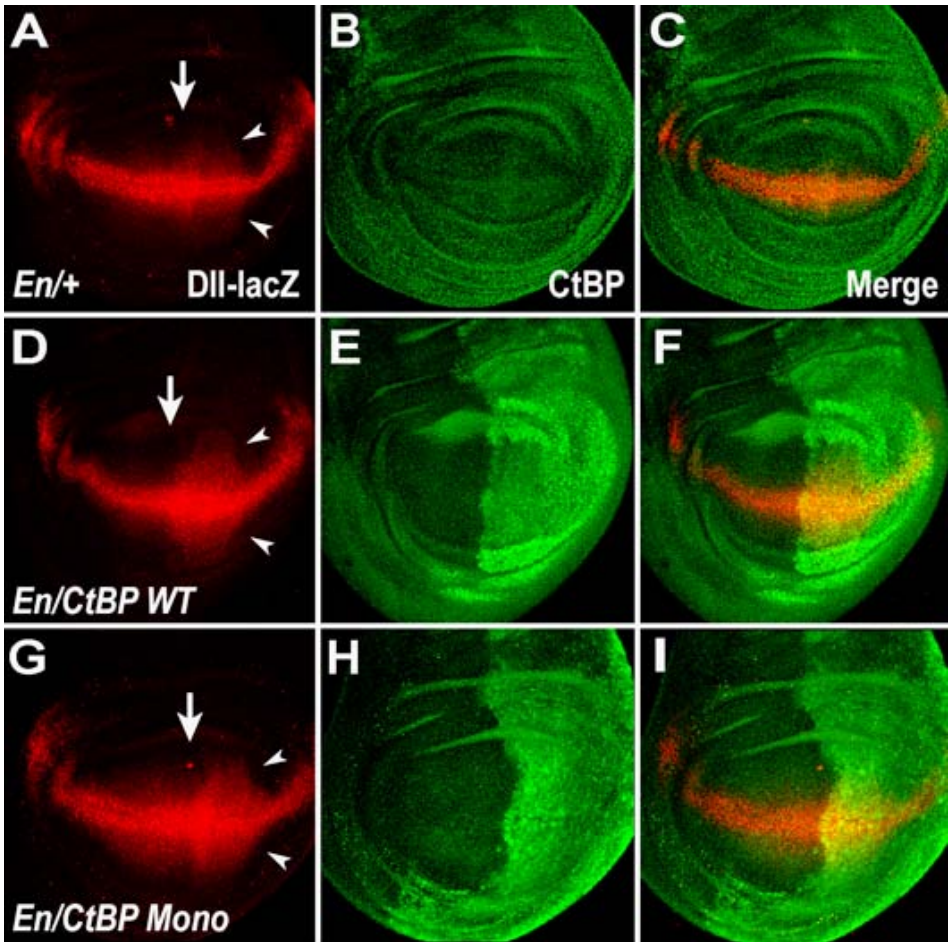

Figure 2.3 CtBP^{Mono} positively regulates the Wg reporter *Dll-lacZ in vivo*. (**A-I**) Confocal images of third instar wing imaginal discs stained for *Dll-lacZ* (red) and CtBP (green) expression from animals containing *En-Gal4* with no transgene (A-C), *UAS-CtBP^{WT}* (D-F) or *UAS-CtBP^{Mono}* (G-I). *En-Gal4* drives CtBP^{WT} or CtBP^{Mono} expression at similar levels (green) in the posterior compartment of the disc (B, E and H; white arrows in A, D and G mark the A/P boundary). Expression of either CtBP^{WT} or CtBP^{Mono} enhances the expression of *Dll-lacZ* (see white arrowheads).

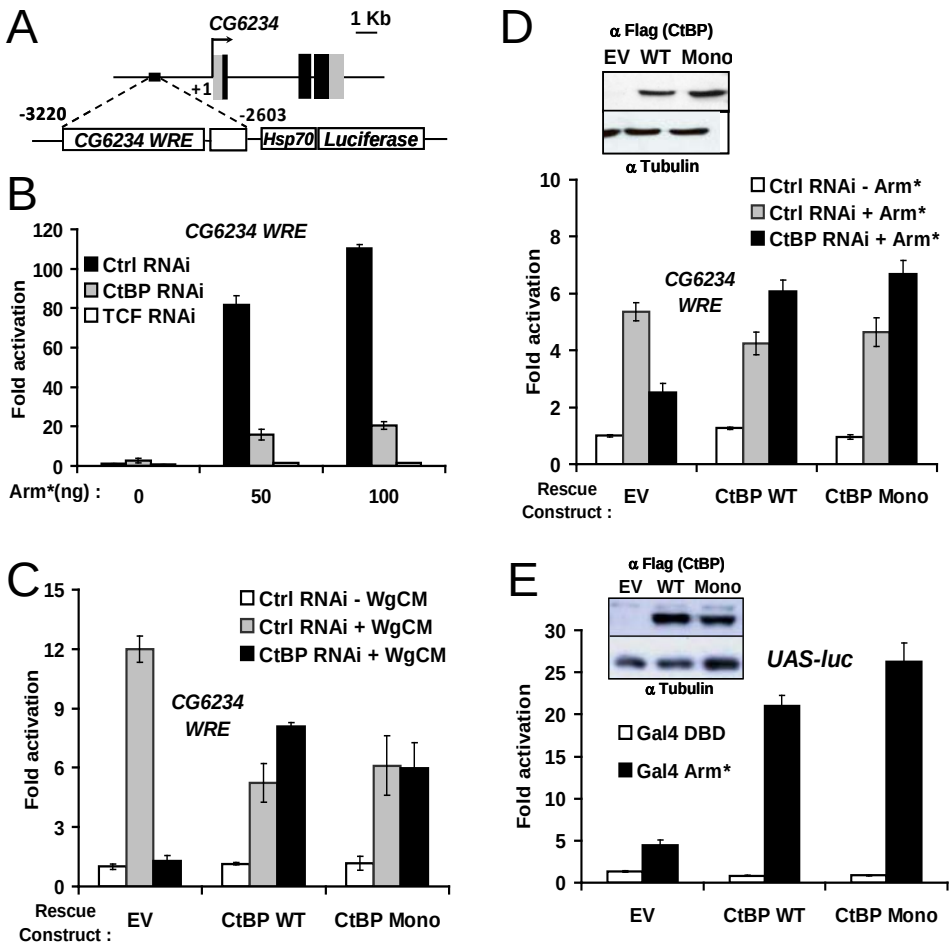

Figure 2.4 CtBP monomers activate Wg targets in Kc167 cells. (**A**) Schematic showing the location of the WRE in relation to the *CG6234* transcription start site and the WRE inserted upstream of a minimal *Hsp70* core promoter/luciferase gene cassette, constituting the *CG6234* WRE reporter. (**B**) Dose dependent activation of *CG6234* WRE reporter by Arm* in Kc cells is attenuated when endogenous *CtBP* or *TCF* is depleted by RNAi to either ORFs in Kc cells. Note that some activation of the *CG6234* WRE reporter still occurs when CtBP is depleted. (**C**) $CtBP^{Mono}$ is able to rescue the Wg-CM mediated stimulation of the *CG6234* WRE reporter when endogenous *CtBP* was depleted. (**D**) $CtBP^{Mono}$ rescued *CG6234* WRE activation by Arm* to a similar level as $CtBP^{WT}$. Both CtBP proteins were expressed at similar levels as judged by immunoblots (inset). (**E**) $CtBP^{Mono}$ was able to enhance the Gal4Arm* mediated activation of *UAS-luc* as efficiently as $CtBP^{WT}$ when expressed at similar levels (inset). Each bar represents a mean of luciferase values from cultures transfected in duplicate (±S.E.), except for panel E, which was in triplicate. Each result is representative of at least three independent experiments, except for panel C, which was performed twice.

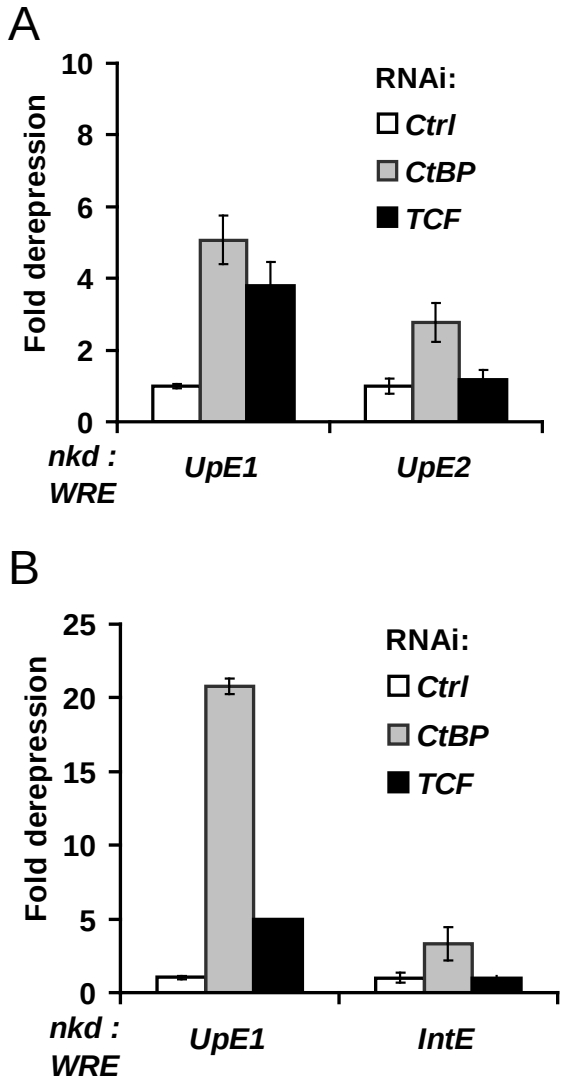

Figure 2.5 ***nkd*** **WREs are repressed by** ***CtBP*** **in the absence of signaling.** **(A)** Reporter assay showing derepression of WREs *nkd-UpE1* and *nkd-UpE2*, derived from the region upstream of the *nkd* transcription start site. In the absence of signaling knockdown of *CtBP* leads to a much higher derepression of *UpE1* compared to *UpE2* and knockdown of *TCF* leads to derepression of *UpE1* but not *UpE2*. **(B)** Reporter assay showing that a WRE from the first intron of the *nkd* gene (*nkd-IntE*) is derepressed to a much smaller degree upon knockdown of *CtBP* or *TCF* when compared to *nkd-UpE1*. Each bar represents a mean of luciferase values from cultures transfected in duplicate (±S.E.) with the result representative of at least three independent experiments.

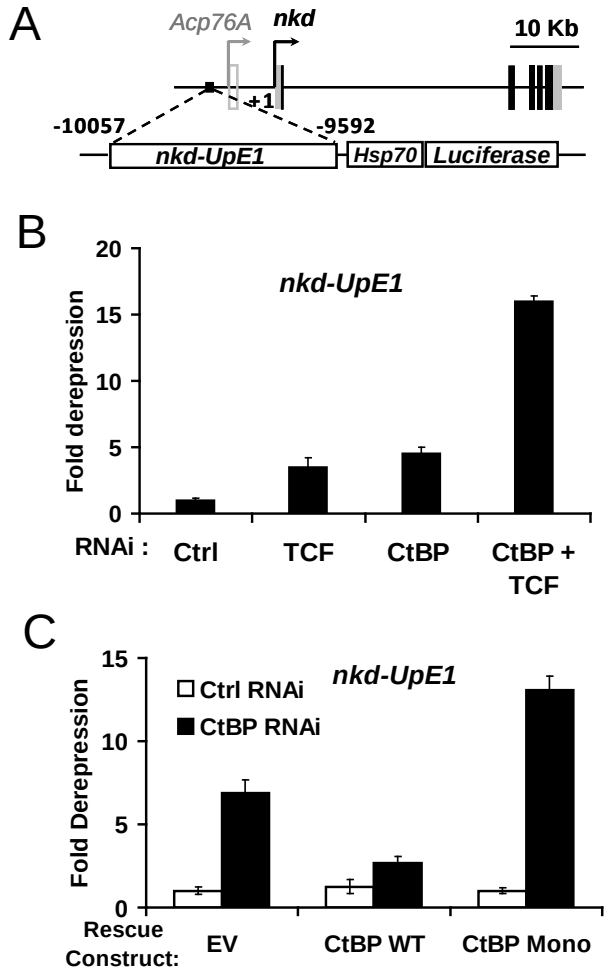

Figure 2.6 CtBP monomers are unable to repress the *nkd-UPE1* WRE in the absence of signaling in Kc cells. (**A**) Schematic showing the location to the *UpE1* WRE in relation to the *nkd* transcriptional start site and outlining the construction of the *nkd-UpE1* luciferase reporter. (**B**) The *UpE1* reporter in Kc cells shows a derepression when endogenous *CtBP* or *TCF* is depleted by RNAi that is greatly enhanced when *CtBP* and *TCF* are knocked down simultaneously. **(C)** Exogenous $CtBP^{Mono}$ cannot rescue the repression of *nkd-UpE1* reporter when endogenous *CtBP* is knocked down, but $CtBP^{WT}$ is able to silence *UpE1* when expressed exogenously in Kc cells. For all experiments, each bar represents a mean of luciferase values from cultures transfected in triplicate (±S.E.). All experiments are representative of at least three independent experiments.

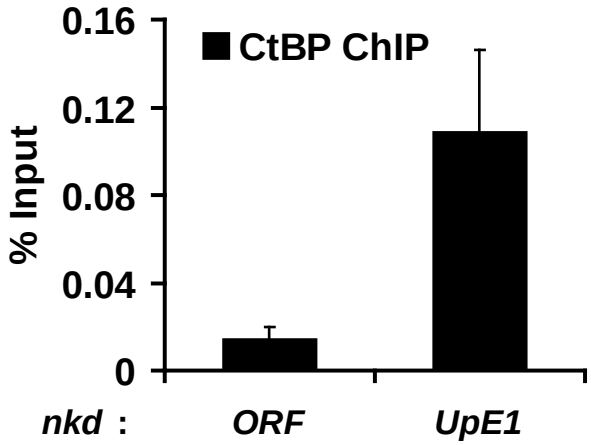

Figure 2.7 CtBP is recruited to the *nkd-UpE1 WRE* in the absence of signaling. CtBP binding to chromatin was assayed by ChIP with an antibody against endogenous CtBP. CtBP is enriched at *UpE1* compared to the coding region (ORF) of the *nkd* gene. Each bar represents a mean of quantitative PCR values in duplicate, from cultures transfected in duplicate (±S.E.). The result shown here is representative of two independent experiments.

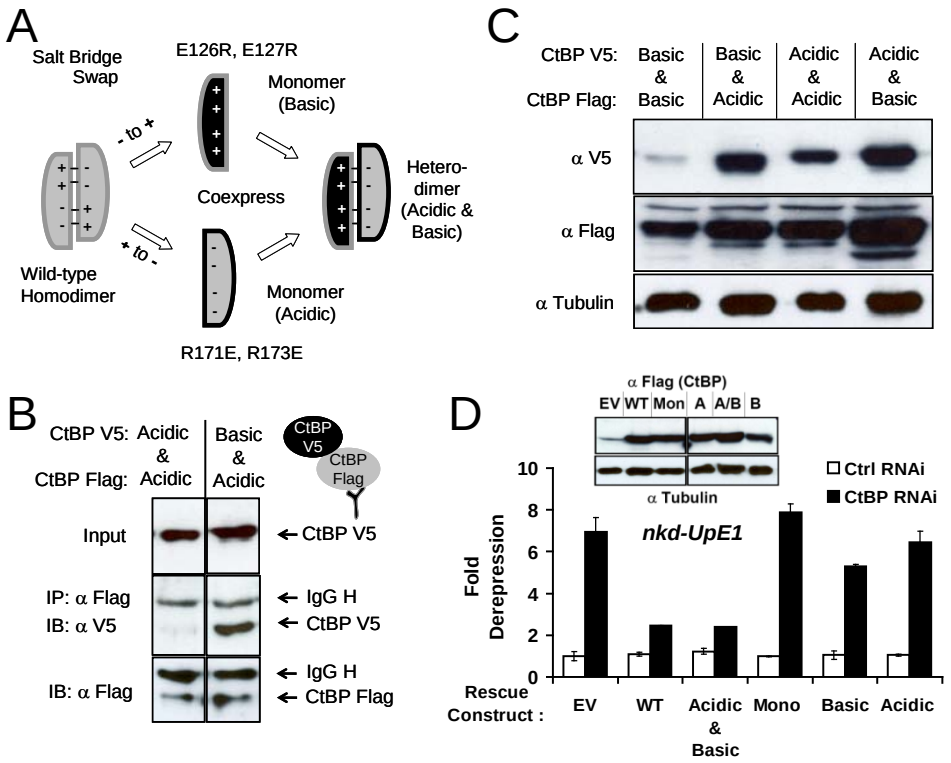

Figure 2.8 CtBP dimerization is required for silencing *nkd-UpE1* reporter in the absence of signaling. (**A**) Cartoon outlining the rationale for creating two monomeric CtBP forms ($CtBP^{Acidic}$ and $CtBP^{Basic}$) which can hetero-dimerize. (**B**) $CtBP^{Acidic}$ cannot co-IP itself but can pulldown $CtBP^{Basic}$. When expressed in Kc cells, a Flag tagged form of $CtBP^{Acidic}$ was unable to pull down a V5 tagged form of $CtBP^{Acidic}$ (lane 1, middle panel) but co-IP was observed with a V5 tagged form of $CtBP^{Basic}$ (lane2, middle panel). Flag tagged forms of $CtBP^{Acidic}$ were pulled down with a similar efficiency (bottom panel). Input (15% of total) are shown in the top panels. (**C**) Immunoblots of Kc cells expressing the indicated CtBP mutants with antibodies against the Flag or V5 epitopes showing that V5 tagged $CtBP^{Basic}$ is stable only when co-expressed with $CtBP^{Acidic}$. V5 and Flag tagged versions of $CtBP^{Acidic}$ were also expressed at higher levels when co-expressed with a complementary $CtBP^{Basic}$. The same amounts of CtBP expression vector were transfected in all lanes. (**D**) Coexpression of $CtBP^{Acidic}$ and $CtBP^{Basic}$ reconstitutes the ability of CtBP to repress the *nkd-UpE1* reporter. Kc cells were depleted of endogenous CtBP by a dsRNA corresponding to the 5' UTR. This resulted in a seven-fold derepression in *nkd-UpE1* reporter activity, which was largely rescued by expression of $CtBP^{WT}$. However, $CtBP^{Mono}$, $CtBP^{Basic}$ and $CtBP^{Acidic}$ are unable to repress *the nkd-UpE1* reporter, and co-expression of $CtBP^{Acidic}$ with $CtBP^{Basic}$ resulted in a similar degree of repression as $CtBP^{WT}$. All the Flag tagged CtBP mutants used were expressed at similar levels (inset). Each bar represents a mean of luciferase values from cultures transfected in duplicate ($\pm$S.E.). For each experiment, the result shown here is representative of at least three independent experiments.

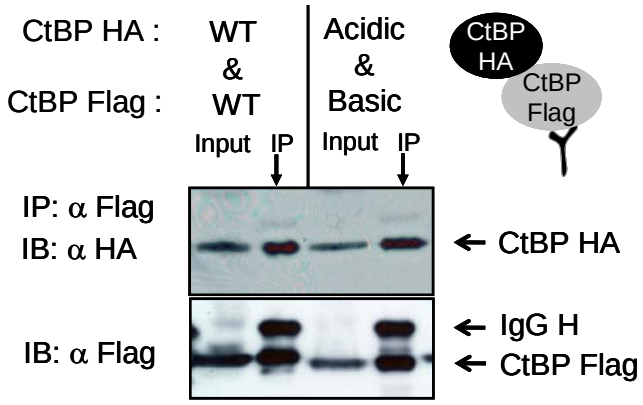

Figure 2.9 $CtBP^{Acidic}$ and $CtBP^{Basic}$ efficiently form heterooligomers. (Top panel) When coexpressed, Flagged tagged $CtBP^{Basic}$ can immunoprecipitate HA tagged $CtBP^{Acidic}$ at comparable levels (lane 4) as similarly tagged versions of $CtBP^{WT}$ (lane 2). (Bottom panel) Flag tagged CtBPWT and $CtBP^{Basic}$ were pulled down at similar levels (compare lanes 2 and 4). Inputs (15% of total) for each co-IP are shown in lanes 1 and 3 of each panel.

***Dpp-Gal4>* *UAS-CtBP* (ºC)**	**Notches (%), n>100**
WT (27)	50.2
Acidic[1]/Basic[1] (27)	43.6
Acidic[2]/Basic[2] (27)	36.0
Acidic[1]/Acidic[2](27)	0
Basic[1]/Basic[2] (29)	0
Mono (29)	0

Figure 2.10 CtBP$^{Acidic/Basic}$ antagonizes Wg signaling during wing development. Percentage of notched wings (n>100 for each genetic background) upon co-expression of CtBP trangenes using *Dpp-Gal4*. Flies were reared at 27˚C or 29˚C to equalize the level of CtBP expression. Two versions of CtBP^{Basic} and CtBP^{Acidic} (1 & 2) were used, so that the transgene copy number was equal when comparing Acidic/Acidic, Basic/Basic and Acidic/Basic wings.

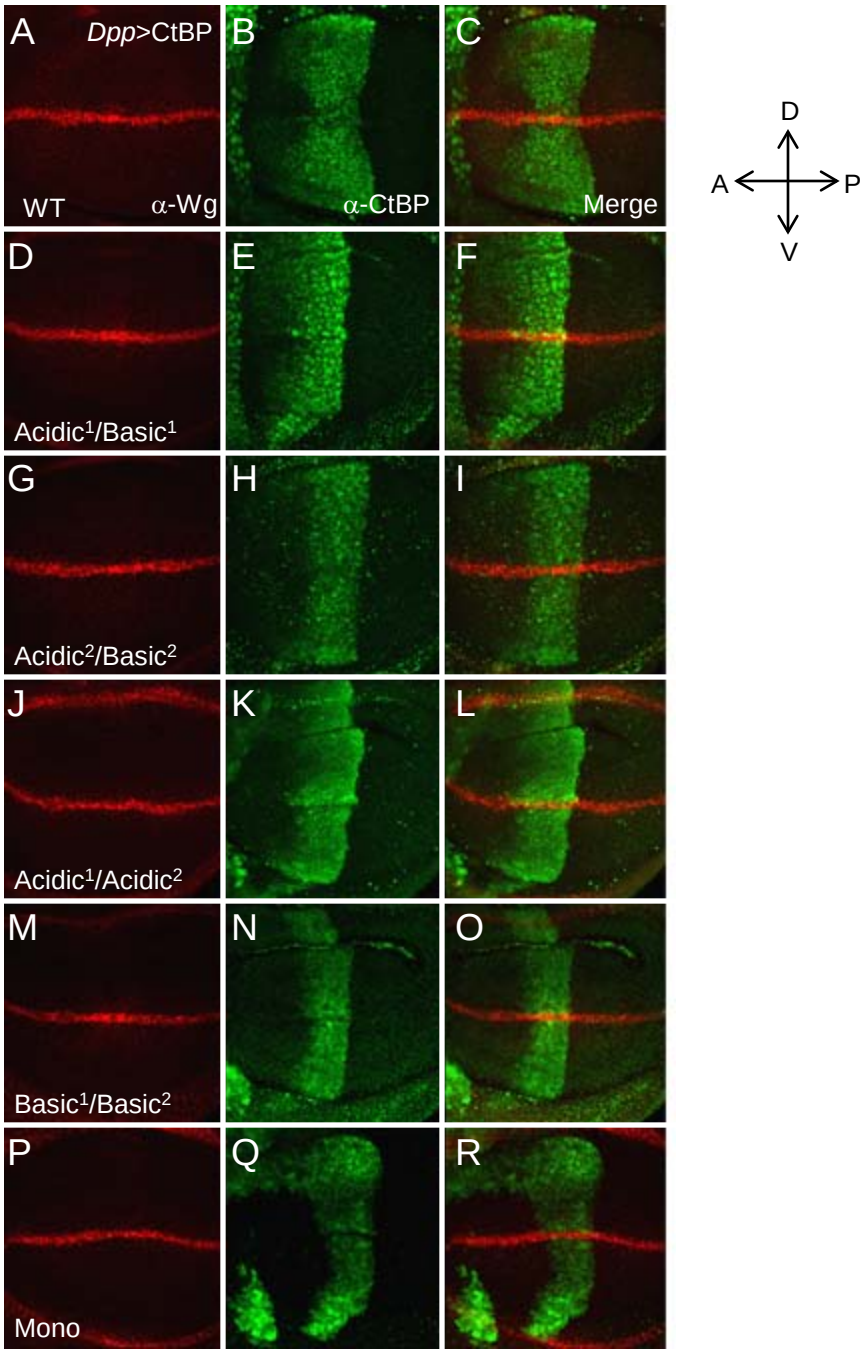
A
Dpp>CtBP
WT
α-Wg
B
α-CtBP
C
Merge
D
A
P
V
D
Acidic1/Basic1
E
F
G
Acidic2/Basic2
H
I
J
Acidic1/Acidic2
K
L
M
Basic1/Basic2
N
O
P
Mono
Q
R

Figure 2.11 Misexpression of CtBP trangenes does not affect Wg expression in the wing primordium. (**A-R**) Confocal images of third instar larval wing imaginal discs showing Wg expression (red) at the D/V boundary of the presumptive wing blade (A, D, G, J, M and P). *Dpp-Gal4* driven expression of $CtBP^{WT}$ (n=21), $CtBP^{Acidic1/Basic1}$ (n=7), $CtBP^{Acidic2/Basic2}$ (n=12), $CtBP^{Acidic1/Acidic2}$ (n=12), $CtBP^{Basic1/Basic2}$ (n=14) and $CtBP^{Mono}$ (n=11) transgenes (green) at the A/P boundary (B, E, H, K, N and Q). Note that $CtBP^{Acidic2/Basic2}$ and $CtBP^{Basic1/Basic2}$ were expressed at lower levels compared to other transgenic combinations (compare H and N to B, E, K and Q) but no combinations affect Wg expression.

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Chapter III

Mutational analysis to investigate the role of CtBP in Wg signaling

Abstract

CtBP is a bimodal transcriptional regulator of Wg signaling. Structural information indicates that CtBP can form dimers and displays high homology to NAD dependent dehydrogenases. CtBP monomers positively regulate Wg targets but are compromised for repression. On the other hand, CtBP oligomers antagonize Wg signaling but it is not known if CtBP dimers can activate Wg targets. In this study, many approaches were employed to 'force' oligomerization of CtBP and test the role of dimers in activation. Futher, role of CtBP monomers in regulation of a Wg target Sens was explored to consolidate the finding that CtBP monomers activate Wg targets. Mutations were also engineered in the catalytic site to test the role of dehydrogenase activity in positive regulation of Wg targets. Mutational analysis indicates that CtBP monomers are not sufficient to activate Sens and catalysis is not required for activation of Wg targets. However, strategies employed to create a 'forced' dimer yielded inconclusive results for the role of CtBP dimers in activation of Wg targets.

Introduction

Development of multicellular organisms is a highly complex process, and cells communicate with each other using several cues, which stimulate events like signal transduction. Remarkably, there are only a few signaling pathways repeated over and over during development, which mediate a variety of processes (Barolo & Posakony, 2002). These pathways follow a common theme of initiating cellular events which include cytosolic and/or nuclear events, and transduce the signal to elicit a transcriptional response. Wnt signaling is one of the highly conserved signaling pathways, which are required for many aspects of animal development (Cadigan & Nusse, 1997). Wnt signaling is also important for adult homeostasis as aberration of the pathway leads to many diseases like colon cancer (Giles et al, 2003; Logan & Nusse, 2004).

The current working model of the pathway is that in the absence of a Wnt ligand, a cytosolic protein Armadillo (Arm; fly β-Cat) is phosphorylated, polyubiquitinated and consequently subject to degradation through the proteasome (Cadigan & Peifer, 2009). Arm is one of the essential co-activators of the pathway and maintaining low levels of Arm is one form of regulation to keep the signaling off. At the nuclear level, the DNA binding anchor of the pathway called T-Cell Factor (TCF) recruits corepressors like Groucho (Gro) which repress targets and keeps them from getting abberantly activated. Although the mechanisms of how the genes are activated in the absence of Arm are not understood, repression of targets is a conserved mechanism to keep the signaling off (Parker et al, 2007).

Signaling is stimulated by the ligand Wingless (Wg; a fly Wnt) which binds to cell surface receptors of the Frizzled family and co-receptors of the LRP 5/6 family (Cadigan & Liu, 2006). This leads to recruitment of some components of the degradation complex to the receptors, and eventual dispersion of the complex (Cadigan & Peifer, 2009). As a result Arm is not degraded, leading to its cytosolic accumulation and nuclear translocation. In the nucleus, Arm interacts with TCF, and recruits cofactors like legless, pygopus and CBP for target gene transcription (Parker et al, 2007).

C-terminal binding protein (CtBP) is another factor which has positive and negative roles in regulating Wg targets (Fang et al, 2006; Hamada & Bienz, 2004). CtBP is also known to act in concert with transcription factors in many other systems, and its role as a corepressor has been extensively explored (Chinnadurai, 2007). The CtBP family of proteins contains a conserved central domain with high homology to NAD^+/NADH dependent dehydrogenases. A weak *in vitro* dehydrogenase activity has been detected in recombinant human CtBP1 (hCtBP1) (Balasubramanian et al, 2003; Kumar et al, 2002). The role of catalytic function in the transcriptional activity of CtBP is controversial. Mutations in the catalytic site compromise co-repressor activity in one case (Kumar et al, 2002), but not in several other contexts (Grooteclaes et al, 2003; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Phippen et al, 2000; Sutrias-Grau & Arnosti, 2004). However, it is not yet known if the catalytic activity of CtBP is required for regulation of Wg targets.

CtBP is a bimodal regulator of Wg signaling and is required for activation of some targets upon Wg stimulation and gene specific repression in the absence of signaling (Fang et al, 2006). It is intriguing how CtBP achieves this differential

regulation. A hypothesis that oligomeric state of CtBP determines its role in differential regulation of Wg targets was tested and the results reported in the previous chapter of this dissertation. In cultured fly cells, CtBP monomers can rescue the activation of some targets when endogenous CtBP is depleted and in flies they enhance Wg dependent phenotypes. However the monomers are incapable of repressing Wg signaling in any context examined so far. This is consistent with a model where CtBP monomers can only play positive roles in Wg target gene transcription.

CtBP oligomers however repress Wg targets, as reported in chapter II of this dissertation. Dimerization of CtBP has also been shown to be indispensable for repression in other contexts apart from Wg signaling (Chinnadurai, 2009; Kumar et al, 2002; Kuppuswamy et al, 2008). For example, CtBP is known to act in concert with a repressor ZEB and chromatin modifying HDACs for repression of *E-Cadherin* (E-Cad) (Kuppuswamy et al, 2008; Shi et al, 2003). Surprisingly, CtBP monomers can form a complex with repressors like ZEB and HDAC2, but are unable to repress *E-Cad* (Kuppuswamy et al, 2008; Zhao et al, 2009). So what are the mechanisms which determine repression only by CtBP dimers, although monomers can interact with these repressors?

ZEB and HDAC2 compete for the same binding site on a CtBP monomer (Zhao et al, 2009), and hence an attractive model is where two monomeric subunits in a dimer, bridge the recruitment of different corepressors. Inspite of having overlapping interaction site, these repressors are bound to different CtBP monomer subunits simultaneously. Consistent with that, other studies have shown that monomers are incapable of repressing (Kumar et al, 2002; Kuppuswamy et al, 2008; Zhao et al, 2009). However in any context

so far, there is no function of CtBP monomer reported. Hence functional interpretations with the CtBP monomer mutants requires caution as the mutations might abrogate its ability to function in any context.

As reported in the previous chapter, a salt bridge swap strategy was used to show that reconstitution of a heterodimer using complementary monomer interactions antagonizes Wg signaling. To demonstrate that, acidic and basic residue pairs, which form inter-molecular salt bridges in a CtBP dimer were swapped. These mutations affect homodimerization of CtBP, but when $CtBP^{Acidic}$ and $CtBP^{Basic}$ mutants are coexpressed, they form heterodimers as the salt bridges are restored. Only the dimers were capable of antagonizing Wg signaling, but not the monomers. This is consistent with the proposed model that dimer formation is essential for repression by CtBP, and the monomers function as repressors only when they self-associate.

However one unresolved questions when it comes to Wg target gene activation by CtBP is can CtBP dimers activate Wg targets? In order to address that, three different approaches were used. Firstly, expression of $CtBP^{Basic/Acidic}$ pair was tested for the enhancement of Arm* mediated reporter activity. Secondly, to eliminate the possibility of a monomeric pool contributing to the activation function of the $CtBP^{Acidic/Basic}$ pair, another mutant with a hydrophobic dimerization interface was engineered to create a more stable dimer ($CtBP^{Dim}$). The third approach was aimed at increasing the probability of dimer formation by covalently linking two CtBP monomeric subunits using a sixty six amino acid glycine-serine linker.

In the first approach, $CtBP^{Basic/Acidic}$ pair was able to positively regulate Arm* mediated reporter expression, and whether this was due to the monomeric pool or heterodimeric, could not be distinguished. The second strategy of increasing hydrophobicity of the interface strategy did not yield conclusive results, as mutations abolished transcriptional activity of CtBP. In the third approach of covalently linking monomeric subunits, the CtBP concatemer was prone to degradation and stabilized to a fragment similar in size to the wildtype CtBP. The degraded concatemer was able to enhance the Arm* mediated reporter activity, but whether this activity was from the degraded fragment or the full length concatemer could not be resolved. Hence the hypothesis that dimers can activate Wg targets could not be tested.

Another unresolved question in the context of regulation of Wg targets by CtBP is the requirement of the NAD dependent catalytic activity. Since this has never been tested, mutations in the catalytic site were engineered and tested for the enhancement of Arm* mediated reporter activation. These mutations did not abolish the activation function of CtBP, as it was able to augment Arm* mediated reporter activation. Results indicate that catalysis may not be required for the biological role of CtBP as an activator of Wg targets.

In the previous report, CtBP monomers were shown to activate Wg targets in cultured fly cells and positively regulate signaling in some fly tissues. To extend the *in vivo* analysis, regulation of a Wg target Sensless (Sens), by a mutant impaired for dimerization known as $CtBP^{Mono}$, was tested in the wing imaginal disc. To that end, a hypomorphic allele of CtBP was sequenced. Previously unmapped lesion was identified to be a non-sense mutation, predicted to generate a truncated protein. Mosaic clones

generated with this strong allele of CtBP did not express Sens and ectopic expression of $CtBP^{Mono}$ could not rescue Sens expression in the clones.

This analysis suggests that expression of Sens may be depdendent on both the repression and activation function of CtBP. Alternatively monomers may not be activating Wg signaling in all contexts. Currently it is not known if Sens is a direct target of Wg signaling. Hence it calls for caution to interpret data from targets which are used as Wg readouts, but may not be directly regulated by Wg signaling.

Results

Coexpression of $CtBP^{Acidic/Basic}$ enhances Arm activity

CtBP oligomers antagonize Wg signaling in some contexts and CtBP monomers can activate but not repress signaling in some contexts. What is not known is if CtBP oligomers can activate Wg targets. In order to test this hypothesis, reconstitution of $CtBP^{Acidic/Basic}$ heterodimers was tested for regulation of Arm* mediated reporter activity. These mutations were in conserved residues aimed at restoring salt bridges (Figure 3.1). Since Arm has atleast two transactivation domains (Fang et al, 2006; Hecht et al, 1999; Stadeli & Basler, 2005), recruitment of Arm* to *cis-elements* using Gal4 DNA binding domain (Gal4Arm*) leads to significant activation of a *UAS-luc* reporter (Fang et al, 2006). Misexpression of $CtBP^{Wt}$ and $CtBP^{Mono}$ led to an enhancement of reporter expression (Figure 3.2). Similarly misexpression of the $CtBP^{Basic}$ or $CtBP^{Acidic}$ also enhanced the reporter expression (Figure 3.2).

This result consolidates the finding that CtBP Monomers positively regulate this Wg target, as mutations in three different regions of the dimerization interface of CtBP lead to an enhancement of the Arm activity (Figure 3.1). However coexpression of $CtBP^{Acidic/Basic}$ pair also enhanced the reporter expression at an extent similar to $CtBP^{Wt}$ and $CtBP^{Mono}$ (Figure 3.2). Hence these data suggest that the co-expression of $CtBP^{Acidic/Basic}$ pair positively regulates Wg signaling in this context.

Nonpolar substituitons in the dimerization interface abrogate the activation and repression functions of CtBP in the context of Wg signaling

It is possible that a monomeric pool in the $CtBP^{Acidic/Basic}$ pair is enhancing Arm activity. Hence, to test the hypothesis that dimers are compromised for activation of Wg targets, a CtBP molecule had to be engineered which upon expression has the equilibrium predominantly shifted to the dimeric form. In order to create this 'forced dimer' molecule, two approaches were undertaken. The first was to make nonpolar substitutions in the dimerization interface to increase the hydrophobicity and stabilize dimer contacts (Figure 3.1). The second was to covalently link two monomeric subunits and increase the probability of dimer formation (Figure 3.4A).

For the first approach, four nonpolar substitutions were made ($CtBP^{Dim}$)(C134V, N138I, T143V, Y144F) to make the dimerization interface more hydrophobic and induce the self-association of CtBP monomers (Figure 3.1). When tested for the enhancement of the Gal4 Arm* activated *UAS reporter*, $CtBP^{Dim}$ was unable to augment reporter

expression although it was expressed at levels similar to $CtBP^{Wt}$ (Figure 3.3). This data suggests that $CtBP^{Dim}$ was unable to activate Wg signaling in this context.

In order to test if the $CtBP^{Dim}$ has a role in Wg signaling *in vivo*, flies carrying the $CtBP^{Dim}$ transgene were tested for regulation of the Wg readouts in the eye and the wing. Ectopic expression of $CtBP^{Wt}$ in the fly eye leads to suppression of Arm* induced small eye phenotype, suggesting that $CtBP^{Wt}$ antagonizes signaling in this context. On the contrary $CtBP^{Mono}$ enhances the small eye suggesting that it positively regulates signaling in this context. A similar regulation was seen in the adult fly wing, where misexpression of $CtBP^{Wt}$ induces wing notches while $CtBP^{Mono}$ alleviates the antagonism of Wg signaling. When tested for regulation of these Wg dependent phenotypes, misexpression of $CtBP^{Dim}$ at levels similar to $CtBP^{Dim}$ had no affect (data not shown). These results suggest that nonpolar substitutions in the dimerization interface, which make it more hydrophobic, abrogate the ability of CtBP to regulate Wg signaling.

CtBP concatemer engineered with a glycine-serine linker is prone to degradation

As an alternative approach to 'force' dimerization of CtBP, a concatemer was engineered using a glycine-serine linker by covalently linking two *CtBP* coding regions. The crystal structure of dehydrogenase domain of CtBP reveals that it forms a homodimer in anti-parallel orientation (Figure 3.4A). The C-terminus of one subunit is about 100 Aº from the N-terminus of another subunit (personal communication; David L. Akey). Hence a sixty six amino acid long linker containing glycine and serine residues

was engineered to covalently link the C-terminus of one subunit with N-terminus of another *CtBP* subunit (Figure 3.4A).

Functional analysis of this CtBP linker chimera, showed that it was able to enhance the Gal4Arm* mediated reporter activation at levels similar to the wildtype CtBP (Figure 3.4B). When tested for expression, the concatemer was degraded and a predominant truncated fragment seen at a size similar to the $CtBP^{Wt}$, as judged by the immunoblot (Figure 3.4B). There were multiple degradation fragments observed between the concatemer and monomeric CtBP suggesting that concatemerization of CtBP rendered it unstable and subject to degradation. The functional analysis was inconclusive and it was undetermined if the truncated fragment, or concatamer or both were contributing to the activity of Gal4 Arm*.

Catalytic activity of CtBP is not required for enhancement of Arm activity

CtBP exhibits high sequence and structural homology to D2-hydroxyacid dehydrogenases and displays weak catalytic activity *in vitro* (Balasubramanian et al, 2003; Kumar et al, 2002). The role of catalysis in the transcriptional activity of CtBP is controversial. Mutations of residues in the catalytic site abolish the repression by CtBP in one context (Kumar et al, 2002) but not in the others (Grooteclaes et al, 2003; Kuppuswamy et al, 2008; Mani-Telang et al, 2007; Phippen et al, 2000; Sutrias-Grau & Arnosti, 2004). However the requirement of catalytic activity for activation by CtBP is not known. To address that, two sets of mutations (H312T and D290A, H312T) were engineered in conserved residues (Figure 3.1), which have been shown to be important

for the catalytic activity of CtBP. When tested for enhancement of the Gal4 Arm* activity, both mutants were able to augment the Arm* mediated activation of the *UAS reporter* (Figure 3.5). These data suggest that mutations in the catalytic site do not abrogate activation of this Wg target by CtBP.

CtBP Mono is unable to compensate for endogenous CtBP to activate Wg target Sens.

Sens is known as one of the short range targets of Wg signaling which is activated in cells adjoining the D/V boundary where levels of secreted Wg are the highest (Parker et al, 2002). During the third instar larval stage, Wg leads to activation of Sens in a double row of cells dorsal and ventral to Wg expressing cells (Figure 3.6 B, F, J). CtBP is required for activation of Sens in the wing imaginal disc as generating mosaic clones using a hypomorphic allele of *CtBP* leads to a loss of Sens (Figure 3.6 B, F, J) (Fang et al, 2006). Although this hypomorphic allele ($CtBP^{87De10}$) (Poortinga et al, 1998) has been used for genetic analysis to study the role of CtBP in various processes during fly development, the mutation has not been characterized.

Sequence analysis of this EMS induced allele revealed that the lesion was a non-sense mutation (Q229 to *) (Figure 3.1), predicted to generate a truncated fragment (see materials and methods). Mosaic clones generated in the wing imaginal disc using this allele of CtBP, had reduced levels of endogenous CtBP as judged by immunostains (Figure 3.6 C, G, K) (Fang et al, 2006). CtBP was detected in adjacent clones expressing GFP, which are wildtype, suggesting that the truncated protein is probably unstable or not

efficiently detected using this polyclonal antisera. Consistent with this protein being severely abrogated for CtBP function, maternal and zygotic expression of this allele using germline clones leads to pleiotropy during embryonic development (Nibu et al, 1998; Poortinga et al, 1998). Hence genetic data suggests that this truncation severely abrogates the function of CtBP.

To test the role of CtBP monomers in regulating Sens, $CtBP^{Mono}$ was ectopically expressed in the posterior region of the wing imaginal disc, which contained mosaic clones of $CtBP^{87De10}$. Ectopic expression of $CtBP^{Wt}$ led to a rescue of the Sens expression consistent with Sens being dependent on CtBP for activation (Figure 3.6 B). Expression of the $CtBP^{Mono}$ at similar levels was unable to rescue Sens expression in these clones when tested with two different transgenic lines (Figure 3.6 F, J). These data suggest $CtBP^{Mono}$ is not sufficient for the activation of Sens, and $CtBP^{Mono}$ cannot compensate the loss of endogenous CtBP for Sens expression.

Discussion

Do CtBP dimers activate Wg targets?

Regulation of Wg targets by CtBP is determined by its oligomeric state. In the previous report, it was shown that monomers are capable of activating Wg targets but are compromised for their ability to repress. Reconstitution of monomers into heterodimers with complementary salt bridges restored the repressive function of CtBP suggesting that dimerization is required for negative regulation of Wg targets. In order to test if dimerization is required for activation of Wg targets, the heterodimers were tested for

regulation of a Gal4Arm* stimulated reporter. Coexpression of the $CtBP^{Acidic/Basic}$ heterodimer augmented the Gal4Arm* mediated reporter activation. This data suggests that either the heterodimers contain a pool of monomers which may be enhancing the reporter activation or dimers are capable of positively regulating Wg signaling. A third possibility is that both dimers and monomer fractions might be capable of activating signaling, but only dimers might be able to repress targets.

Co-Immunoprecipitation of differentially tagged forms of CtBP shows that $CtBP^{Acidic/Basic}$ self-associates at levels similar to the wildtype CtBP (Figure 2.9, Chapter II). Hence $CtBP^{Acidic/Basic}$ also has a similar ratio of monomers and dimers, as the wildtype CtBP. Since $CtBP^{Wt}$ is able to both activate and repress targets, but CtBP monomers are only capable of activating targets, it is likely that the monomeric pool of the $CtBP^{Acidic/Basic}$ pair augments Arm activity. However if the dimers are also capable of activating targets, it is consistent with a model where repression is solely a function of CtBP dimers but activation is a co-ordinated effort of monomers and dimers. A mutant which is predominantly a dimer would be required to further distinguish between the different possibilities.

In an attempt to 'force' dimerization of CtBP, the first approach taken was to create a hydrophobic dimerization interface similar to the one found in molecules like Gro, p53 and Chibby (Chen et al, 1998; Mofunanya et al, 2009; Song et al, 2004). Gro forms tetramers in solution mediated by a Leucine Zipper Like domain (LZL) at the N-terminus, which has been shown to be important for its repression in non-Wnt related contexts (Chen et al, 1998). The LZL tetramerization domain of Gro contains two amphipathic alpha helices with buried polar patches implicated in stabilizing the tetramer

(Chen et al, 1998). Replacing the LZL domain with the heterologous tetramerization domain of p53 does not impair the repression function of Gro (Chen et al, 1998). This demonstrates that the only function of this domain is to form quartenary structures. Such a strategy to stabilize a dimer can be used for Gro because the tetramers adapt a parallel orientation. In the case of CtBP, each monomeric subunit adopts an anti-parellel confirmation to form a dimer. Hence an approach of engineering a hydrophobic interface was taken in order to further stabilize the dimer interactions when compared to the wild-type CtBP.

Functional data suggests that the nonpolar substitutions in the dimerization interface impair the transcriptional activity of CtBP in several contexts. The loss of function is not due to instability as the mutant is expressed at levels similar to the $CtBP^{Wt}$ in cultured cells and in flies. One of the several possibilities is that the polar patch in the dimerization interface, which was mutated affects the dimerization of CtBP. At the same time it also impairs the interface contacts of the monomer with its interacting partners, hence rendering it non-functional.

A second possibility is that this molecule is not localized in the nucleus and hence impaired for its nuclear functions. Mutations in the dimerization interface of hCtBP2 (R141A/R163A) have been shown to impair the nuclear localization (Kuppuswamy et al, 2008). While none of these residues were mutated in the version engineered for this study, it is possible that these mutations could be impairing its nuclear localization, since no information is available on the nuclear localization signals of *Drosophila* CtBP. Fusion of a heterologous nuclear localization signal and examining the mutant activity in the transcriptional assays would be required to further address this possibility.

Another approach taken to enhance the probability of CtBP monomers forming dimers was to covalently link two monomeric subunits. Structural analysis of CtBP shows that it forms an anti-parallel dimer under conditions of crystallization (Kumar et al, 2002; Nardini et al, 2003). So the C-terminus of one monomeric subunit is ~100 A from the N-terminus of another subunit (personal communication; David L Akey). A sixty six amino acid long glycine-serine linker was used in an attempt to provide flexibility to the monomeric subunits for dimerization. However the concatemer was subject to degradation and of the many degraded fragments, the predominant one was in the size range of the wildtype monomer. This suggests that the linker is most likely subject to cleavage, and leads to a stabilized monomeric molecule instead of the concatemer.

The degraded concatemer was also capable of activating the Gal4Arm* mediated reporter. This could be because of the CtBP concatemer and/or the truncated fragments enhancing the Gal4Arm* mediated reporter activation as monomers or dimers. Hence I was unable to distinguish if it was the monomer or the forced concatemer which is able to activate the reporter. Considering that a mutant form of CtBP, which predominantly remains a dimer could not be engineered, the hypothesis that dimers can activate Wg targets could not be tested.

Catalytic activity of CtBP is not required for positive regulation of the Arm responsive reporter

CtBP shows a high homology to NAD+/NADH dependent dehydrogenases and displays a weak dehydrogenase activity *in vitro* (Balasubramanian et al, 2003; Kumar et

al, 2002). It has a conserved Rossman fold which contains four key residues mediating catalysis - a histidine, an aspartate, a glutamate and an arginine (Kumar et al, 2002; Nardini et al, 2003) (Figure 3.1). Mutation of the four residues or the histidine alone has been shown to abolish the weak dehydrogease activity of CtBP *in vitro*.

In the context of transcriptional activity, mutation of the four residues abolishes repression in one case (Kumar et al, 2002), although it has not been determined if this mutant is impaired in nuclear localization. However mutation of the single histidine does affect its repressive function in several non-Wg contexts *in vitro* and *in vivo*. As it is yet unknown if catalysis is required for activation by CtBP, a catalytic site mutants with one and two residues mutated were tested for enhancement of the Gal4Arm* activity. Both mutants were able to augment the Gal4Arm* mediated reporter activation suggesting that the catalytic activity of CtBP is not required for positive regulation of this reporter.

CtBP monomers are not sufficient to rescue Sens expression

CtBP has been shown to activate Wg targets in cultured cells and *in vivo* (Fang et al, 2006). In the wing primordium , CtBP partially activates two Wg targets, Distal-less (Dll) and Sens (Fang et al, 2006). CtBP is required for spatial expression of Dll, as expression of Dll is lost in clones away from the D/V boundary, hence not making it a very penetrant phenotype for rescue analysis. To test the role of CtBP monomers in regulation of Wg targets *in vivo*, the loss of Sens expression was examined, as that is completely penetrant during early stages of Sens expression.

Ectopic expression of $CtBP^{Mono}$ could not rescue Sens expression in clones with a strong allele of CtBP. This data raises the possibility that Sens is a target which might require both the repression and activation function of CtBP. Hence CtBP dimers and monomers may act in concert directly or indirectly with a combination of repression and activation for expression of Sens, as it is not known if Sens is a directly or indirectly regulated by Wg. Alternatively, the mutations in the interface which force monomerization of CtBP, might affect its interaction with factor or factors which might be essential for Sens activation. It is also likely that CtBP Monomers may not be sufficient for activation of all Wg targets or the activation of Wg targets by CtBP might be gene specific for monomers and dimers. Finally, as it is not known if Sens is a direct target of Wg signaling, the data for such readouts needs to interpreted with caution, as Sens may be an output of activation and repression by Wg signaling.

Materials and Methods

Plasmids and reporter assays

Kc167 cell culture was performed as reported previously (Fang et al, 2006). *pAcCtBP*^{short} with 2x Flag tags at the C-terminus (kindly provided by Dr. D. Arnosti) was used for all reporter assays. Site directed mutagenesis of *pAcCtBP*^{short} (hereafter referred to as $CtBP^{WT}$) was used to introduce mutations in the dimerization interface to generate $CtBP^{Mono}$ (C134Y, N138R, R141A, R142A), $CtBP^{Basic}$ (E126R, E127R), $CtBP^{Acidic}$ (R171E, R173E) and $CtBP^{Dim}$ (C134V, N138I, T143A, Y144F). The sixty six amino acid glycine-serine linker was generated by PCR from *pET52B* (kindly provided by Dr. J.

Bardwell) and cloned into the KpnI site of $CtBP^{WT}$, followed by cloning another CtBP ORF upstream of the linker using a KpnI and NotI site (Figure 3.3A). The start codon downstream of the linker was mutated to avoid an alternative translation start. *pAcGal4DBD, pAcGal4Arm*, pUAS-luc* and *pActinlacZ* constructs have been described elsewhere . For transgenic lines, cDNAs for the $CtBP^{WT}$ and mutants with two C-Terminal flag tags were subcloned into *pUAST* vector using the KpnI and XbaI sites. Gal4Arm* reporter assays were performed as described in Chapter II.

Drosophila **genetics**

Transgenic *UAS-CtBP* lines were generated using the injection facility at BestGene Inc. (Chino Hills, CA). w^{1118}, *En-Gal4, 5188* ($p[FRT]^{82B}$, *p[GFP])* and $CtBP87^{De10}$ were obtained from Bloomington Stock Center. The EMS generated $CtBP^{87De10}$ (Poortinga et al, 1998) chromosomal region (3R:8837388 to 8851905) was sequenced by extracting genomic DNA (Purgene DNA purification, Gentra systems Inc.) from $CtBP87^{De10}$/*TM6* and $CtBP87^{De10}$/*p[GFP]* adults and compared with sequence obtained from genomic DNA of *p[GFP]* and w^{1118} adults. All contigs were aligned with the available annotated genomic sequence from Flybase (www.flybase.org).

Out of the 10 single nucleotide polymorphisms (SNPs) found over the entire locus, two SNPs were unique to the two $CtBP87^{De10}$ chromosomes sequenced, and showed overlapping peaks in the chromatogram, since the flies were heterozygous. One of this was a silent mutation in the coding region and the other was a non-sense mutation (CAG (Q229) to TAG (*)) in the third exon. Since the regions upstream and downstream

of the transcription start site were not sequenced, there are possibilities of mutations in those regions, although the probability is low as *CtBP87*^{De10} was an EMS generated allele.

Clones of *CtBP*^{87De10} were generated by mitotic recombination in lines carrying CtBP transgenes using *hsFLP* and a *p[FRT]*^{82B} , *p[GFP]* chromosome via 1h 37ºC heatshock and 24-48 h after egg laying. Experiments with *En-Gal4* were carried out at 25ºC.

Immunoblots and immunostains

For western blot analysis, anti-Flag (1:2500, Sigma) were used followed by HRP conjugated anti-Mouse (Jackson Immunochemicals). Signal was detected using ECL kit (Amersham). Immunostaining of wing imaginal discs was performed as described previously using anti-Sens (1:1000) and anti-CtBP (1:1000) (Fang et al). Alexa 488, Cy3 and Cy5 conjugated secondary antibodies were obtained from Molecular Probes and Jackson Immunochemicals. Samples were examined using a Leica triple channel confocal microscope DM6000B-CS and processed using Adobe Photoshop 8.0.

Figure 3.1. Mutation scheme of conserved residues in *Drosophila* CtBP. Alignment of *Drosophila* CtBP (dCtBP) with Human CtBP1 (hCtBP1) depicting the high homology. Residues in black and bold represent the ones targeted for mutation and ones on top represent the residues in the mutants, which are $CtBP^{Basic}$,$CtBP^{Mono}$,$CtBP^{Dim}$,$CtBP^{Acidic}$ and $CtBP^{Cat}$. The lesion in a hypomorphic $CtBP^{87De10}$ allele is represented as a non-sense mutation (*).

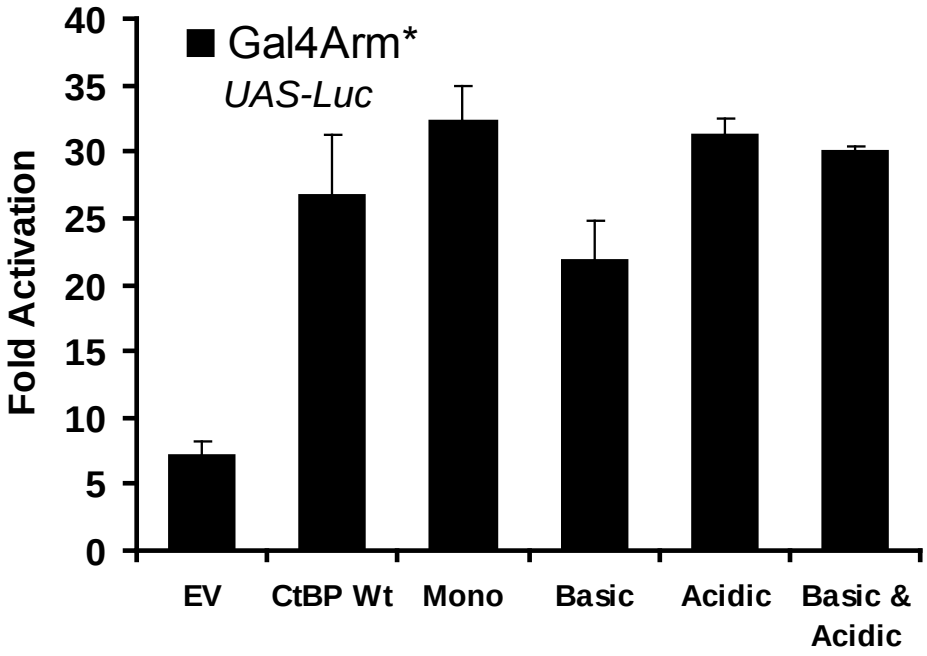


Figure 3.2. CtBP Mono Acidic & Basic coexpression enhances Gal4Arm* mediated activation of a *UAS-Reporter*. Histogram showing luciferase activity of a *UAS-reporter*. Gal4 Arm* was co expressed with empty vector (EV) or CtBP Wildtype (Wt), Monomer (Mono), Basic, Acidic or Basic & Acidic mutants. CtBP Basic & Acidic coexpression enhances the reporter activation to a similar extent as CtBP Wt. Each bar represents a mean of triplicates (±S.E.) and the data shown here is representative of three independent experiments.

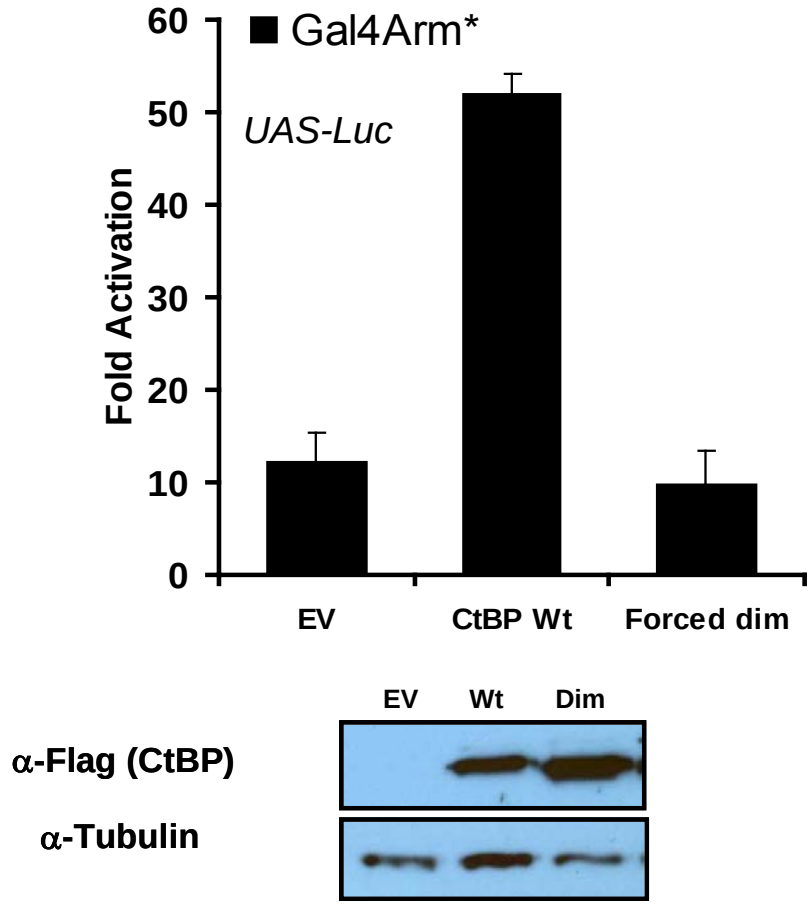
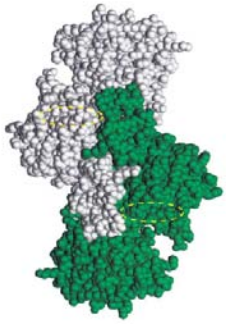
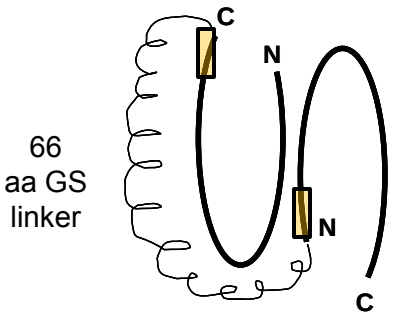


Figure 3.3. Mutations in the dimerization interface to make it hydrophobic abolish the activation function of CtBP. Histogram showing luciferase activity of a *UAS-reporter*. Gal4 Arm* was co expressed with empty vector (EV) or CtBP Wildtype (Wt) or Forced Dim. Forced Dim is unable to enhance the Gal4 Arm* mediated reporter activation although expressed at levels similar to Wt (bottom panel). Each bar represents a mean of triplicates (±S.E.) and the data shown here is representative of three independent experiments.

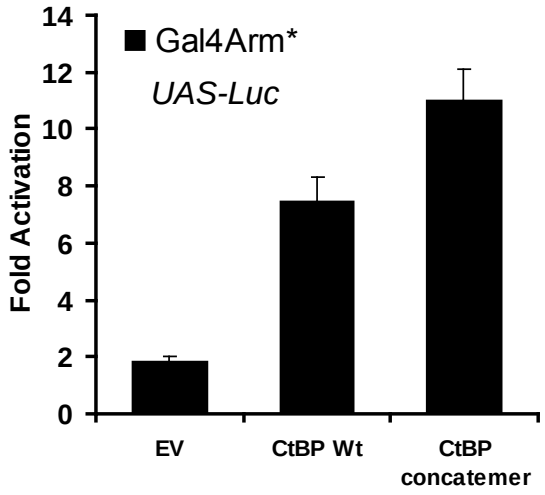
A





CAA	ATG	ATA	TCA	AAT	CAA	GAA	AAG	GCG	GCC	GCA	GGT	GGT	GGT	GGT	TCT	GGT
Q	M	I	S	N	Q	E	K	A	A	A	G	G	G	G	S	G
GGT	GGT	GGT	TCT	TCC	TCA	GGT	TCA	GGT	GGC	GGG	GGA	TCT	GGT	GGT	GGT	GGC
G	G	G	S	S	S	G	S	G	G	G	G	S	G	G	G	G
TCA	GGA	TCC	GGT	GGC	TCG	AGT	TCC	GGG	AGC	GGG	AGC	TCT	TCT	GGT	TCC	GGA
S	G	S	G	G	S	S	S	G	S	G	S	S	S	G	S	G
GGC	GGT	GGA	GGA	TCA	GGC	GGT	GGC	GGA	TCA	GGA	AGT	GGG	AGC	GGA	GGC	GGC
G	G	G	G	S	G	G	G	G	S	G	S	G	S	G	G	G
GGA	TCA	GGC	GGA	GGT	GGA	AGC	GGT	AGT	CTG	GAC	AAA	AAT	CTG	ATG	ATG	CCG
G	S	G	G	G	G	S	G	S	L	D	K	N	L	M	M	P

B



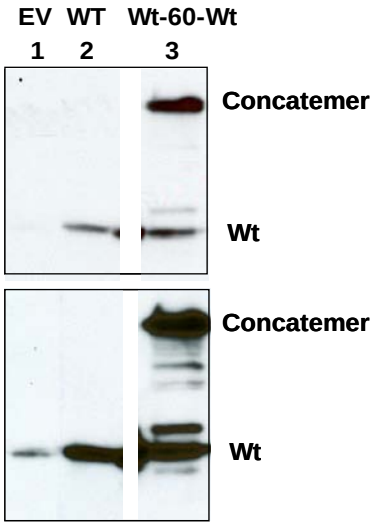


Figure 3.4. Covalent linkage of two CtBP monomers using a Glycine-Serine linker makes it prone to degradation and positively regulates Gal4Arm* activity. (A) Space fill model of CtBP Wt showing the anti-parellel orientation of a CtBP dimer (left) (adapted from Kumar et al., 2002). Strategy used to covalently link two CtBP Monomers with a sixty-six amino acid (aa) Glycine-Serine (GS) linker. The linker sequence is shown with boxed regions showing the C-terminus of a monomer linked to the N-terminus of another. (B) Histogram showing luciferase activity of a *UAS-reporter*. Gal4 Arm* was co expressed with empty vector (EV), CtBP Wt or CtBP Concatemer (Wt-60-Wt). CtBP concatemer enhances the Gal4 Arm* mediated reporter activation. Each bar represents a mean of triplicates (±S.E.) and the data shown here is representative of two independent experiments. The panels on the right are immunoblots which show that the concatemer (lane 3, top panel) gets degraded to a smaller fragment which is similar in size to the Wt protein (lane 2, top panel). At a higher exposure, more degradation fragments are detected (bottom panel).

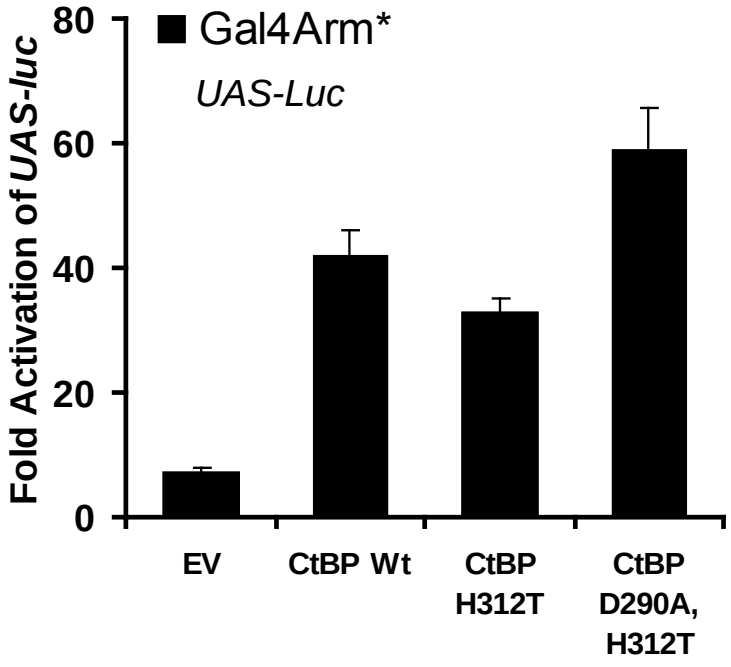


Figure 3.5. Mutations in the catalytic site do not abolish the enhancement of Gal4 Arm* mediated reporter activation. Histogram showing luciferase activity of a *UAS-reporter*. Gal4 Arm* was co expressed with empty vector (EV) or CtBP Wildtype (Wt) or catalytic mutant with a one (H312T) or two (H312T, D290A) residues mutated . Both catalytic mutants enhance the reporter activation to a similar extent as CtBP Wt. Each bar represents a mean of triplicates (±S.E.) and the data shown here is representative of three independent experiments. *This data was published in Fang et al., 2006.*

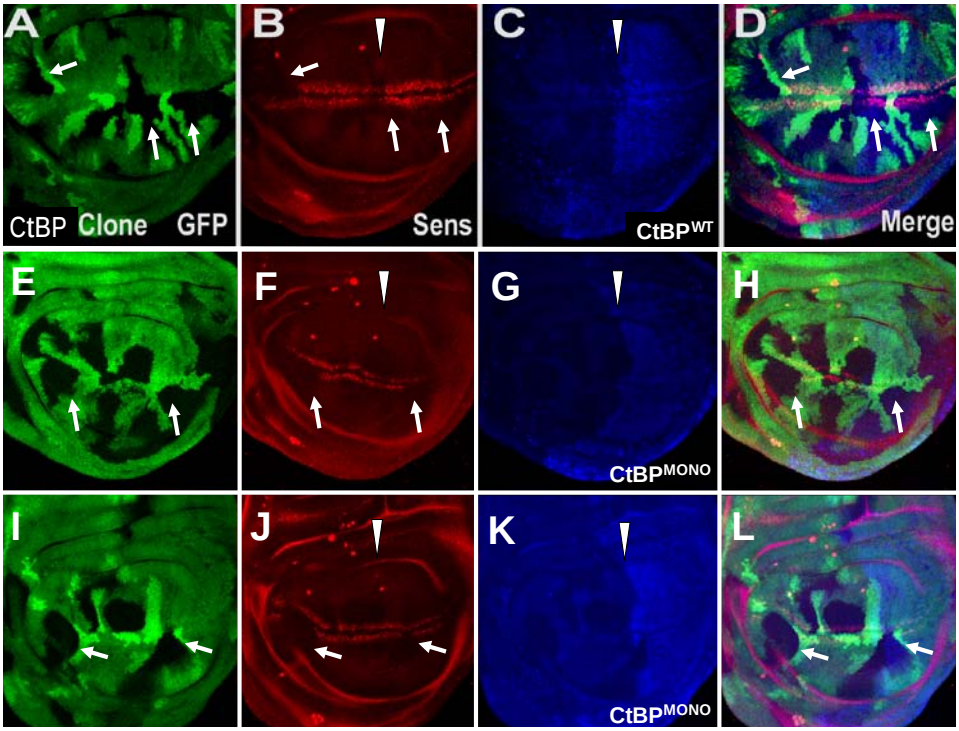


Figure 3.6. CtBP Monomers are not sufficient for the activation of a Wg target Sens in the wing primordium. CtBP activity was removed by creating mitotic clones of *CtBP*^{87De10}, a strong allele of *CtBP*. Clones (white arrows) were marked by a loss of GFP (green) and monitored for Sens expression (red). Ectopic expression of CtBP (blue) using En-Gal4 was used to rescue the loss of Sens expression. The anterior/posterior axis is marked by a arrowhead Note the reduced levels of endogenous CtBP in the clones. (A-D) Confocal images of third instar wing discs. Ectopic expression of CtBP^{WT} in the posterior rescues the loss of Sens. Clones in the anterior with no ectopic CtBP^{WT} do not express Sens. (E-H) Ectopic expression of CtBP^{Mono} is unable to rescue Sens expression. (I-L) Similar results were obtained using another line expressing the CtBP^{Mono} transgene.

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Chapter IV

Exploring POP1 oligomerization and investigating the role of Helper-like *cis-elements* in binding of POP1

Abstract

Wnt/Wg signaling regulates target gene expression through a DNA binding mediator TCF. *Drosophila* TCF and human TCF E-isoforms display a bipartite DNA binding thorough the highly conserved HMG box and C-Clamp domains. In fly Wnt/Wg response elements (WREs), the HMG binding sites and the C-Clamp binding Helper sites are required for activation of several target genes. While the HMG sites are extensively studied in many systems, there is little known about the importance of Helper sites in other organisms. Further, the HMG-Helper pairs in the fly WREs do not show a stringent requirement for orientation and spacing. In this report, I show that Helper like sequences are required for the binding and Wnt mediated response of *C. elegans* TCF homolog POP1. I also show that POP1 forms oligomers in cultured cells and propose a model where POP1 attains the flexibility to bind variably spaced and oriented HMG-Helper pairs by forming oligomers.

Introduction

Wnt signaling is a highly conserved signaling pathway important for several aspects of animal development and aberration of the pathway is a cause for many diseases (Cadigan & Nusse, 1997; Logan & Nusse, 2004). The pathway is regulated by controlling the nuclear levels of β-Catenin (β-Cat), which is one of the key effectors of the pathway (Cadigan & Peifer, 2009). β-Cat is known for its function in linking the cell-adhesion molecules Cadherins to the cytoskeleton but a cytosolic pool of β-Cat transduces Wnt signals (Cadigan & Peifer, 2009) .

In the absence of Wnt stimulation, low cellular levels of β-Cat are maintained as it is subject to degradation upon phosphorylation and subsequent polyubiqitination . The phosphorylation is mediated by a complex of proteins which include adenomatous polyposis coli, Axin, glycogen synthase kinase3 and caesin kinase I (Kennell & Cadigan, 2009). Signaling is initiated by a group of highly conserved ligands called Wnts, which lead to several downstream events including target gene transcription. Binding of Wnts to the frizzled group of serpentine receptors and LRP 5/6 single pass membrane coreceptors leads to stabilization of β-Cat (Cadigan & Liu, 2006). The phosphorylation of β-Cat is inhibited, through less understood events involving Dishevelleds. This inturn leads to increase in nuclear β-Cat levels, which regulates Wnt target genes.

The transcriptional changes induced by Wnt signaling are mediated by a group of conserved proteins called T-Cell Factors (TCFs) (Parker et al, 2007). In the nucleus, TCFs repress target gene transcription in the absence of signaling but activate targets upon signal stimulation (Parker et al, 2007). Hence apart from regulating nuclear levels

of β-Cat, another prominent mode of Wnt regulation in the nematode *C. elegans* is to titrate the nuclear levels of worm TCF homolog POP1 (Phillips & Kimble, 2009). This Wnt initiated event inturn relieves the repression by POP1 and in order to activate signaling. This is a consequence of a phosphorylation event which is Wnt/MAPK dependent as it is mediated by one of the four worm β-Cat s called wrm-1, which does not bind POP1. In worms, this is one of the key signaling events in establishing Anterior/Posterior asymmetry from embryonic to larval stages (Phillips & Kimble, 2009). However two of the other three β-Cat homologs, SYS1 and BAR1 act in a canonical signaling pathway and mediate POP1 dependent activation of Wnt targets. Hence apart from the canonical branch, worms have a TCF regulatory branch which mediates Wnt signaling events.

TCFs bind DNA through a High Mobility Group (HMG) DNA binding domain and recruit transcriptional regulators to target loci (Arce et al, 2006). The *cis-elements* recognized by the HMG box have been extensively studied in several contexts (Parker et al, 2007). However based on their high frequency of occurrence in the genomes, how TCFs locate their specific targets still remains a mystery. Algorithms which predict Wnt targets based on a particular distribution of HMG binding sites (Hallikas et al, 2006) have not had a high success rate in identifying a majority of experimentally validated Wnt targets (Chang et al, 2008; Hatzis et al, 2008; Yochum et al, 2007). Furthermore, it has been demonstrated experimentally in fly cells for a Wnt target *naked cuticle*, that having a cluster of HMG sites is not sufficient to 'fish out' a *bona fide* WRE (Chang et al, 2008).

The discovery of a novel cis-element termed the 'Helper' site has provided insight into mechanisms of target gene selection by fly TCF (Chang et al, 2008). These

additional elements are required for activation of several Wingless (Wg; a fly Wnt) targets and Wnt response elements (WREs) examined so far (Chang et al, 2008). Helper sites bind to another DNA binding domain in TCF known as the C-Clamp (Chang et al, 2008). Mammals have four TCF genes and majority of isoforms do not express the C-Clamp (Arce et al, 2006). However flies and worms contain one TCF gene which codes for the C-Clamp (Arce et al, 2006). In flies, the C-Clamp has been shown to significantly contribute to the binding and function of TCF, which complements the requirement of Helper sites for Wg target activation (Chang et al, 2008). Hence fly TCF follows a bipartite mechanism of binding to DNA. Since Helper sites are crucial for activation of several fly targets, it is an intriguing question if this mechanism is conserved among other invertebrates.

In worms, not many directly regulated Wnt targets have been identified, and the three known targets are *ceh22* , *psa3* and *end1* (Arata et al, 2006; Calvo et al, 2001; Lam et al, 2006; Shetty et al, 2005). In this report, I have investigated the requirement of putative Helper sites for binding of POP1 to a *ceh22* WRE. Two Helper-like sites in the *ceh22 WRE* were identified based on sequence similarity to the fly Helper sequence, and tested for binding to full length POP1. *In vitro* binding assay indicates that these helpers are important for binding of POP1 to the *ceh22* WRE. Further in cultured fly cells, helper sites in the *ceh22* WRE reporter are required for a maximal response to Wnt signaling. Hence the mechanism of bipartite binding may be conserved amongst flies and worms, as helper sites are required for binding of POP1 to the *ceh22* WRE.

In many fly WREs examined, the functional HMG and Helper sites are clustered (Chang et al, 2008) but do not have a fixed orientation with respect to each other, nor

have a rigid spacing requirement. There don't seem to be stringent rules for spacing and orientation of the Helper sites in reference to the HMG sites. It is attractive to hypothesize that oligomerization of POP1 on the WREs bridges the variable interactions with the HMG and Helper sites, which would make the orientation and spacing requirements less stringent. Consistent with a model of homo-oligomerization, I report that POP1 self-associates in cultured fly cells. On native gels, bacterially purified full length POP1 and a fragment missing the N-terminal β-Cat binding domain migrate at a range suggestive of a high molecular weight complex with DNA. Hence oligomerization may be required for binding of POP1 to the different HMG-Helper pair combinations, and consequently regulate Wnt target genes.

Results:

Full length POP1 expressed in E.Coli, binds to the *ceh22, psa3* and end1 WRE fragments *in vitro*

ceh22, psa3 and *end1* are three known targets of Wnt signaling, which have been shown to be regulated in a HMG site dependent manner. A truncated fragment of recombinant POP1 containing only the HMG box has been shown to bind the *ceh22 WRE* probe, but binding with full length POP1 has not been reported for any WREs to date. Probes derived from the *ceh22, psa3* and *end1 WRE* containing known functional HMG sites were tested for binding to the full length POP1. *ceh22* has two HMG sites which are important for binding of a truncated POP1 fragment containing the HMG box. However, it is not known if both sites contribute individually to the Wnt mediated response of

ceh22. Two probes containing each of the two HMG sites termed *ceh22-1* and *ceh22-2* were tested for binding to POP1. Binding was detected for both probes and competed with increasing molar excess of unlabelled probe (Figure 4.1). Similar results were seen for the *psa3* and *end1* probes (Figures 4.2 & 4.3) indicating that full length POP1 specifically interacts with these WRE fragments *in vitro*.

HMG and Helper like sequences are important for binding of POP1 to the *Ceh22-2 WRE*

Previously, novel cis-elements have been identified termed the Helper sites, which were shown to be required for the function and binding of fly TCF to the WREs (Chang et al, 2008). Helper site mediated activation has been shown to be through a DNA binding domain called the C-Clamp which is conserved among flies and worms. To investigate the role of helper sites in binding of POP1 to worm WREs, a sequence scan based on similarity to fly Helper sites and their distance from the HMG site were used to find putative helper sites. This led to the identification of two candidate *cis-elements* termed Helper1 and Helper2 in *ceh22-2* (Figure 4.1).

To test if HMG and Helper sites in these WREs are important for the interaction of POP1, labeled probe with mutated HMG site or mutated Helper was tested for binding to POP1. There was no detectable binding of POP1 to the HMG mutant or Helper mutant probes (Figure 4.4). In a competetion assay, molar excess of the wildtype unlabelled probe was able to compete with the *ceh22-2* labelled probe for binding to POP1. The HMG and Helper mutant probes did not compete significantly (Figure 4.5) with the

wildtype labeled probe. These results indicate that the HMG site and putative Helper sites are important for binding of POP1 to the *ceh22-2 WRE in vitro*.

To define the minimal sequence in the Helper sites required for POP1 binding, the first four bases comprising the GCCG motif in both helper sites were mutated. These mutants were termed as S_h1 and S_h2. In a second set of mutations, the last three bases AAA and CTT of the Helper sites were mutated and termed h1_S and h2_S respectively. In a third set of mutations, all seven bases on Helper1 (H1 mut) and Helper2 (H2 mut) were mutated. When tested in the competition assay, the S_h1, h1_S , S_h2 and h2_S were strong competitors for POP1 binding when compare to the H1 mut and H2 mut (Figures 4.6 and 4.7). These results indicate that each Helper is important for binding of POP1 to the *ceh22-2 WRE in vitro* and the entire seven base pair sequence is required for strong binding of POP1.

Helper sites are important for BAR1 mediated activation of the *ceh22 WRE* reporter in cultured fly cells

In order to investigate the functional importance of the Helper sites for activation of the *ceh22 WRE* , a 0.5 kb genomic fragment was tested for a response to Wnt signaling in the cultured fly cells KC167 (KC). Misexpression of the worm β-Cat/BAR1 and TCF/POP1 led to a weak activation of *ceh22 WRE* reporter (Figure 4.8). Misexpression of POP1 alone did not activate this WRE (data not shown). Mutation of the individual HMG sites did not significantly affect the reporter activation (Figure 4.8). However mutation of Helper 1 or Helper 2 significantly affected the BAR1/POP1 mediated

reporter activation (Figure 4.6). This data suggests that the Helper sites are important for activation of the *ceh22 WRE* reporter in this heterologous system.

POP1 forms oligomers in KC cells

The Helper and HMG sites in fly WREs do not show a rigorous orientation and spacing constraint but fly TCF binds the WREs in a bipartite fashion. One way to achieve that is if TCF can form oligomers which might bridge the binding to individual HMG and Helper sites. Hence self-association of POP1 was tested in KC cells by co-Immunoprecipitation (co-IP). Flag and HA tagged forms of POP1 were co-expressed and antibody directed to the Flag tag was used to immunoprecipitate POP1-Flag from whole cell lysates. POP1 HA was co-IPed only from cells co-expressing POP1 Flag (Figure 4.9). These results indicate that more than one POP1 molecules form a complex in KC cells.

Bacterially purified full length POP1 and a N-Terminal truncated fragment migrate as a high molecular weight complex when bound to DNA

In vitro binding of full length POP1 to WREs has never been reported before. However when tested for binding to the *ceh22-2* WRE probe, POP1 seemed to migrate as a high molecular weight DNA-protein complex (Figure 4.10). This was further evident when compared to migration of a truncated GST (Glutathione S-Transferase) tagged fly TCF fragment, containing only the HMG box and C-Clamp, on a native gel (Figure 4.10).

The predicted molecular weight of this GST-HMG-C fragment is ~ 41 KDa (GST ~26 KDa & HMG-C ~ 15 KDa). Although not demonstrated in this report, GST is known to dimerize (Lim et al, 1994). Hence the predicted molecular weight of GST-HMG-C dimer under native conditions would be ~82 KDa.

The predicted molecular weight of full-length recombinant POP1 expressed in E.Coli is ~ 64 KDa. Average molecular weight of a DNA base pair is 0.66 KDa and hence the 69 bp *ceh22-2* WRE probe is ~45 KDa. So when in complex with the *ceh22* WRE probe, the predicted molecular weight of the GST-HMG-C dimer fragment is ~127 KDa, and the POP1 monomer fragment is ~109 KDa. However the POP1 in complex with the WRE probe migrates at a higher molecular weight range compared to the GST-HMG-C clamp fragment (Figure 4.10). Hence POP1 might form oligomers in complex with the *ceh22-2* WRE *in vitro*.

A similar complex was observed for a truncated POP1 fragment missing the N-terminal region 1-112 (ΔN POP1) (Figure 4.11). The predicted size of ΔN POP1 in complex with the *ceh22-2* probe is ~97 KDa. Under denaturing conditions, ΔN POP1 and the full length POP1 migrate at the predicted size range (~43 KDa and 64 KDa respectively) (Figure 4.11B). However under native conditions in complex with the *ceh22-2* WRE, ΔN POP1 migrates at a high molecular weight range similar to the full length POP1 (Figure 4.11C). This result suggests that ΔN POP1 might form oligomers when in complex with the *ceh22* WRE and the N-Terminal β-cat binding domain of POP1 is not be required for that.

Discussion

Conserved role of Helper sites in bipartite binding of TCF

DNA binding motif in the E-tail of TCFs called the C-Clamp is highly conserved from invertebrates to vertebrates (~86% similarity) (Arce et al, 2006). Bipartite binding of fly TCF through HMG box and C-Clamp is dependent on HMG sites and Helper sites and C-Clamp is important for the activation function of TCF (Chang et al, 2008). Functionally, Helper sites are essential for Wg mediated regulation of WREs in cultured cells and in flies. Consistent with a conserved role, this analysis shows that the Helper sites are important for binding of POP1 to the *ceh22-2 WRE*.

The mammalian C-Clamp of human TCF1E has also been shown to bind an extended sequence RCCG (R=A/G) similar to the helper site, although this sequence has not been functionally validated (Atcha et al, 2007). However the bipartite binding of TCF is required for activation of Wg targets and may be conserved among worms, considering the only TCF gene in worms *POP1* contains a conserved C-Clamp. Functionally mutation of the Helper sites significantly affects the activation of *ceh22-2 WRE* in cultured fly cells (Figure 4.8) . Hence a rigorous functional validation *in vivo* is required to test if these Helpers are physiologically important for Wnt target gene activation. In addition, putative Helper sites need to be validated in the context of other known Wnt targets *PSA-3* and *end-1*, which contain functional HMG sites.

Oliogmerization of POP1

The architecture of the fly WREs indicates that the HMG-Helper pairs do not have a very stringent orientation and spacing requirement (Chang et al, 2008). This has also been tested *in vitro* for the fly TCF fragment where Helper site augments the binding of HMG-Clamp to the HMG site in different orientations (Mikyung Chang; unpublished data). So what provides TCFs the flexibility to bind HMG-Helper with variable orientation and spacing?

p53 is an example of DNA binding proteins that are dimers in solution, however in complex with DNA they form tetramers (Riley et al, 2008). Each dimer binds a 10 bp consensus termed 'half site' which is separated from another half site by 0-21 bp (el-Deiry et al, 1992; Funk et al, 1992). Hence oligomerization is one way which provides flexibility in recognizing DNA motifs with variable spacing.

Another way this can be achieved is through a flexible linker which joins the HMG and C-Clamp. An example of such a linker based bipartite DNA binding family of factors is POU-1 (Pit, Oct and Unc) (Herr et al, 1988). A member of the POU-1 family of proteins called Oct-1 has two DNA binding domains and act co-operatively to bind an octamer sequence 5'ATGCAAAT3' (Herr et al, 1988). However the two DNA binding domains of POU known as the POU-specific and POU-homeo domain can bind independently to the ATGC and AAAT motif respectively. Structural studies suggest that these domains contact DNA on opposite faces of the octamer sequence (Klemm et al, 1994). These domains are joined by a flexible linker (25 aa), the length of which varies

amongst the POU domain family, which aids them in contacting opposite faces of the octamer.

A similar mechanism might impart the DNA binding to TCFs as the HMG and C-Clamp in worms is separated by 19 amino acids and flies by 27 amino acids. A crystal structure of the HMG-Clamp with a HMG-Helper pair might partly address how many A^{o} apart are the HMG-Helper pairs are to make conclusions that the region between the HMG and C-Clamp can act as a linker. However considering the variable orientation and spacing of HMG-Helper pairs in the fly WREs and the two helpers in *ceh22* WRE there are likely to be other mechanisms which TCF utilizes bind to the HMG-Helper pairs.

One example of an alternative mechanism comes from another member of the POU family of proteins known as Pit-1. The POU domain of Pit-1 does not have a defined DNA binding consensus. Structural information indicates that these molecules form homodimers lets them adapt different confirmations due to flexibility of the linker, and as a consequence recognize more degenerate binding sites (Jacobson et al, 1997). Pit-1 contains the shortest linker amongst the POU family of proteins (15 aa), but recognizes a 12 bp consensus (Jacobson et al, 1997). In this case, homodimerization and a flexible linker aids in the Pit-1 protein recognizing a longer consensus.

Considering that both the helpers in the *ceh22* WRE are important for binding of POP1 and the Helper2 is 31 bases away from the HMG site, one possibility is that the oligomerization of TCF leads to the binding of HMG box and C-Clamp of different subunits to the HMG and Helper sites. While there are possibilities where the POP1 is already present as a dimer or in a higher order form in solution, it is also possible that the

oligomerization is induced upon binding to the DNA probe. Further biochemical analysis like sedimentation velocity, native PAGE or gel filtration of the recombinant POP1 in presence and absence of the DNA probe can help distinguish between those possibilities.

Co-Ip of differentially tagged forms of POP1 in cultured fly cells supports a model that POP1 forms oligomers on the WREs to contact the HMG-Helper pairs (Figure 4.9). Consistent with that, full length recombinant POP1 and the truncated fragment, migrate at a higher molecular weight range than expected for a monomer-DNA complex (Figures 4.10 & 4.11). Although this is not indicative of oligomer formation because the globular structure of a protein determines its mobility in native gels, it is suggestive of a higher order complex formation. Alternatively, this could also be a result of DNA bending as the HMG boxes can bend DNA (Behrens et al, 1996; Giese et al, 1992; Love et al, 1995), which alters its mobility in the native gels.

What could be another functional relevance of TCF oligomers?

One of the key requirements for a transcription factor to provide the required functional output is the ability to find the right *cis-elements*. In the case of TCF, finding the WREs is the goal and the Helper sites have been shown to provide a level of specificity in differentiating a WRE from a region that is not responsive to Wg signaling (Chang et al, 2008). There might be many factors which are important for the architecture of these elements, e.g. combination of other transcription factor sites, spacing requirement or position of the HMG and Helper sites in the WRE. However there could

also be additional regulation at the transfactor level, which prevents it from binding to other loci which may be 'attractive' or putative WREs.

In the case of fly TCF, *in vitro* analysis suggests that the mutation of the C-Clamp greatly enhances the binding of the HMG box to the HMG site. This is consistent with a model where the C-Clamp prevents the exposure of HMG box to regions containing only the HMG sites, and only lets it bind to regions which have both HMG and Helper sites. So this self inhibition can help keep a check on binding of TCF to 'attractive' but 'spurious' sites. Oligomerization of TCF could be one mode of action where the HMG and Clamp on different molecules interact with each other to achieve that. The domain of oligomer formation might also lie at a different region than the HMG and C-Clamp

How conserved is the self-association of POP1?

Considering that the helper versus HMG orientation and spacing does not follow very stringent rules in fly WREs, it may be highly likely that the fly TCF also forms oligomers to interact with these sites simultaneously. A majority of the vertebrate TCF isoforms do not express the C-Clamp which suggests that the C-Clamp may not be essential for functions of the vertebrate TCFs. However if the oligomer formation of TCFs is conserved amongst vertebrates, then it is possible that the isoforms containing the C-Clamp might form hetero-oligomers with isoforms which do not express the C-Clamp. This could regulate some targets which have the Helper like sites, and are dependent on isoforms not expressing the C-Clamp for activation.

Materials and Methods

Electrophoretic Mobility Shift Assay

EMSA was performed using 5' Biotinylated probes from Integrated DNA Technologies. The assays were performed on 6% Tris-Borate-EDTA Native gels using the LightShift Chemiluminescent EMSA Kit (Pierce) and the Chemiluminescent Nucleic Acid Detection Module (Pierce) as per the manufacturer's instructions. Recombinant proteins and probes were incubated in Binding buffer (10 Mm Tris-HCl pH 7.5, 50 mM KCl, 1mM DTT) and 50 µg/ml poly (dI-dC), 0.05% NP-40, 5mM $MgCl_2$ and 5% Glycerol (final). The mixture was incubated on ice for 5 min and room temperature for 30 min. For competition, the unlabeled probes were added to the protein mix 10 min prior to the labeled probes. The concentrations of proteins and probes used in each experiment have been specified in the figure legends. Each EMSA was performed atleast twice and representative shown.

Vectors and Plasmids

Recombinant POP1 was induced in BL23 Codon plus strain (Stratagene). E.Coli incubated with overnight culture in 2.4 litres of Luria Broth and induced using 100 mMIPTG at $O.D._{600}$ of 0.6 at 37º C. After 4 hours of induction, cells were spun down at 5000 rpm for 15 min. Resuspension buffer (PBS+1%Glycerol+20mM Immidazole) were added to the cells and they were subject to lysis in the French press. Lysates were spun at 12,000 rpm for 10 min and the supernatant incubated with 2 ml Nickel coated beads from Sigma and loaded onto a column. After washing the beads with 20 column volumes of

Wash buffer (PBS+1%Glycerol+40mM Immidazole), the proteins were eluted using 5 ml of Elution buffer (PBS+10%Glycerol+200mM Immidazole). Complete Protease inhibitor- EDTA free mix was added to all buffers as per manufacturer's instructions and the recombinant proteins stored at -80ºC.

Co-IP

For co-IPs, 6-10 $x10^6$ Kc cells were seeded with 1µg pAcCPOP1/10^6 cells for 3 days before harvesting. Cells were resuspended in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.9, 1% CHAPS, 10% glycerol, 0.1 mM EDTA with Complete mini-EDTA free protease inhibitor cocktail, (Roche)) and sonicated thrice on ice in pulses of 6 seconds. Lysates were pre-cleared using Protein A/G sepharose beads. Total protein concentration was measured using *DC* protein assay (Bio-Rad). Lysates corresponding to 3mg total protein was used for each IP. 5 % of this lysate was saved as input. The remainder was incubated with 5µg primary antibody for 2 hours at 4ºC followed by incubation with Protein A/G sepharose beads for 30 minutes at 4ºC. The antibody-antigen complexes were washed 4 times with lysis buffer and eluted in 60µl of Laemmli sample buffer for western blot analysis. Results shown are representative of two independent experiments.

Plasmids and reporter assays

pAC POP1 with 2x Flag and 4X HA tags at the C-terminus was made using POP1 cDNA kindly provided by Dr. David Eisenmann. A PCR POP1 amplicon (Forward

5’ATCCGGTACCATGGCCGACGAAGAGG3’, Reverse 5’TGATTTGATATCGCAG-TACACATCGATTCC3’ was cloned into the KpnI and EcoRV sites in *pAC 5.1* containing 2 Flag Tags or 4 HA Tags as described in chapter II. BAR1 cDNAa was obtained by Yuji Kohara and cloned into KpnI and NotI sites of Pac 5.1. *pGL3ceh22 WRE was* made by PCR of a 0.5 kb region (Forward 5’ ACATCGAC-GCGTCCGATTGTTTCCTACAAT3’, Reverse 5’ CATCCGCCCGGGGGTGAAATG-TGAAGGAGTCGGAC3’) upstream of the splice acceptor site in the first *ceh22 intron* and the amplicon cloned into MluI and XmaI sites of *pGL3* as described in Chapter II . *pAclacZ* construct has been described in chapter II.

ceh22 WRE and reporter assays were performed by transiently transfecting 50 ng of the reporter and 500ng POP1 along with 500 ng of empty vector or BAR1 expression plasmids in 2.5 x 10^5 cells/well. For all reporter assays 5 ng of *pAcLacZ* was transfected for normalization and *pAC5.1* (Invitrogen) to control for DNA amounts. Luciferase and LacZ assays were performed as described in chapter II.

Immunoblots

For western blot analysis, anti-Flag (1:2500, Sigma) and anti-HA (1:1000, Roche) were used followed by HRP conjugated anti-Mouse or anti-Rat IgG (Jackson Immunochemicals). Signal was detected using ECL kit (Amersham).

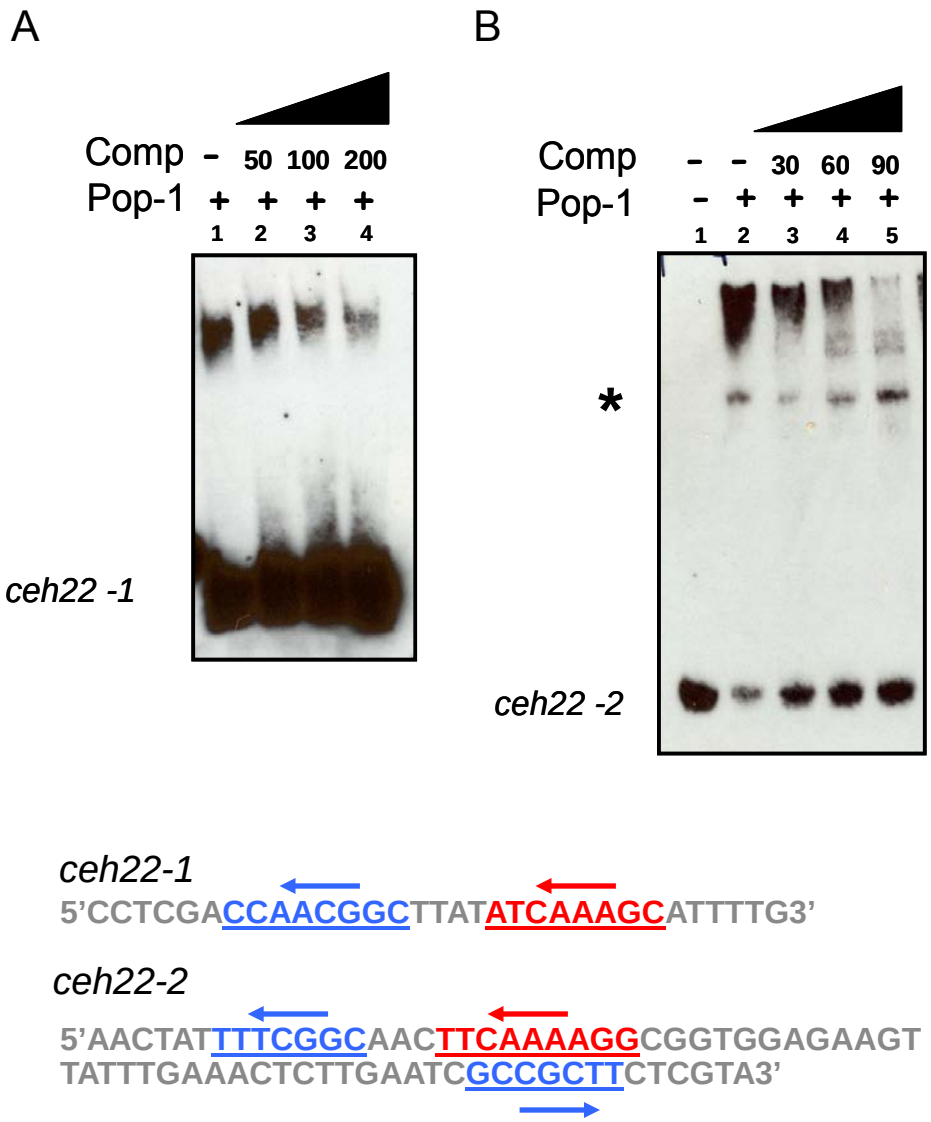
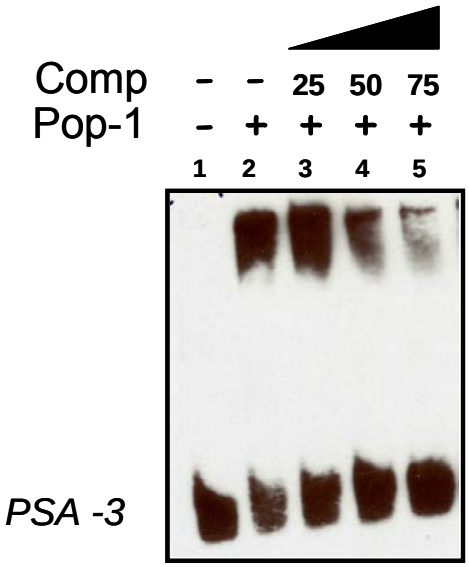
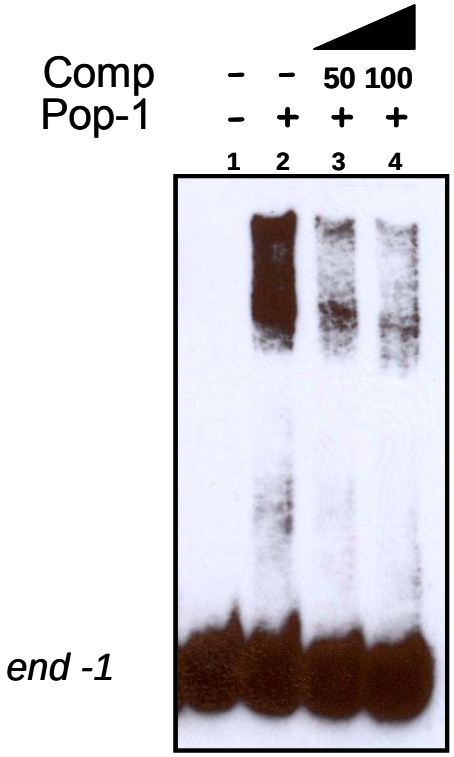


Figure 4.1. POP1 binds to fragments of *ceh22* WRE *in vitro.* EMSA showing the binding of of recombinant POP1 to *ceh22* WRE biotinylated probes. POP1 (1.5 µg) binds to a probe containing the HMG1 site (*ceh22-1*) (A) and 400 ng to HMG2 site (*ceh22-2*) (B). Increasing molar excess of the unlabelled competitor was added to the reaction mix followed by the labelled probe to compete for POP1 binding (Lanes 2-4 in A) and (Lanes 3-5 in B). Asterisk(*) in (B) marks a non-specific band. Probe sequences are shown below with HMG sites (red) and putative helper sites (blue) and arrows showing the orientation with respect to the coding strand.



5’ACCGTTATGGCCGTGGAGGCTCTTTTGATGTGCA
GCCGACAAGTACG3’

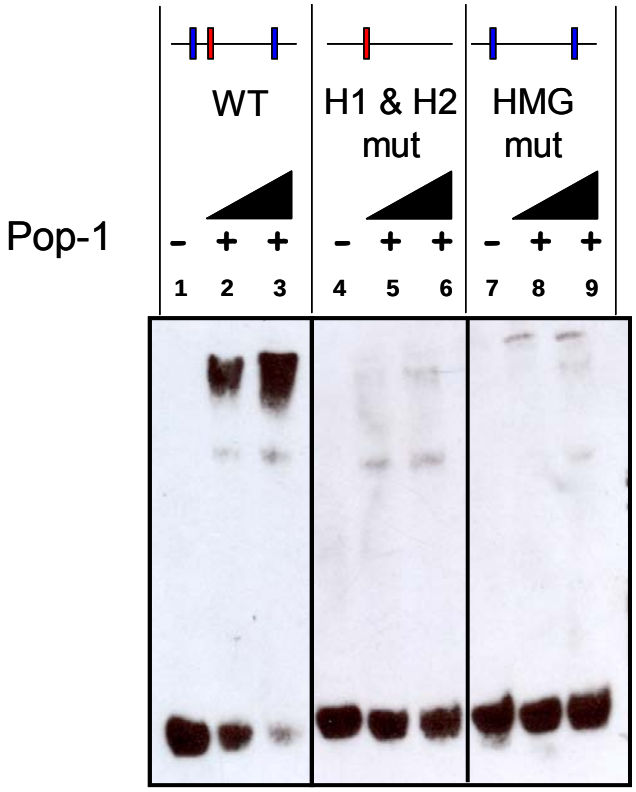
Figure 4.2. POP1 binds to a fragment of *psa-3* WRE *in vitro.* EMSA showing the binding of 1 µg of recombinant POP1 to *psa-3* WRE biotinylated probe. Increasing molar excess of the unlabelled competitor was added to the reaction mix followed by labelled probe to compete for POP1binding (Lanes 3-5). Probe sequence is shown below with HMG site (red) and putative helper sites (blue) and arrows showing the orientation with respect to the coding strand.



end-1

5’TACCTTTTGGCGGACAGGTTCTGTATTTCTTTGAACAAT
GCCAAGTTGAACTGTATACTTCCCGCCACACGAGTTCGA
AGACCATCACCCTATACAGTTTGCTCCGCCCTACCTTAAA
TATAGGCGGAGCTTGCAA3’

Figure 4.3. POP1 binds to a fragment of *end-1* WRE *in vitro.* EMSA showing the binding of 1.5 µg of recombinant POP1 to *end-1* WRE biotinylated probe. Increasing molar excess of the unlabelled competitor was added to the reaction mix to followed by the labelled probe compete for POP1binding (Lanes 2-4). Probe sequence is shown below with HMG site (red) and putative helper sites (blue) and arrows show the orientation with respect to the coding strand.



ceh22 -2

WT

5'AACTATTTTCGGCAACTTCAAAAGGCGGTGGAGAAGTTATTTGAAA CTCTTGAATCGCCGCTTCTCGTA3'

H1&H2 mut

5'AACTATgggattaAACTTCAAAAGGCGGTGGAGAAGTTATTTGAAACT CTTGAATCtaataggCTCGTA3'

HMG mut

5'AACTATTTTCGGCAACccggggGGCGGTGGAGAAGTTATTTGAAACT CTTGAATCGCCGCTTCTCGTA3'

Figure 4.4. HMG site or Helper site mutations abolish binding of POP1 to *ceh22-2* WRE fragment *in vitro*. EMSA showing the binding of recombinant POP1 to *ceh22-2* WRE fragment. Increasing amounts of POP1 (0.4 µg and 0.8 µg) were tested for binding to biotinylated probes which were Wildtype (WT) (Lanes 2, 3), HMG site mutant (Lanes 5, 6) or Helper site 1 and 2 (H1&H2) mutant (Lanes 8, 9). Lanes 1, 4, 7 show probes with no protein. Probe sequences are shown below with HMG sites (red) and helper sites (blue).

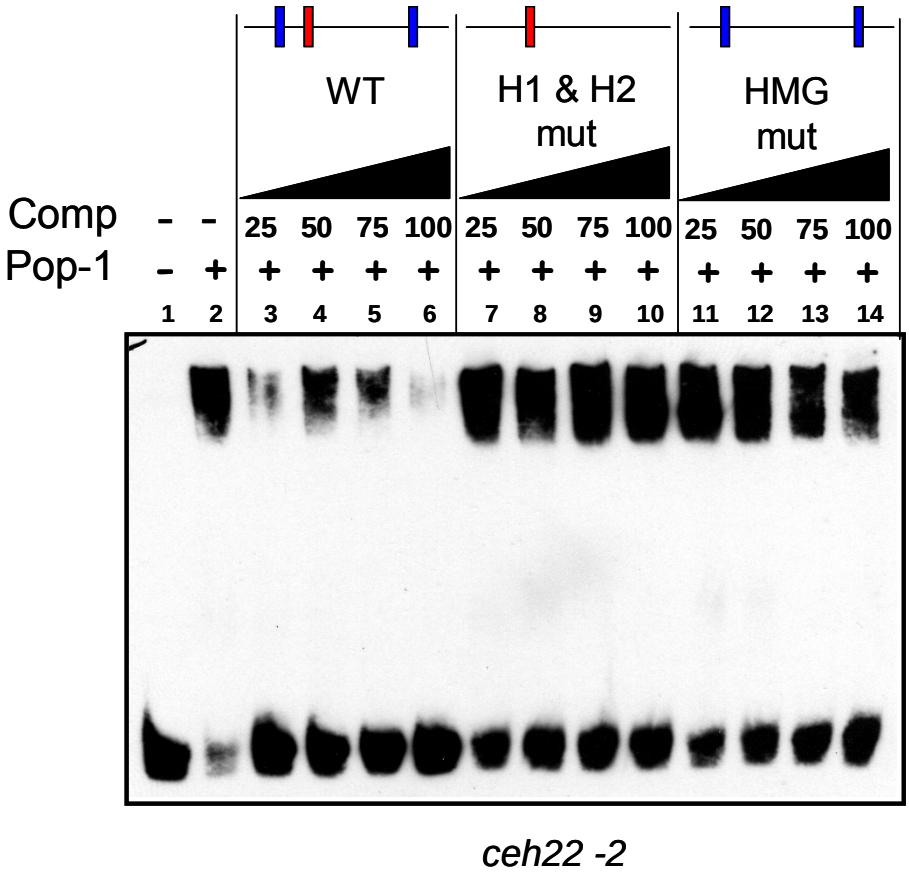


Figure 4.5. HMG site and Helper sites are required for binding of POP1 to *ceh22-2* WRE fragment *in vitro.* EMSA showing the binding of recombinant POP1 (0.5 µg) to *ceh22-2* WRE biotinylated probe. Increasing molar excess of unlabelled competitor was tested for binding to POP1. Wildtype (WT) competitor can compete for binding to POP1 (Lanes 3-6), Helper site 1 and 2 (H1&H2) (Lanes 7-10) mutant competitors are unable to compete the binding and HMG site mutant (Lanes 11-14) is extremely weak for competing with POP1. Lane 1 is no protein and Lane 2 is no competitor.

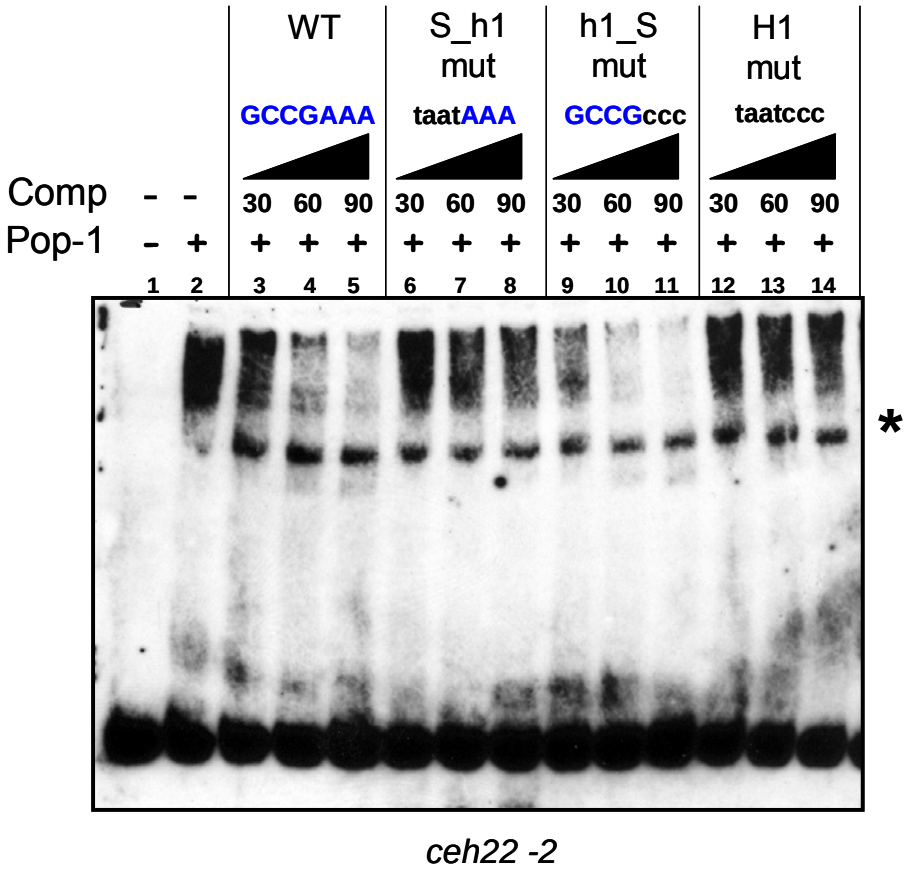


Figure 4.6. Helper site 1 is required for binding of POP1 to *ceh22-2* WRE fragment *in vitro.* EMSA showing the binding of recombinant POP1 (0.5 µg) to *ceh22-2* WRE biotinylated probe. Increasing molar excess of unlabelled competitor was tested for binding to POP1. Wildtype (WT) competitor can compete for binding to POP1 (Lanes 3-5). GCCG mutant (h1_S)(lanes 6-8) and AAA mutant (h1_S) (lanes 9-11) are stronger competitors when compared to Helper site 1 (H1) mutant (Lanes 12-14) for binding to POP1. Lane 1 is no protein and Lane 2 is no competitor.
* represents a non-specific band.

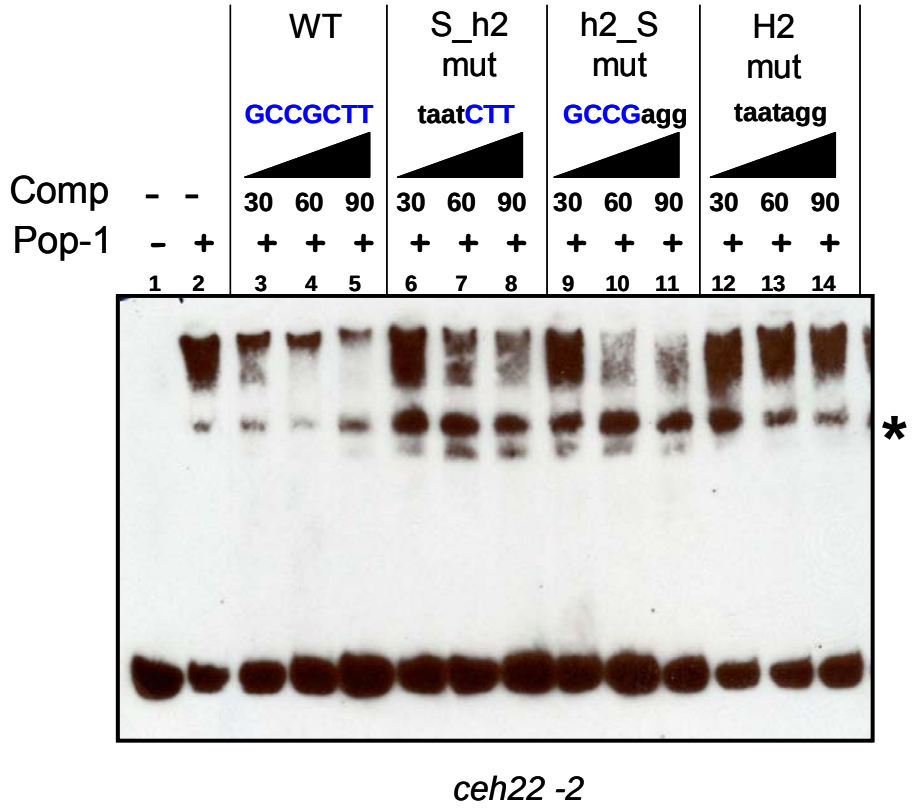


Figure 4.7. Helper site 2 is required for binding of POP1 to *ceh22-2* WRE fragment *in vitro*. EMSA showing the binding of recombinant POP1 (0.5 µg) to *ceh22-2* WRE biotinylated probe. Increasing molar excess of unlabelled competitor was tested for binding to POP1. Wildtype (WT) competitor can compete for binding to POP1 (Lanes 3-5). GCCG mutant (h2_S) (lanes 6-8) and CTT mutant (h2_S) (lanes 9-11) probes are stronger competitors when compared to the Helper site 2 mutant (H2) (Lanes 12-14) for binding to POP1. Lane 1 is no protein and Lane 2 is no competitor.
* represents a non-specific band.

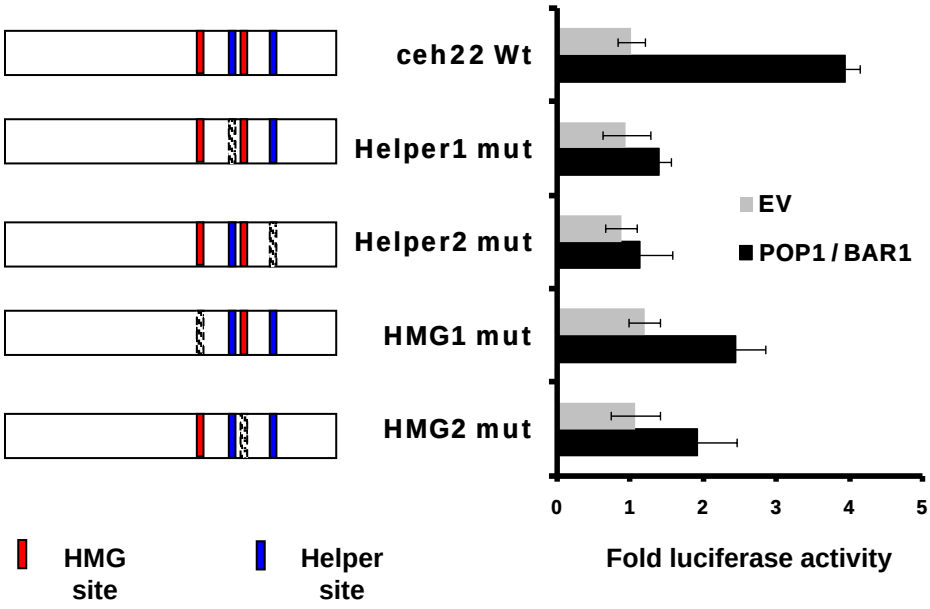


Figure 4.8. Helper sites are important for response of *ceh22* WRE to Wnt signaling. Luciferase activity of the *ceh22* WRE reporter with empty vector or BAR1/POP1. Helper1 or Helper2 mutation significantly affects the reporter activation but HMG1 or HMG2 mutation does not. Data shown here is an average of two independent experiments (±S.E.).

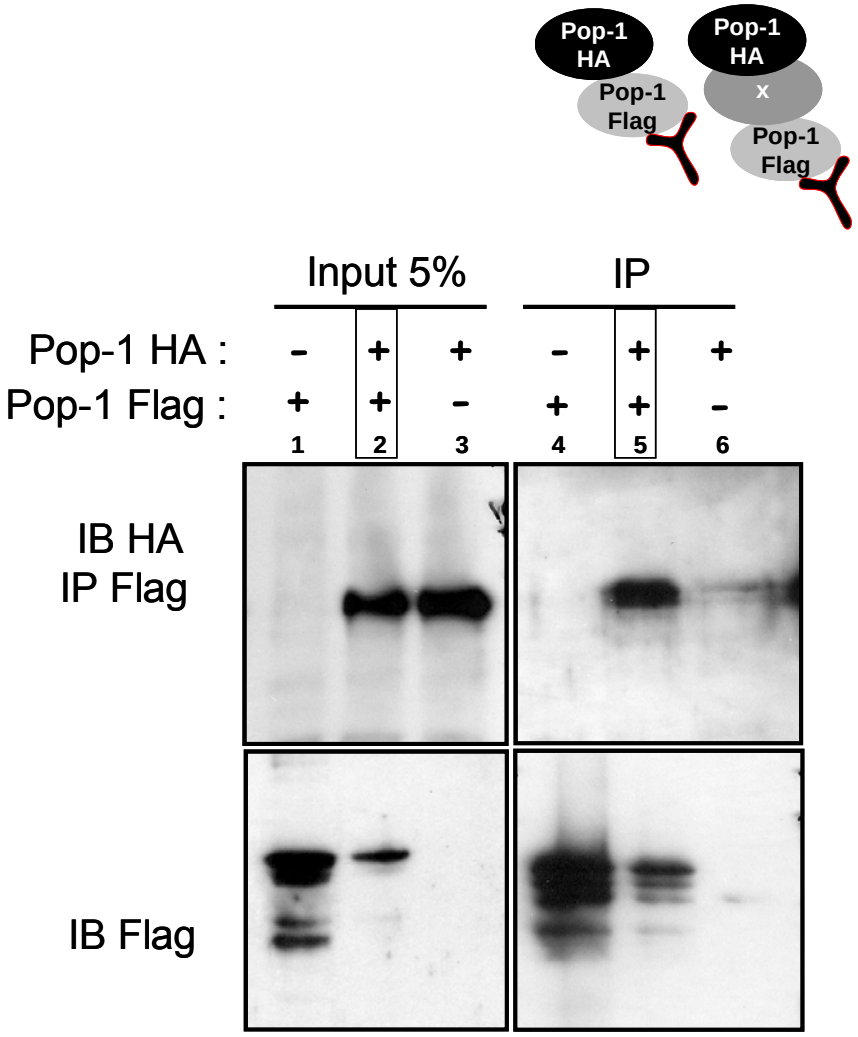


Figure 4.9. POP1 self-associates in KC cells. Co-immunoprecipitation of POP1 HA with POP1-Flag using a anti-Flag antibody. POP1 HA and POP1 Flag were misexpressed in KC cells. Immunoblot for HA shows that POP1 Flag can pull down POP1 HA (lane 5, top panel) only from lysates co-expressing POP1 HA and Flag (lanes 2, top panel) and not from lysates which do not express POP1 HA (lanes 1 and 4, top and middle panels) or POP1 Flag (lanes 3 and 6, bottom panel). Bottom panel shows expression of POP1 Flag from whole cell lysates (lane 1 and 2, bottom panel) and immunoprecipitated fraction (lane 4 and 5, bottom panel).

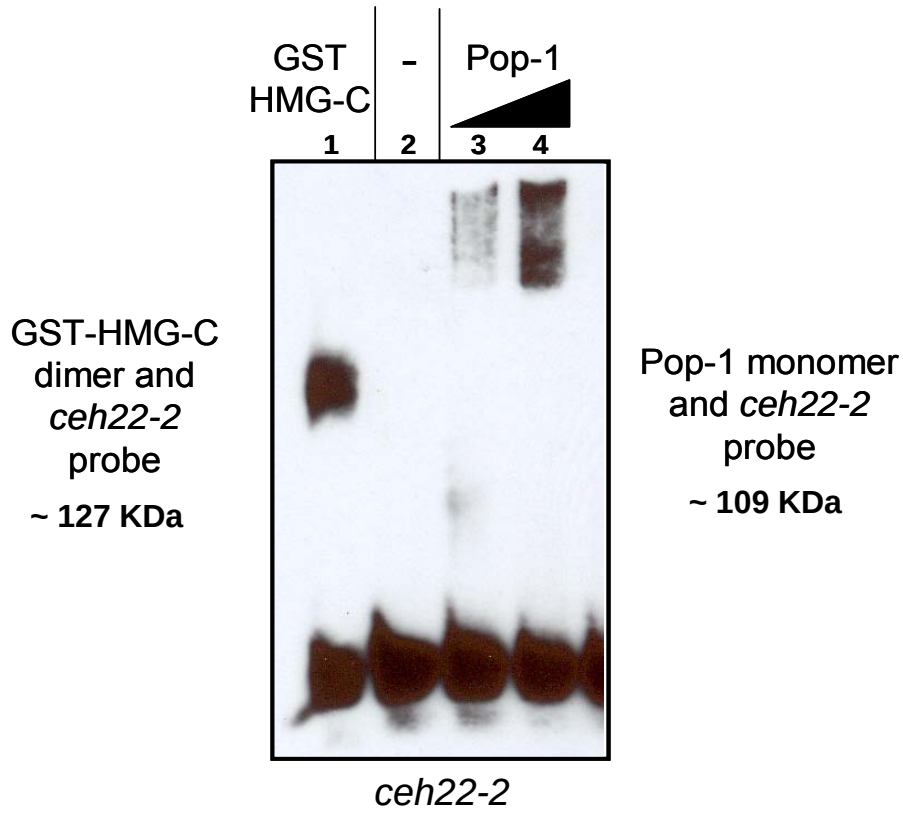


Figure 4.10. POP1-DNA complex migrates in the high molecular weight range. EMSA showing the binding of recombinant GST-HMG-C Clamp (0.5 µg) (lane 1) and POP1 (0.5 µg) (lane 3,4) to *ceh22-2* WRE biotinylated probe. POP1 migrates in a high molecular weight range than the expected monomer-DNA complex, when compared to the possible GST-HMG-C dimer.

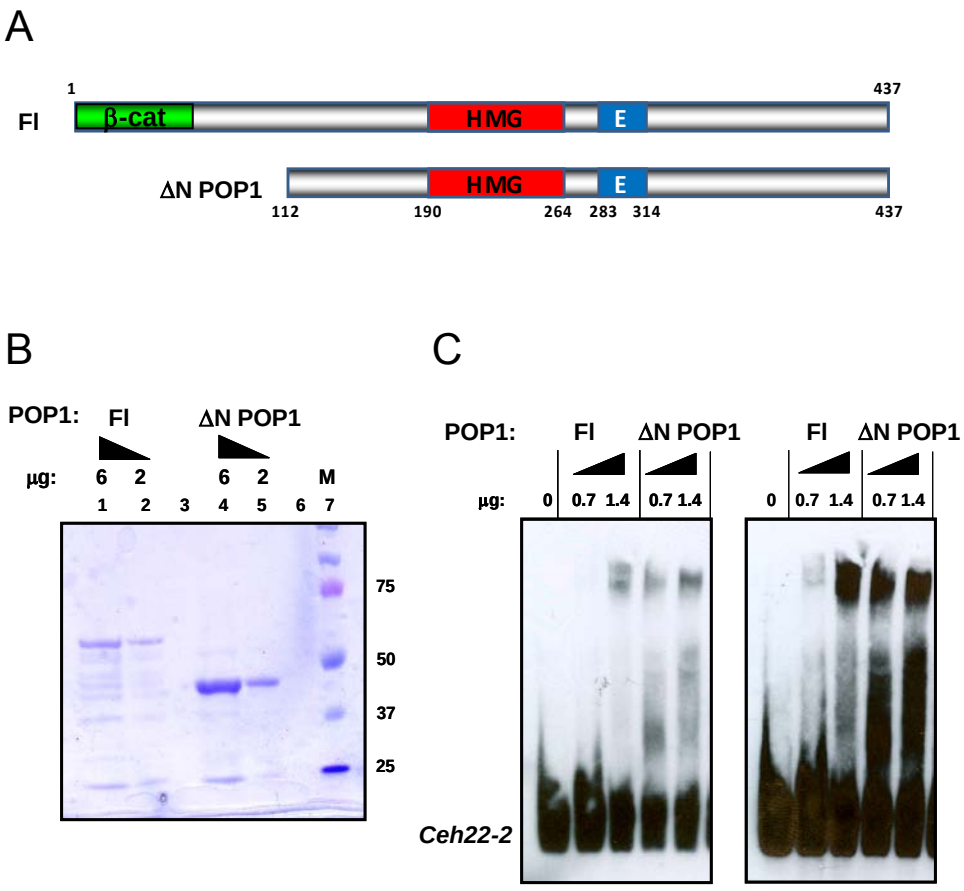


Figure 4.11. N-terminal deletion does not affect the migration of POP1 as a high molecular weight protein-DNA complex. (A) Schematic of the full length POP1(Fl) and N-terminal truncated fragment (ΔN POP1) missing the β-cat binding domain. (B) Commassie stained SDS-PAGE of the Fl (lanes 1&2) and ΔN-pop1 (lanes 4&5) with the non-degraded bands showing migration at the expected monomer size range. (C) EMSA showing the binding of recombinant Fl and ΔN POP1 in increasing concentrations to *ceh22-2* WRE biotinylated probe. ΔN POP1 migrates in a molecular weight range similar to Fl POP1 as seen in the shorter (left) and longer (right) exposure.

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Chapter V

Conclusions and Future directions

Summary of contributions

Wnt/β-cat signaling cascade is highly conserved throughout the animal kingdom and is required for many aspects of development and adult homeostasis (Cadigan & Nusse, 1997; Logan & Nusse, 2004). About two decades of work has gone into understanding the physiology and molecular mechanisms underlying this pathway. However there are several aspects which are not well understood and many questions remain unanswered.

Protein oligomerization is a widely known phenomenon which controls many cellular events. My dissertation is focused on the role of protein oligomerization in two different aspects of Wnt signaling. In one case, I have shown that oligomerization plays a role in the differential activity of CtBP. This will have many implications both in the Wnt and the CtBP fields, as CtBP has many diverse roles apart from regulating Wnt targets. As a contribution to these fields, I have investigated a previously unexplored role of CtBP monomers in Wg target gene transcription. I have also shown that oligomerization of CtBP is required for repression, through a 'salt-bridge swap' strategy which can be very useful in the context of structural biology.

In the second aspect, I have reported a novel finding that the worm TCF homolog POP1 can form oligomers. Further having tested the requirement of Helper like *cis-elements* for binding of POP1 to DNA, I report that these elements play a conserved role in the TCF-DNA interaction amongst flies and worms.

The Oligomeric state of CtBP determines its role as a transcriptional coactivator and corepressor of Wingless targets

CtBP plays dual roles in regulation of Wg target gene transcription by contributing to the nuclear switch of TCF from a repressor to an activator (Fang et al, 2006). Regulation of Wg targets by CtBP is gene specific. Using Wg readouts in flies and cultured Kc167 (Kc) cells, I have tested a two-part hypothesis that CtBP monomers positively regulate Wg signaling and CtBP oligomers antagonize Wg signaling.

The role of CtBP monomers has never been investigated in the context of Wg signaling. Hence based on the available structural information, which had a very important role in this analysis, I tested different sets of mutations in the dimerization interface for regulation of Wg signaling. $CtBP^{Mono}$ mutant enhanced the Arm* induced small eye phenotype in the fly eye and alleviated the antagonism of Wg signaling in the adult wing. In the wing primordium, ectopic expression of $CtBP^{Mono}$ led to enhancement of a Wg responsive reporter *Dll-lacZ*. All these data are consistent with CtBP monomers playing a positive role in Wg signaling.

In cultured Kc cells, CtBP was required for the activation of the reporter derived from a Wg target *CG6234*. When endogenous *CtBP* levels were depleted, $CtBP^{Mono}$

mutant, which is predominantly a monomer, was able to restore the activity of the Wg response element (WRE) of *CG6234* (*CG6234 WRE*). In another assay, $CtBP^{Mono}$ was able to enhance activity of Arm*, when Arm* was directly recruited to DNA. These data are consistent with the hypothesis that CtBP monomers play a positive role in regulating Wg target gene transcription.

The second part of the hypothesis was tested by assaying for a reporter derived from a Wg target *naked cuticle* (*nkd*) in the absence of signaling. Depletion of *CtBP* led to a deprepression of a WRE reporter *nkd-UpE1* in the absence of signaling. $CtBP^{Mono}$ was unable to repress *nkd-UpE1* when endogenous was *CtBP* was depleted. To test if oligomer formation was required for the repression of *nkd-UpE1*, complementary mutations were engineered in the dimerization interface. Acidic and basic residue pairs, which form inter-molecular salt bridges in a CtBP dimer, were targeted for a swap. Engineering the $CtBP^{Acidic}$ and $CtBP^{Basic}$ mutants was aimed at disrupting homodimerization, but restoring salt bridges and a consequent heterodimerization when the $CtBP^{Acidic}$ and $CtBP^{Basic}$ mutants were coexpressed.

The success of the strategy was demonstrated by Co-IP, when $CtBP^{Acidic}$ could not form homo-oligomers but formed hetero-oligomers with $CtBP^{Basic}$. When expressed as a pair, $CtBP^{Basic}$ and $CtBP^{Acidic}$ were able to repress the *nkd-UpE1* in the absence of signaling. However when expressed as monomers, they were unable to repress the *nkd-UpE1*. A similar regulation was seen in the adult wing where CtBP monomers were unable to antagonize Wg signaling. However when expressed as a pair, the $CtBP^{Acidic/Basic}$ heterodimer induced wing notches, thus antagonizing signaling. This was consistent with the second part of the hypothesis that CtBP oligomers repress Wg targets.

Hence this study has elucidated a previously unexplored mechanism which imparts dual roles to CtBP for differential regulation of some Wg targets. CtBP monomers activate Wg targets and CtBP oligomers repress Wg targets (Figure 5.1).

Mutational analysis to investigate the role of CtBP in Wg target gene regulation

In the previous report, the role of CtBP dimers in regulating Wg targets is not clear. In this study, I describe three approaches which were aimed at testing the hypothesis that CtBP dimers are unable activate Wg targets, but could not address the question conclusively.

In order to address if CtBP dimers can activate Wg targets, expression of $CtBP^{Basic/Acidic}$ pair was tested for the enhancement of Arm* mediated reporter activity. $CtBP^{Basic/Acidic}$ pair was able to positively regulate Arm* mediated reporter expression. However to eliminate the possibility of a monomeric pool contributing to the activation function of the $CtBP^{Acidic/Basic}$ pair, a hydrophobic dimerization interface was engineered to create a more stable dimer. The mutations abolished transcriptional activity of CtBP when tested in several Wg readouts.

As an alternative strategy to increase the probability of dimer formation, two CtBP monomeric subunits were covalently linked using a sixty six amino acid glycine-serine linker. However the CtBP concatemer was prone to degradation and stabilized to a fragment similar in size to the wildtype CtBP. The degraded concatemer was able to enhance the Arm* mediated reporter activity, but whether this activity was from the

degraded fragment or the full length concatemer could not be resolved. Hence the hypothesis that dimers cannot activate Wg targets could not be tested.

Exploring POP1 oligomerization and investigating the role of Helper-like *cis-elements* in binding of POP1

In this study, the requirement of Helper like *cis-elements* was explored in a Wnt target *ceh22 WRE*. Worm TCF homolog POP1 was tested for the binding and activation of a *ceh22 WRE*. Putative Helper like sequences were identified based on sequence similarity to the fly Helper sites. *In vitro* binding assays were performed to test if the helper like sequences were important for the POP1and DNA interaction. Two Helper sites were identified and mutation of each Helper site significantly abrogated the binding of POP1 to the *ceh22WRE*.

To test the functional importance Helper sites for Wnt mediated activation of the *ceh22 WRE*, mutant reporter constructs were generated and tested in a heterologous fly cell culture system. The wildtype reporter responded to stimulation by the worm β-cat, while Helper mutants showed a significant reduction in the reporter activity. Hence, this analysis suggests that the Helper sites are important for the activation of *ceh-22* in response to Wnt signaling.

The architecture of the fly WREs with respect to the HMG-Helper pairs suggests that the HMG and Helper sites do not have a very stringent orientation and spacing requirement. Hence I tested a part of the hypothesis that TCFs bind in a bipartite manner to HMG-Helper pairs and find that in cultured cells, POP1 self-associates. This could

explain how POP1 attains flexibility in recognizing HMG-Helper pairs with variable orientations, by forming oligomers on the WREs.

Future directions

I have tested the hypothesis that quaternary structure of CtBP determines its role in activation and repression of Wg targets. CtBP monomers are regulating activated Wg targets and CtBP oligomers are repressing Wg targets (Figure 5.1). Therefore dimerization can provide a differential activity to CtBP. Currently there is no insight into the mechanism(s) which govern the dual roles of CtBP in regulating Wg targets. So what is the mechanism?

Mechanism of activation of Wg targets by CtBP

As Arm/β-Cat is a key effector of the Wg pathway (Cadigan & Peifer, 2009), and has been shown to recruit CtBP by Chromatin-IP (Fang et al, 2009), the possibility of CtBP interacting with Arm was explored using co-IP. However in multiple attempts, the association $CtBP^{Wt}$ or the $CtBP^{Mono}$ with Arm could not be detected suggesting that interaction might be weak, or CtBP is present in complex with Arm only on the chromatin. It is also possible that there are other factors which bridge the association of Arm and CtBP, making it technically challenging to co-IP the complex. Exploring the interaction of CtBP with other factors, which are required for activation by Arm, will help elucidate the mechanism of activation by CtBP.

Arm is known to have two transactivation domains; an N-terminal and a C-Terminal transactivation domain (Fang et al, 2006; Hecht et al, 1999; Stadeli & Basler, 2005). These domains can independently activate reporter genes when directly recruited to the chromatin. A chimera of full length Arm* with the Gal4 DNA binding domain (Gal4Arm*) was shown to require CtBP for its maximal activity (Fang et al, 2006). When tested for transactivity of the two domains, only the N-terminal of Arm was dependent on CtBP (Fang et al, 2006). Consistent with this, CtBP is recruited by Gal4Arm* N-terminus to *cis-elements* of a *UAS-luc* reporter (Fang et al, 2006). It is possible that CtBP interacts with one of the known components required for the transactivity of the N-Terminus of Arm.

There are two other cofactors Pygopus (Pygo) and Legless (Lgs), which are required for the N-terminal transactivity of Arm (Stadeli & Basler, 2005). As key components of the Wg pathway, both Pygo and Lgs are essential for many Wg directed developmental events downstream of Arm stabilization (Belenkaya et al, 2002; Kramps et al, 2002; Parker et al, 2002; Thompson et al, 2002). In the nucleus, Lgs bridges the interaction between Pygo and Arm (Kramps et al, 2002; Stadeli & Basler, 2005). Lgs is thought to merely be an adaptor, as misexpression of an Arm-Pygo fusion can bypass the need for Lgs in flies (Kramps et al, 2002). Pygo on the other hand has been implicated in being the nexus of Arm to the basal transcriptional machinery and impart the transactivity to the N-terminus of Arm (Carrera et al, 2008; Stadeli & Basler, 2005). In order to explore the mechanism of activation, the functional and biochemical interaction of CtBP with Pygo was tested.

Does CtBP interact with Pygo?

Pygo has two conserved domains known as the N-Terminal Homology Domain (NHD) and the Plant Homeo Domain (PHD) (Parker et al, 2002). Pygo-PHD interacts with Lgs and is required for its recruitment to the Arm activation complex (Kramps et al, 2002; Stadeli & Basler, 2005). Pygo-NHD interacts with components of the mediator complex, which is a multi-subunit complex essential for RNA-PolII mediated transcription (Carrera et al, 2008). Pygo has a transactivation function, which is independent of its association with Arm (Stadeli & Basler, 2005). A Gal4-Pygo chimera can activate the *UAS-luc* reporter and this transactivation is dependent on the NHD of Pygo, but not the PHD (Stadeli & Basler, 2005). Hence to test if CtBP is required for the activity of Pygo, I assayed the transactivation of Gal4-Pygo under conditions where endogenous *CtBP* is depleted in KC cells.

CtBP is required for the maximal activity of Gal4-Pygo and CtBP monomers associate with Pygo in KC cells

Preliminary data suggests that I may have found a missing piece of the puzzle and that CtBP activates Wg targets by interacting with Pygo. Based on the *UAS-luc* reporter analysis, CtBP is required for the maximal activity of Pygo. A dose dependent activation of the *UAS-luc* by Pygo was significantly affected under conditions where endogenous CtBP was depleted by RNAi (Figure 5.2). Further, in support of CtBP monomers activating Wg targets, CtBP ^{Mono} was found to weakly interact with Pygo when co-

expressed with Arm*, in a Co-IP assay (Figure 5.3). Experiments are underway to confirm this genetic and biochemical interaction of CtBP with Pygo.

However ,there are a few key questions which need to be answered, to learn more about the interaction of CtBP with Pygo. Those are:

1. Is the transactivation by Pygo through CtBP monomers?

   This can be addressed by testing if $CtBP^{Mono}$ can rescue the activity of Gal4-Pygo, when endogenous *CtBP* is depleted. A similar gene replacement strategy has been successfully used for the *CG6234 WRE* reporter activation.

2. Does $CtBP^{Mono}$ interact directly with Pygo?

   This can be addressed using *in vitro* binding assays, for e.g., testing if bacterially purified Pygo and CtBP interact.

3. Does Pygo have the PXDLS or RRT motifs which are commonly found in proteins which interact with CtBP? Conversely, does interaction of CtBP depend on the residues known to be important for binding to the PXDLS and RRT motifs?

   The answer to the common interaction motifs is probably 'no' as the polypeptide sequence of Pygo does not have any regions which might be a PXDLS or RRT motif. There are a few interacting partners of CtBP which do not have the 'signature' motifs for e.g., tramtrak69 (Wen et al, 2000), and Pygo may belong to this class. However if mapped, mutating the interaction domains will be important to validate the function and interaction of CtBP with Pygo.

The available data suggests that CtBP monomers are acting in concert with Pygo to activate Wg targets (Figure5.1). Answering the remaining questions will be important to understand this interaction.

Is the positive role of CtBP monomers in Wnt signaling conserved?

CtBP knockout mice show temporal loss of expression of a Wnt target *Brachyury* (*T*) (Hildebrand & Soriano, 2002). Similarly requirement of Wnt signaling for activation of *T* is stage specific (Galceran et al, 2001). However the regulation of *T* has never been tested in CtBP null mouse embryonic fibroblasts (MEFs). These cultured fibroblasts respond to Wnt signaling (Hamada & Bienz, 2004) and so activation of *T* can be tested in *CtBP* mutant MEFs.

A WRE reporter of the *T* gene, which has the functional TCF sites (Galceran et al, 2001), is another target which can be tested for activation by Wnt signaling in MEFs. If active, then *T* transcript levels can be compared between the wildtype and CtBP null MEFs to test the requirement of CtBP for Wnt stimulated activation of *T*. To test if CtBP is recruited to the WRE of *T* for activation, Chromatin-IP can be performed in wildtype and CtBP mutant cells. Finally a rescue with the $CtBP^{Wt}$ and different monomeric mutants can be used to explore the role of CtBP monomers in activation of T. However, this is contingent to the *T* gene responding to Wnt signaling in MEFs. Further, a $CtBP^{Mono}$ mutant knock-in strategy can be employed, where the endogenous CtBP is replaced by $CtBP^{Mono}$ to test if monomers can rescue the loss of T expression in CtBP null mice.

Do CtBP monomers play a positive role in transcriptional regulation, in non-Wg related contexts?

CtBP has been shown to play varied roles in animal development (Chinnadurai, 2007). In flies, CtBP is expressed both maternally and zygotically (Poortinga et al, 1998), and in the early embryonic stages the maternal component has the most contribution to the function of CtBP. Embryos lacking the zygotic and maternal component of CtBP display a severely disrupted segmentation pattern at the level of pair-rule genes (Nibu et al, 1998b; Poortinga et al, 1998). For example, in wildtype embryos, a pair-rule gene *even-skipped* (*eve*) is expressed in a pattern of 7 stripes across the anterior/posterior (A/P) axis, but in *CtBP* mutant embryos there is a fusion of *eve* stripes 2 and 3 (Poortinga et al, 1998).

Some of these defects are due to repression by CtBP as it has been shown to interact with the gap genes *Knirps, Kruppel* and *Giant* (Keller et al, 2000; Nibu et al, 1998a; Strunk et al, 2001) and regulate the *eve stripe* enhancers. For example, ectopic expression of Knirps represses the *eve stripe2* enhancer and this repression is dependent on the CtBP binding PXDLS motifs in Knirps (Nibu et al, 1998a). Hence repression by CtBP is required for some of the pair-rule patterning during embryonic development.

Although repression plays an important role, CtBP mutant phenotypes suggest that CtBP has both inductive and suppressive roles in the regulation of pair-rule gene patterning. This is evident by the loss of striped pattern for a number of pair-rule genes, for e.g., *CtBP* mutant fly embryos show an expansion of *eve* stripe 2 and 3 but a loss of stripes 4-6 (Nibu et al, 1998a; Poortinga et al, 1998). In the case of another pair-rule

gene *Runt*, the effect is more dramatic as there is no expansion of stripes, but a loss of stripes 3-7 (Poortinga et al, 1998). Hence, CtBP has an inductive role in the pair-rule patterning.

The molecular mechanisms of the pair-rule pattern regulation by CtBP are not understood and could be result of an indirect regulation. In one instance, CtBP has been shown to interact both genetically and biochemically with a repressor Hairy, and reduction of *CtBP* gene activity can suppress the Hairy mutant phenotypes (Phippen et al, 2000). Hence this is suggestive of CtBP repressing a repressor to activate target genes. In another instance, *Knirps* mutant embryos also show a loss of *eve* stripes 4-6 similar to that seen in CtBP mutant embryos (Nibu et al, 1998a). Since Knirps binds CtBP directly, it may be activating these stripes in concert with CtBP.

In Chapter II of this dissertation, I have reported that CtBP monomers positively regulate Wg signaling in several contexts. Mutations in the dimerization interface separate the activation and repression functions of CtBP. Hence ectopic expression of $CtBP^{Mono}$ from transgenes generated in this study can be used to test the rescue of pair-rule stripe patterning in CtBP mutant embryos. If CtBP monomers play a positive role in other contexts apart from Wg signaling, a rescue of pair-rule patterning due to loss of activation by CtBP is expected. In *CtBP* mutant embryos, ectopic expression of $CtBP^{Mono}$ might restore the expression of stripes 4-6 as a result of activation by CtBP monomers. However, the fusion of stripes 2 and 3 might not be restored if it is a result of repression by CtBP oligomers.

Mechanism of repression of Wg targets by CtBP

In the absence of signaling, the cofactors Groucho (Gro), HDACs, ISWI/Acf1 and Kaiso act in concert with TCF for global or gene-specific repression of Wnt/Wg targets (Parker et al, 2007). In KC cells, CtBP has been shown to repress a Wg target *nkd* (Fang et al, 2006) and a WRE *nkd-UpE1* in the absence of signaling. In the wing imaginal disc, depletion of *CtBP* by RNAi leads to a derepression of *nkd-UpE1* (Figure 5.4). Depletion of *CtBP* and *TCF* has an additive effect on the derepression of *nkd-UpE1* suggesting that they might act independent of each other. In support of this, CtBP recruitment to a *nkd WRE* is independent of TCF and Gro (Fang et al, 2006) and ISWI/Acf1 (Yan Liu; unpublished data). CtBP does not have a known DNA binding domain and hence is likely to be recruited by some unknown transcription factor(s) to repress *nkd* in the absence of signaling.

Of the other factors which repress Wg targets in the absence of signaling, Kaiso is an interesting candidate which might recruit CtBP to repress *nkd*. Kaiso is a DNA binding protein and has a TCF independent mode of repression in *xenopus* embryos (Park et al, 2005). The fly Kaiso homolog Tramtrak69 (ttk69) has been shown to interact with CtBP genetically and biochemically (Wen et al, 2000), although this interaction has not been explored in the context of Wg signaling. In the wing imaginal disc, *nkd-UpE1*1 is repressed by CtBP (Figure 5.4). Hence misxpression of transgenic *ttk69* (Wen et al, 2000), can be tested for repression of *nkd-UpE1* in KC cells and the wing primordium, when endogenous *CtBP* is depleted. If misexpression of ttk69 can compensate for the depleted levels of *CtBP*, it will imply that CtBP represses *nkd-UpE1* expression through *ttk69*.

CtBP has been widely implicated to exercise its repression function through class I HDACs (Chinnadurai, 2007) and this has never been tested in the context of Wg signaling. Although there is no evidence for a functional interaction between CtBP and the fly Class I HDACs (Rpd3 and HDAC3), there is some evidence that the HDAC activity of a immunoprecipitated Knirps complex is dependent on the CtBP binding sites of knrips (Struffi & Arnosti, 2005). Misxpression of Rpd3 and HDAC3 can be tested for repression of *nkd-UpE1* in KC cells, when endogenous CtBP is depleted. A similar strategy can be applied to test if Rpd3 or HDAC3 repress *nkd-UpE1*1 in the wing imaginal disc. If depletion of Rpd3 and HDAC3 leads to derepression of *nkd-UpE1*1, misexpression of CtBP will be tested for the rescue of the repression of *nkd-UpE1*1 in this background. These genetic interactions will suggest that CtBP exercises the repressive function on *nkd-UpE1* through class I HDACs.

In the next section, I will discuss the future directions for investigating the role of Helper sites in Wnt mediated response of worm targets and role of POP1 oligomers in Wnt signaling.

Conserved role of Helper sites for TCF binding and activity

One central question for gene regulation by transcription factors is how do they locate their targets? The specific *cis-elements* recognized by the DNA binding domain of transcription factors serve as a platform for different complexes. How do the *cis-elements* 'array' themselves to 'attract' transcription factors?

A study from the Cadigan lab identified novel *cis-elements* called the 'Helper' sites, which along with the known HMG sites are required for bipartite binding of TCF (Chang et al, 2008b). A bioinformatics based search and functional analysis indicates that these elements provide a high level of specificity for identification of Wg targets (Chang et al, 2008b). *In vitro* analysis in this dissertation shows that Helper sites are important for binding of worm TCF homolog POP1 to the *WRE* of a Wnt target *ceh22*. In cultured fly cells, Helper sites are important for activation of the *ceh22 WRE*. To determine the biological relevance, it is important to know if the Helper sites play an essential in regulation of *ceh22 in vivo*.

In worms, *ceh-22* is required for distal tip cell specification and is expressed in the somatic gonadal precursors at the early larval stages (Lam et al, 2006). The HMG sites are important for activation of a 1.2 kb *ceh22 WRE* reporter in worms (Lam et al, 2006). Mutation of the Helper sites in the context of this reporter is required to determine their role in activation of the *ceh22 WRE*. Furthermore, the requirement of Helpers needs to be tested in two other known Wnt targets, *psa-3*(Arata et al, 2006) and *end-1*(Calvo et al, 2001; Shetty et al, 2005). Preliminary data suggests that two putative helpers in the *psa-3* WRE affect POP1 binding *in vitro* (data not shown). The next step is to be examine the *in vivo* role of Helpers, in activation of the Wnt targets. If the Helper sequences are functional, it would indicate that they play a conserved role amongst flies and worms for activation of Wnt targets, possibly by bipartite binding of TCFs.

Role of C-Clamp in worms

TCFs which display bipartite binding have an additional DNA binding domain called the C-Clamp (Atcha et al, 2007; Chang et al, 2008b). An allele of TCF (*pan*^{13a}), which is a point mutation in the C-Clamp domain has a weak segment polarity phenotype (van de Wetering et al, 1997) suggesting that it is important for the role of TCF in Wg signaling. The analysis of Helper sites in *ceh22 WRE* suggests that C-Clamp might be important for binding of POP1 to the Helper sites. Mutation of 5 conserved residues in C-Clamp abrogates the activation function of fly TCF and human TCF1E , when examined in cultured cells (Atcha et al, 2007; Chang et al, 2008b). Similar mutations can be engineered in POP1 and the mutant protein can be tested for the rescue of a *POP1* mutant phenotype. This will help decipher the role of C-Clamp in Wnt signaling in *C. elegans*.

Role of POP1 oligomers in Wnt signaling

The architecture of the fly WREs with respect to the HMG-Helper pairs suggests that the HMG and Helper sites do not have a very stringent orientation and spacing requirement (Chang et al, 2008a). In addition, when tested *in vitro* the Helper site augments binding of the fly HMG-C-Clamp to the HMG-Helper pair in different orientations (Mikyung Chang; unpublished data). So what provides TCFs the flexibility to bind HMG-Helper pairs which are in different orientations and have variable spacing?

p53 is an example of DNA binding proteins that are dimers in solution, and form tetramers when in complex with DNA (Riley et al, 2008). Each dimer binds a 10 bp

consensus known as the 'half site' with a sequence separated by 0-21bp from the other 'half site', providing a flexibility in spacing (el-Deiry et al, 1992; Funk et al, 1992). Another way the flexibility can be achieved is through a linker which joins two the DNA binding domains. An example of such a linker based bipartite DNA binding family of factors is POU-1 (Pit, Oct and Unc) (Herr et al, 1988). A member of the POU-1 family of proteins called Oct-1 has two DNA binding domains joined by a flexible linker (25 amino acids), and recognizes an 8 bp consensus (Herr et al, 1988). Structural studies suggest that these domains contact DNA on opposite faces of the octamer sequence, and this is achieved by the flexible linker (Klemm et al, 1994).

An example of a protein which combines both these mechanisms is another POU domain family member called Pit-1. Structural information indicates that these molecules form homodimers which lets them adopt different confirmations in complex with DNA, due to flexibility of the linker (Jacobson et al, 1997). Surprisingly Pit-1 contains the shortest linker amongst the POU family of protein (15 amino acids), but recognizes a 12 bp sequence (Jacobson et al, 1997). Hence in this case, homodimerization certainly aids in the Pit-1 protein recognizing a longer DNA binding site.

Both the Helpers in the *ceh22 WRE* are important for binding of POP1 and one of the Helper sites is 31 bases away from the HMG site. Hence one possibility is that the oligomerization of TCF leads to the binding of HMG box and C-Clamp of different subunits to the HMG-Helper pairs. The co-IP of differentially tagged forms of POP1 in cultured fly cells supports a model in which POP1 self-associates on the WREs to contact the HMG-Helper pairs. Consistent with that the full length recombinant POP1 migrates in

a higher molecular weight range than expected for a monomer-DNA complex. Hence mapping the oligomerization domain using systematic deletions and co-IPs will be key in determining the biological relevance of oligomer formation. If the domain is mapped, residues in that domain can be mutated to test if the mutant can compensate for the functions of wildtype POP1. Some key issues that can be addressed are:

1) Is the oligomerization of POP1 required for the binding to the *ceh22 WRE in vitro* or for its activity in cultured cells?

2) Can the dimerization defective mutant rescue loss of function phenotypes of POP1 in worms?

3) Does disrupting POP1 oligomers make the spacing and orientation rigid? In other words does only one orientation of HMG and Helper sites, with a limited spacing, generate a Wnt response with POP1 monomers?

How conserved is the oligomer formation of POP1?

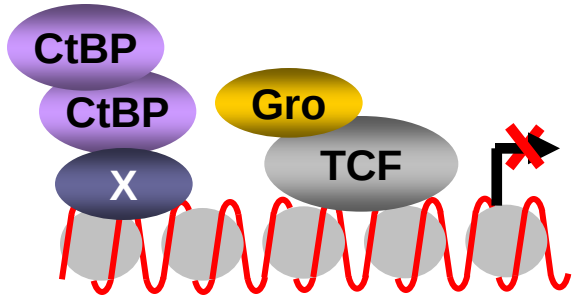
Considering that the architecture of the fly WREs has a variable distribution of HMG-Helper pairs in terms of spacing and orientation (Chang et al, 2008b), the role of POP1 oligomerization might be conserved in flies. However, a majority of the vertebrate TCF isoforms do not express the C-Clamp (Arce et al, 2006) which suggests that the C-Clamp may not be essential for all functions of the vertebrate TCFs. It is possible that *in vivo*, the isoforms coding for the C-Clamp and the ones which do not express the C-Clamp form hetero-oligomers to regulate some targets. This might serve as one of the mechanisms for TCF isoforms which do not have another known DNA binding domain,

to specifically locate the WREs. Hence the self-association of differentially tagged fly TCF and the four vertebrate TCFs, e.g., human TCFs can be tested in KC cells.

Oligomerization of TCFs may have other roles than bridging complexes on the HMG and Helper sites. While there are several possibilities, oligomerization might be one of way of recruiting different factors for formation of activation and repression complexes. A differential regulation based on oligomeric state has been demonstrated for CtBP in this dissertation. Hence identifying the dimerization domain may be key to separate the diverse functions of TCF monomers and oligomers, if any.

A

Repression by CtBP dimers in the Wnt off state



B

Activation by CtBP monomers through Pygo in the Wnt on state

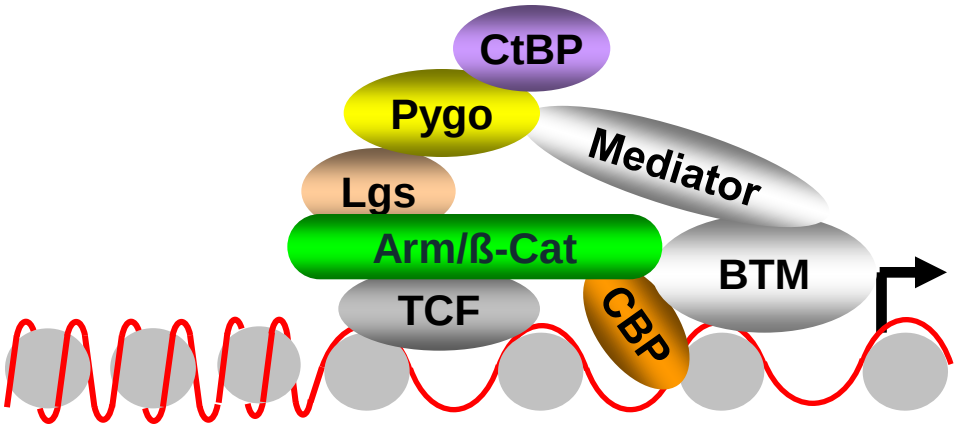


Figure 5.1. Models for repression and activation of Wg targets by CtBP. (A) Cartoon showing the model for repression of Wg targets by CtBP dimers in the absence of signaling. (B) Cartoon showing the model for activation of Wg targets. CtBP monomer is recruited to the N-terminus of Arm through Pygo, in the Wnt on state..

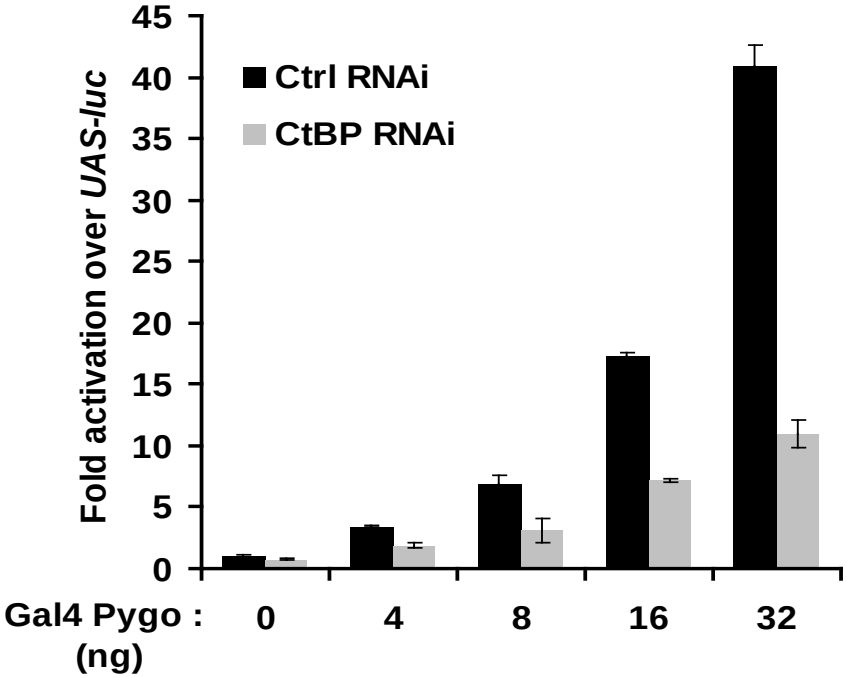


Figure 5.2. Transactivity of Pygo is partially dependent on CtBP. Histogram showing luciferase activity of a *UAS-reporter*. Gal4 Pygo was expressed in cells treated with control (ctrl) RNAi or CtBP RNAi. Depletion of CtBP leads to a significant decrease in activation of the *UAS-luciferase* (l*uc*) reporter by Gal4 Pygo.

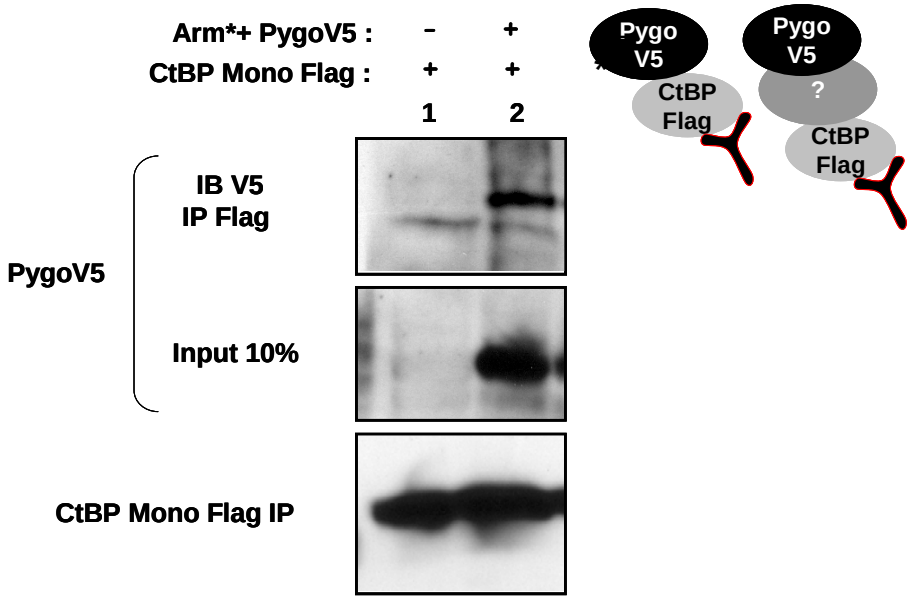


Figure 5.3. CtBP Mono associates with Pygo in KC cells in the presence of signaling. Co-immunoprecipitation of Pygo V5 with CtBP Mono-Flag using an anti-Flag antibody. Arm*, PygoV5 and CtBP Mono Flag were misexpressed in KC cells. Immunoblot for V5 shows that CtBP Mono Flag can pull down Pygo (lane 2, top panel) only from lysates expressing Pygo (lane 2, middle panel) and not from lysates which do not express Pygo (lane 1, top and middle panels). Arrow points to the Pygo band and * represents a non-specific band. CtBP Mono Flag is immunoprecipitated at similar levels (lanes 1 & 2, bottom panel).

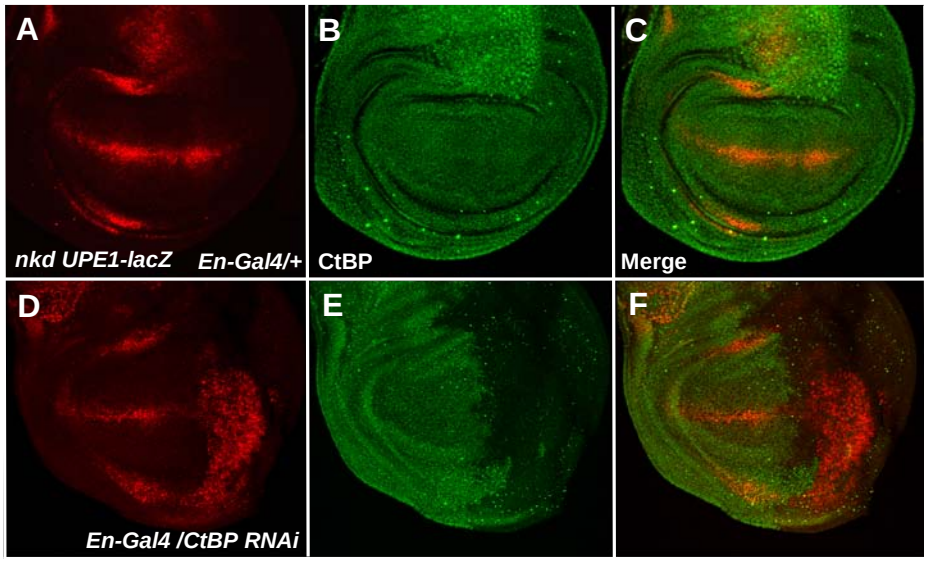


Figure 5.4. *nkd UPE1* is repressed by CtBP in the posterior compartment of the wing imaginal disc. Confocal images of third instar wing discs where CtBP (green) was depleted in the posterior compartment of the wing imaginal disc by RNAi. *nkd UPE1* reporter expression was monitored by staining for lacz (red). (A-C) *nkd UPE1* pattern in the wildtype discs. (D-F) Broader expression of lacZ in *UPE1* discs where *Engrailed-Gal4* (*En-Gal4)* is driving the expression of *CtBP RNAi* transgene in the posterior compartment.

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