HOW A NOVEL ZINC FINGER DOMAIN IMPROVES TARGET GENE SELECTION BY TCF IN WNT/β-CATENIN SIGNALING

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular, Cellular and Developmental Biology)
in the University of Michigan
2014

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To my family

ACKNOWLEDGEMENTS

My mentor, Ken Cadigan, has been a great source of encouragement and guidance. I appreciate his support through the many ups and downs during my journey through graduate school. I admire his scientific acumen and value the training I have received from him.

I'd like to thank my thesis committee, Steve Clark, Gyorgyi Csankovszki and Scott Barolo, for the many thoughtful discussions and ideas during and outside of my committee meetings. I am also grateful for support and funding from the MCDB department.

I thank current and former members of the Cadigan lab, particularly Hilary Archbold, Chen Zhang, Yaxuan Yang, Chandan Bhambhani, Lisheng Chen and Pete Burby, for being great colleagues. Special thanks to Hilary, Chen and Yaxuan for their inputs towards my dissertation. I am grateful to several other labs, particularly the Csankovszki, Chapman, Buttita and Bardwell labs, who have been very generous with their resources and advice.

I am thankful for the friendship of many friends both in Ann Arbor and back home. For the innumerable dinners, movie marathons and nerdy debates, I thank Karthik Kumar, Vignesh Suresh and Kartheek Gangadhara. For their great company and the many adventures, I thank Krittika Mittal and Akshaya Srinivasan. For making my final year in Ann Arbor so memorable, I thank my house-mates at the Gamma Alpha Co-op:

Tara Clancy, Hannah Maier, Laura Berry, Emily Lauderdale, Nat Lichten, Miles Ellenberg, Brendan O'Neill, Chuan Li, Jared Kabara, Katherine Crocker and Damian Wassel. And a big thank you to Sarada Anne and Shilpa Kanthamneni, just for being awesome.

Finally I dedicate my dissertation to my family, without whose endless encouragement I would not have been able to make it so far. I am so very thankful for their patience and love. To Gandharv Kashinath, for being my family away from home and always being there for me. To my brother, Abhimanyu, for being a constant source of entertainment. And most of all to Amma and Appa, for inspiring me and always believing in me.

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CHAPTER I

GENERAL INTRODUCTION

THE WNT/β-CATENIN PATHWAY

Wnt/β-catenin signal cascade

The Wnt/β-catenin (Wnt/β-cat) pathway is a highly conserved cell-cell signaling pathway that is implicated in diverse developmental and homeostatic processes across metazoans (Archbold et al., 2012; Clevers 2006; Logan and Nusse, 2004). The pathway is able to achieve this diversity of outputs through transcriptional regulation of various target genes, downstream of its signaling cascade (Archbold et al., 2012; Cadigan and Waterman, 2012).

The Wnt/β-cat pathway is dependent on the stability and abundance of cytoplasmic β-cat (Figure 1.1). In the absence of a Wnt signal, low cytoplasmic levels of β-cat are maintained by a complex of Axin, Adenomatous polyposis coli (APC) protein, Glycogen synthase kinase 3 (GSK3) and Casein kinase I (CKI), collectively referred to as the "destruction complex". Phosphorylation by this complex, results in the ubiquitination followed by proteosomal degradation of β-cat in the cytoplasm (Valenta et al., 2012; Cadigan and Peifer, 2009). Wnt signaling is initiated by the recognition of secreted Wnt ligands by the co-receptors Frizzled (Fz) and Low density receptor related protein 5/6

(LRP 5/6). The activated receptor complex in turn inhibits the "destruction complex", allowing cytoplasmic β-cat levels to increase (Cadigan and Peifer, 2009; MacDonald et al., 2009). The accumulation of β-cat in the cytoplasm results in its translocation into the nucleus, where it binds specific transcription factors to mediate transcription of Wnt target genes. The TCF family of transcription factors are major nuclear mediators of the pathway (Archbold et al., 2012; Cadigan and Waterman, 2012). TCF bound by β-cat initiates transcriptional activation of target genes, with the aid of co-activators such as Pygopus and CREB-binding protein (CBP/p300). In certain contexts, TCF can also act as a switch where it recruits co-repressors, such as Groucho/ transducin-like Enhancer of split (Gro/TLE), to represses target genes in the absence of a Wnt signal (Cadigan and Peifer, 2009; MacDonald et al., 2009).

Role of Wnt/Wg signaling in *Drosophil*a epidermal patterning and wing margin specification

Wingless (Wg) is the best characterized of the seven Wnts in *Drosophila* (Llimargas and Lawrence, 2001; Nusslein-Volhard and Wieschaus, 1980; Bejsovec et al., 2006; Zaffran and Frasch, 2002). It is critical in the development of a number of tissues, including patterning of the epidermis and wing margin.

Wnt/Wg signaling plays multiple roles at different stages of epidermal patterning (Bejsovec, 2013). In the early phase, it is required for establishing the anterior-posterior (A-P) boundary of segments. A single row of cells in the anterior of each segment expresses *wg* (Figure 1.2). Wg induces expression of *engrailed* (*en*) in adjacent cells (DiNardo et al., 1988; Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991).

However, the presence of *sloppy-paired* (*slp*), a repressor of *en*, in the anterior restricts the expression of *en* to cells in the posterior of each parasegment (Cadigan et al., 1994). En in turn activates *hedgehog* (*hh*) transcription. Hh is thought to help set up a positive feedback loop that maintains *wg* and *en* expression at the A-P boundary (Cadigan et al., 1994; Lee et al., 1992). At the end of embryogenesis, the embryonic epidermal cells secrete a cuticle with alternating bands of hook-shaped structures, called denticles, and naked cuticle on its ventral surface. Wg specifies this naked cuticle, by repressing the expression of *shaven baby* (s*vb*), a transcription factor whose target genes encode denticle structural elements (Payre et al., 1999). Each segment gives rise to six rows of denticles, each of which have a characteristic shape, size and polarity. Wnt/Wg signaling also contributes to this denticle diversity, by influencing *en*-expressing cells (Doughan and Dinardo, 1992) and other signaling pathways, such as those of Notch and EGF (Alexandre et al., 1999; Bejsovec, 2013).

Wnt/Wg signaling is also required for specifying the *Drosophila* wing margin. Patterning of the wing margin, specifically the determination of sense organs and hairs along the margin, takes place during the third larval instar and early pupal development stages (Couso et al., 1994). By mid third instar, *wg* is expressed along the presumptive wing margin in a 3-6 cell wide stripe on either side of the dorso-ventral (D-V) boundary (Figure 1.3) (Couso et al., 1994; Phillips and Whittle, 1993). Wg refines its own expression along the D-V border through a negative autoregulatory loop (Rulifson et al., 1996). Wg is thought to act as a morphogen (Martinez Arias et al., 2003; Cadigan et al., 2002) driving expression of *acheate* and *senseless* on either side of *wg* expressing stripe. This establishes the proneural region within which sensory mother cells (SMCs) give rise

to chemosensory margin bristles (Couso et al., 1994; Jaffar-Nejad et al., 2006; Romani et al., 1989; Parker et al., 2002).

Wnt/β-cat signaling pathways in *C.elegans*

In *C.elegans*, Wnt/β-cat signaling works through at least two pathways: the canonical Wnt/β-cat pathway and the Wnt/β-cat asymmetry pathway. While the asymmetry pathway regulates most asymmetric cell divisions throughout development, the canonical pathway is known to control a handful of cell fate specifications in the nematode (Phillips and Kimble, 2009). Both pathways share a number of common signaling components, but differ mainly in the existence of a POP-1 regulatory branch, that promotes POP-1 nuclear export, in the asymmetry pathway (Phillips and Kimble, 2009; Harding and King, 2008).

Another area of difference is in the involvement of different β-cat homologs in each pathway, as *C.elegans* has four known β-cat homologs: HMP-1, WRM-1, BAR-1 and SYS-1 (Phillips and Kimble, 2009; Robertson and Lin, 2012). Unlike *Drosophila* and vertebrates, which have a single β-cat that carries out both adhesion and signaling functions, the four *C.elegans* β-cat homologs each have divergent roles. HMP-1 is dedicated to adhesion, while the latter three homologs mediate the Wnt signaling pathway (Robertson and Lin, 2012; Korswagen et al., 2000). BAR-1 acts as a co-activator of POP-1 in the canonical pathway analogous to β-cat's interactions with TCF in *Drosophila* and vertebrates (Korswagen et al., 2000). WRM-1 and SYS-1 are involved in the asymmetry pathway, which is called so because it involves the asymmetric nuclear localization of POP-1 and SYS-1 across asymmetrically dividing daughter cells (Phillips

and Kimble, 2009). WRM-1 works with the transforming-growth-factor-β-activated kinase MOM-4 and the Nemo-like kinase LIT-1 to promote POP-1 nuclear export in the POP-1 regulatory branch (Lo et al., 2004), while SYS-1 is a transcriptional co-activator of POP-1 (Kidd et al., 2005).

HMP-1, WRM-1 and BAR-1 were identified through sequence homology (Costa et al., 1998; Rocheleau et al., 1999; Eisenmann et al., 1998). SYS-1, however, has negligible sequence identity to canonical β-cat and was initially identified as a co-activator of POP-1 through a functional screen (Kidd et al., 2005). Structural analysis later revealed that it contained 12 Armadillo repeats, which is characteristic of β-cat (Liu et al., 2008). The discovery of SYS-1 is interesting as it suggests the existence of other Wnt signaling components with highly divergent sequences that remain to be discovered both in *C.elegans* and other organisms. It also indicates a potential shortcoming in current methods that use sequence homology to identify novel signaling molecules.

Role of Wnt/β-cat signaling in regulating homeostasis of mouse intestinal epithelium

The epithelium of the small intestine in mice is a rapidly self-renewing tissue that is organized into crypts and villi. Stem cells reside near the bottom of the crypt, and give rise to a steady stream of rapidly proliferating transit-amplifying (TA) progenitor cells that are capable of differentiating into four cell types: enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Figure 1.4). After differentiation at the crypt-villus junction, the former three types of cells migrate upwards into the villi, while Paneth cells settle down at the crypt bottom (Clevers, 2006; Pinto and Clevers, 2003).

Wnt/β-cat signaling has been found to play a major role in regulating the proliferation, differentiation and maintenance of these intestinal epithelial cells. Several

genetic studies in mice have established the contribution of various Wnt signaling components, including β-cat, TCF4, APC and Lgr5, in this homeostatic process (reviewed in Pinto and Clevers, 2003). The conditional knock-out of either β-cat or TCF4 from the intestinal epithelium results in the loss of crypts, despite differences in Cre drivers used in each experiment (van Es et al., 2012; Ireland et al., 2004). While such loss of function mutations reduce the number of proliferating cells in the intestine (van Es et al., 2012; Kuhnert et al., 2004; Pinto et al., 2003; de Lau et al., 2011), gain of function mutations (e.g. deletion of the Wnt antagonist APC) have the opposite effect causing an expansion of the proliferating compartment (Sansom et al., 2004; Kim et al., 2005).

Interestingly, both loss and gain of function mutations in the Wnt pathway result in the mislocalization of Paneth cells from the crypt base (van Es et al., 2012; Ireland et al., 2004; Kuhnert et al., 2004; Pinto et al., 2003). This observation makes sense considering how the receptor EphB3 and its ligand Ephrin-B1, both of which play a role in Paneth cell positioning, are regulated by Wnt signaling. Microarray analysis revealed that EphB3 expression is upregulated, while Ephrin-B1 expression is downregulated by the Wnt/β-cat pathway (Batlle et al., 2002), potentially making Paneth cell positioning sensitive to both over and under activation of the pathway. In addition to regulating Paneth cell positioning, Wnt signaling plays a role in Paneth cell differentiation and maturation through Wnt induced expression of Sox9 and the Mmp-7/crytidin program, respectively (van Es et al., 2005; Bastide et al., 2007).

Target genes in the Wnt/β-cat pathway and the implications of their discovery

Although the role of the Wnt/β-cat pathway in diverse biological processes has been established, there remains a lack of evidence linking the pathway to direct target genes that are regulated by the pathway. A direct target is one that is not dependent on the expression of an intermediate factor to be regulated by Wnt/β-cat signaling. For example, genetic studies suggest that *svb* is repressed by Wg giving rise to bands of naked cuticle in the *Drosophila* epidermis (Payre et al., 1999). However, whether *svb* is a direct transcriptionally repressed target of Wnt/Wg signaling remains to be established. Similarly, *acheate* and *senseless*, which are implicated in determining the proneural fate of wing margin cells, are Wg dependent (Martinez Arias, 2003; Jaffar-Nejad et al., 2006; Parker et al., 2002), yet no Wnt Cis Regulatory Module (W-CRM) has been isolated for either genes.

In addition to augmenting our understanding of Wnt biology, knowledge of genes that are directly regulated by Wnt/β-cat signaling could provide potential targets for therapeutic intervention against disorders caused by misregulation of the pathway. Work by Clevers and colleagues on the "intestinal Wnt/ TCF signature" provides an example of how target gene information can be leveraged to explore therapies. The "intestinal Wnt/ TCF signature" comprises 208 genes that are regulated by the Wnt/β-cat pathway in several colorectal cancer cell lines (van der Flier et al., 2007). While many of these genes have been studied, the best characterized is the stem cell marker, leucine-rich-repeat-containing G protein-coupled receptor 5 (Lgr5). Lgr5 has been found to play an important role in epithelial stem cell biology as well as in cancer progression (Barker et al., 2007; Barker et al., 2009; Barker et al., 2013). These findings have prompted research on its applications in regenerative medicine (Sato et al., 2009) and in cancer diagnostics, as a

marker for cancer stem cells in intestinal tumors (Kemper et al., 2012; Schepers et al., 2012).

An understanding of how the Wnt/β-cat pathway recognizes target genes and regulates their transcription is critical for the identification of novel direct Wnt targets in the future. For example, knowledge of transcription factor binding site consensus and affinities can help fine-tune bioinformatic tools, such as the Enhancer Element Locator (Hallikas et al., 2006), to better search for and identify potential W-CRMs. These putative W-CRMs could be further validated using ChIP and reporter gene mutagenesis in cell culture or transgenic organisms. In addition, an understanding of the interactions between different transcription factors could be applied towards methods like genome-wide ChIP. Unlike many non-systems level approaches that have been used most often in the past to identify the majority of known Wnt targets, these systems level methods could help increase the scope of our search for novel Wnt target genes.

TCF IS A MAJOR TRANSCRIPTION FACTOR IN THE WNT/β-CAT PATHWAY

Historical perspective

The TCF family of proteins are the best characterized DNA-binding transcription factors in the Wnt/β-cat pathway. TCFs were originally discovered as T-cell factors that regulate T-cell specific genes, such as CD3ε (Travis et al., 1991; Waterman et al., 1991; van de Wetering et al., 1991), lending to their name. Their role in Wnt/β-cat signaling was discovered with multiple studies that revealed their ability to bind the fly β-cat, Armadillo (Arm) (Behrens et al., 1996; Molenaar et al., 1996, Brunner 1997, van de

Wetering 1997). β-cat had already been established as an important mediator of the Wnt pathway (Noordermeer et al., 1994; Peifer et al., 1991), but the mechanism of transcriptional regulation downstream was still a mystery since β-cat was primarily known as an adhesion molecule (Peifer et al., 1992). Studies that revealed both physical and functional interactions between TCF and β-cat helped create a more complete picture of the Wnt/β-cat signaling circuit.

Two groups initially established the functional contribution of TCF in the Wnt/β-cat pathway in Xenopus and *Drosophila*. In Xenopus embryos, misexpression of LEF1 caused induction of a secondary axis, a classic Wnt over-activation phenotype (Behrens et al., 1996). In addition, axis duplication induced by β-cat overexpression was blocked by LEF1 or TCF3 lacking the N-terminal β-cat binding domain, which had been previously mapped (Behrens et al., 1996; Molenaar et al., 1996). *TCF/Pangolin (Pan)* mutants in *Drosophila* caused a segment polarity phenotype, indicative of a lack of Wnt/Wg signaling. Conversely, a hyperactive β-cat/Arm phenotype was blocked in a *TCF/Pan* mutant background, suggesting TCF acts downstream of β-cat (Brunner et al., 1997, van de Wetering et al., 1997). Over the years, many other groups have established the functional role of TCF in Wnt signaling in other organisms, including mice, zebrafish, and Hydra (Galceran et al., 1999; Kim et al., 2000; Duffy et al., 2010).

The TCF family and their conserved domains

The TCF family is highly conserved across metazoans. Most invertebrates have a single TCF gene, with *TCF/Pan* from *Drosophila* and *POP-1* from *C.elegans* being the most well characterized. Most vertebrates have four TCF genes for *TCF1, TCF3, LEF1*

and *TCF4* (Archbold et al., 2012). All TCF family members share two highly conserved domains: the β-cat binding domain and the DNA-binding HMG domain (Figure 1.5).

The N-terminal region of TCF has been mapped as the β-cat binding domain. This conserved region shows around 60% sequence identity across different TCFs (Arce et al., 2006). The functional relevance of this domain has been well established. TCFs lacking this domain have dominant negative effects and antagonize endogenous Wnt signaling (Molenaar et al., 1996; Behrens et al., 1996; ven de Wetering et al., 1997). In addition, a single amino acid mutation within this domain suppressed a hyper-active Wnt phenotype in *Drosophila* (Brunner et al., 1997). Structural studies of β-cat bound to the TCF β-cat binding domain further validate this interaction (Graham et al., 2000; Poy et al., 2001)

The very well characterized high mobility group (HMG) domain is a DNA binding domain in TCF. Studies with the HMG domain of LEF1 revealed that this domain interacts with DNA through minor groove contacts and causes DNA bending (Love et al., 1995; Giese et al., 1992). The basic tail adjacent to the HMG domain was also found to contribute to both DNA binding and bending functions (Love et al., 1995). The HMG domain binds specific DNA sequences (Giese et al., 1991; van de Wetering et al., 1991). In vitro studies have determined a high affinity consensus sequence of CCTTTGATS (S=G/C) (van de Wetering et al., 1997; van Beest et al., 2000; Hallikas et al., 2006; Atcha et al., 2007; Archbold et al., 2012).

Another DNA binding domain in TCF, called the C-clamp, is C-terminal to the HMG domain and also highly conserved across invertebrate TCFs and E-tail containing vertebrate TCF isoforms, TCF1E and TCF4E (Atcha et al., 2007). The structure and function of the C-clamp will be elaborated on separately.

Other transcription factors that regulate the Wnt/β-cat pathway

In *Drosophila*, *TCF/Pan* mutants qualitatively resemble *Wnt/Wg* mutants for many phenotypic readouts (Brunner et al., 1997; van de Wetering et al., 1997; Schweizer et al., 2003), suggesting that TCF/Pan is the primary transcription factor for most Wnt/Wg signaling, at least at the embryonic and larval stages. TCF was also found to be required for Wnt signaling mediated head regeneration in the cnidarian Hydra (Duffy et al., 2010). However, none of these studies unambiguously rule out the possibility of TCF-independent Wnt signaling in either organism. The conditional knockout of β-cat in mice revealed several mutant phenotypes, but not all could be correlated with a TCF (reviewed in Zhang and Cadigan, 2014). This result could be explained by redundancy due to the existence of multiple vertebrate TCFs, but could also hint at the involvement of transcription factors besides TCF in the Wnt/β-cat pathway.

Several groups have identified other transcription factors that bind β-cat and activate transcription (reviewed in Archbold et al., 2012; Cadigan and Waterman, 2012; Valenta et al., 2012; Zhang and Cadigan, 2014). These include Sox proteins, such as Sox17 (Sinner et al., 2004), FOXO (Esser et al., 2005), Pitx2 (Kioussi et al., 2002), HIF1 alpha (Kaidi et al., 2007), Yap1 (Rosenbluh et al., 2012) and nuclear hormone receptors such as the androgen receptor (Mulholland et al., 2002) and the Vitamin D receptor (Shah et al., 2006). However, most of these and other studies that explore alternate transcription factors are not very robust as they are primarily based on overexpression assays in cell culture and protein-protein binding experiments with exogenously expressed proteins.

Two of these transcription factors, the Forkhead box O (FOXO) protein and Sox17, have been studied utilizing in vivo models providing stronger evidence for their involvement in the Wnt/β-cat pathway (Esser et al., 2005; Sinner et al., 2004). FOXO proteins are oxidative-stress induced transcription factors that, under oxidative stress conditions, display an increased capacity to bind β-cat and activate expression of stress-induced FOXO targets such as *MnSOD/sod-3.* In transgenic *C.elegans,* both *daf-16/FOXO* and *bar-1/β-cat* mutants showed a reduced ability to activate an *MnSOD/sod-3* reporter despite stress conditions, suggesting a requirement for both proteins. Moreover, the *MnSOD/sod-3* reporter activity was rescued in *bar-1/β-cat* mutants with the expression of wild type BAR-1 (Esser et al., 2005). The HMG box transcription factor, Sox17, regulates endodermal gene expression in Xenopus. This regulation was found to be β-cat dependent, as β-cat depletion using morpholino treatment decreased activation of a number of endogenous target genes in Xenopus, in the presence of Sox17. Expression of a stabilized β-cat however rescued this activity (Sinner et al., 2004). Although this assay involved overexpression of Sox17, the fact that these experiments were performed in vivo better supports a role for Sox17 compared to other transcription factors that have been tested only in cell culture.

Studies involving the bicoid related transcription factor, Pitx2, have been limited to mammalian cell culture, but found that Pitx2 bound β-cat even at endogenous levels (Kioussi et al., 2002). An antibody block against either Pitx2 or β-cat reduced the activation of the Pitx2 target, *Cyclin D2*, indicating a requirement for β-cat. In addition, chromatin immuno-precipitation (ChIP) found that both Pitx2 and β-cat, but not LEF-1,

localized to the *Cyclin D2* promoter after GSK3 inhibition, providing more direct evidence for the involvement of Pitx2 in regulating Wnt targets (Kioussi et al., 2002).

Although FOXO, Sox17 and Pitx2 have been tested more robustly than other transcription factors, a major piece of the puzzle that is still missing across all non-TCF transcription factors is mechanistic evidence linking these proteins to the Wnt/β-cat pathway. This would ideally be specific point mutations in the β-cat binding domain of these transcription factors that disable their regulatory ability in vivo. Such evidence is available for TCF, where the knock-in of a six amino acid LEF-1 mutant displayed the same developmental defect as a LEF-1 null mutant in mouse embryos (Kratochwil et al., 2002). This mutation had previously been found to abrogate binding between LEF-1 and β-cat, suggesting it was disrupting the β-cat binding domain of LEF-1 (Hsu et al., 1998). The best evidence among non-TCF transcription factors is for Sox17, where a three amino acid mutation in the β-cat binding region reduced activation of endogenous target genes in Xenopus when overexpressed (Sinner et al., 2004). However, a knock-in of this mutant in vivo remains to be tested.

Whether these interactions between β-cat and other transcription factors is TCF-independent is also debatable. Many studies have not tested this sufficiently while others have discovered potential interactions involving TCFs. The DAF-16/FOXO regulated *MnSOD/sod-3* reporter activity was not affected in the mutant TCF *pop-1(hu-9)* background (Esser et al., 2005). However, the viability of homozygous *pop-1 (hu9)* worms suggests this hypomorphic allele may be too weak to sufficiently affect TCF interactions. Depletion of TCF3 did not affect Sox17 target gene expression (Sinner et al., 2004), and ChIP failed to detect LEF1 at the Pitx2-regulated Cyclin D2 promoter

(Kioussi et al., 2002), suggesting Sox17 and Pitx2 are acting independently of TCF. However, other studies have found that both transcription factors are capable of binding certain TCFs (Zorn et al., 1999; Sinner et al., 2007; Vadlamudi et al., 2005; Amen et al., 2007), making it difficult to rule out the involvement of TCFs.

INSUFFICIENCY OF HMG DOMAIN-HMG SITE INTERACTIONS IN TARGET SELECTION

The consensus HMG domain binding site of CCTTTGATS (S=G/C) reflects sites that have displayed high affinity binding to TCFs in various in vitro studies (van de Wetering et al., 1997; van Beest et al., 2000; Hallikas et al., 2006; Atcha et al., 2007). Many functional sites that fit this consensus have been found in various W-CRMs (He et al., 1998; Yamaguchi et al., 1999; Jho et al., 2002). Genome wide studies in colorectal cancer cell lines (such as LS174T and HCT116 cells) found that 70% of TCF4 bound regions and 84% of β-cat bound regions contained the consensus HMG motif (Yochum et al., 2008; Hatzis et al., 2008). In addition, multimerized consensus HMG sites upstream of a minimal promoter respond to Wnt/β-cat pathway stimulation in cell culture (Korinek et al., 1997; Chang et al., 2008) and recapitulate Wnt signaling in many tissues in transgenic mice, Xenopus and zebrafish (DasGupta and Fuchs, 1999; Geng et al., 2003; Dorsky et al., 2002).

However, strict adherence of an HMG site sequence to the consensus is not necessarily correlated with its functional relevance. Some functional HMG sites in *c-myc* and *naked cuticle (nkd)* W-CRMs deviate from the consensus at two or three positions (Tuupanen et al., 2009; Wright et al., 2010; Chang et al., 2008). Systematic analysis of in

vitro binding of TCF4 to DNA with single base pair variations from the consensus revealed that many substitutions only reduced and did not completely eliminate binding (Hallikas et al., 2006). Also, the HMG domain was found to be able to recognize a large number of lower affinity sites in addition to high affinity sites (Badis et al., 2009). Some of these sites are functional in W-CRMs, such as those of *even-skipped (eve)* and *sloppy paired 1 (slp1)* (Knirr and Frasch, 2001; Lee and Frasch, 2000).

Conversely, the presence of HMG sites does not guarantee regulation by the Wnt/β-cat pathway. Genomic regions near the Wnt target gene *nkd* that contain HMG sites do not always recruit TCF and do not necessarily constitute functional W-CRMs (Parker et al., 2008). Many inconsistencies have been observed with the responsiveness of multimerized HMG sites. Synthetic reporters containing multimerized HMG sites derived from endogenous W-CRMs fail to activate in *Drosophila* (Riese et al., 1997; Barolo et al., 2006). Such synthetic reporters have also been known to sometimes not express in tissues known to have Wnt signaling, in both mice and other organisms (reviewed in Barolo, 2006; eg. Dessimoz et al., 2005; Geng et al., 2003).

The degeneracy in HMG domain-HMG site interactions suggests the existence of millions of potential HMG sites in the genome (Archbold et al., 2012). This leads to the question: how does TCF distinguish between functional and non-functional sites in this sea of possibilities to accurately locate its target? One possible solution is additional sequence information that TCF can recognize either by itself or when in complex with other transcription factors or co-factors. The finding that synthetic reporters containing only HMG sites are not always Wnt responsive (Riese et al., 1997; Barolo et al., 2006; Geng et al., 2003; Dessimoz et al., 2005; Fathke et al., 2006) also supports this possibility

of additional binding sites that improve TCF's specificity. An understanding of what this additional sequence information is and how it is regulated by TCF and the Wnt/β-cat pathway can aid in the discovery of direct Wnt targets using both in vitro (ChIP etc.) and computational approaches in the future.

MECHANISMS FOR TARGET GENE SELECTION

Bipartite recognition by TCF

The discovery of GC rich motifs called Helper sites in W-CRMs provided direct evidence of sequence information, in addition to HMG sites, that helps TCF locate its targets. Helper sites have a consensus of GCCGCCR (R=A/G) in *Drosophila* and GCSGS (S=G/C) in vertebrates (Chang et al., 2008, Atcha et al., 2007, Hoverter et al., 2012). Helper sites were found to be critical for Wnt dependent activation of multiple W-CRMs in *Drosophila* (Chang et al., 2008). In mammalian cells, the presence of Helper sites in synthetic reporters with multimerized HMG sites increased TCF-mediated activation (Hoverter et al., 2012). In addition, genome wide chromatin immunoprecipitation (ChIP) studies of human TCF4 revealed an enrichment of a GC rich motif similar to the Helper site in multiple Wnt targets (Hatzis et al, 2008). Together, this data indicates an important functional role for Helper sites in Wnt/β-cat signaling.

A direct link between TCF and Helper sites was established with the discovery of the C-clamp (Atcha et al., 2007). The C-clamp is a highly conserved domain C-terminal to the HMG domain. It is present in most invertebrate TCFs as well as in the E-tail containing isoforms of vertebrate TCF1 and TCF4 (TCF1E and TCF4E) (Archbold et al., 2012) (Figure 1.5B). The C-clamp was required for TCF to recognize Helper sites in vitro

(Chang et al., 2008; Atcha et al., 2007). In addition, the activation of Wnt/Wg targets in *Drosophila* cell culture was dependent on the presence of a C-clamp (Chang et al., 2008). In mammalian cells, certain W-CRMs, such as those of *LEF1* and *cdx1*, were specifically only activated by the C-clamp containing TCF1E and TCF4E isoforms (Atcha et al., 2007).

While cell culture data suggests an important role for the C-clamp in Wnt signaling, the role of the C-clamp has not been validated sufficiently in vivo. In vivo studies have been limited to the finding that a point mutation in the C-clamp (A374V) resulted in a weak segment polarity defect in *Drosophila*, indicative of reduced Wnt/Wg signaling. This mutation also suppressed a Wnt/Wg overactivation phenotype caused by constitutively active β-cat/Arm (van de Wetering et al., 1997).

Both C-clamp dependent in vitro binding of fly TCF/Pan to Helper sites (Chang et al., 2008) and the enrichment of Helper-like GC-rich sites in in vitro DNA binding studies with human TCF1E (Atcha et al., 2007; Hoverter et al., 2012) led to the development of the bipartite recognition model for TCF. This model suggests that TCF recognizes DNA through simultaneous HMG domain-HMG site and C-clamp-Helper site interactions (Figure 1.6). Considering the presence of Helper sites along with HMG sites doubles the amount of sequence information available to TCF, this model could likely explain a mechanism for TCF to improve its specificity in recognizing its targets.

In addition to TCF, a conserved C-clamp has been found in three other proteins in humans (Figure 1.7), where it is also thought to have an important DNA binding function. Huntington's disease binding protein 1 (HDBP1) (also known as GLUT4 enhancer factor or SLC2A4 regulator) and 2 (HDBP2) (also known as papilloma virus binding factor or

zinc finger 395) contain a C-clamp that is sufficient to bind a Helper-like GC rich motif in vitro (Tanaka et al., 2004; Sichtig et al., 2007). The third C-clamp containing protein is glucocorticoid induced gene 1 (GIG1) (also known as ZNF704) (Yamamoto et al., 2007). These transcription factors play a role in several different processes, such as adipogenesis (HDBP2, Hasegawa et al., 2013) and myogenesis (GIG1, Yamamoto et al., 2007), suggesting a wider contribution by the C-clamp, beyond processes regulated by TCF and the Wnt/β-cat pathway.

Bipartite recognition in other transcription factors

Bipartite recognition of DNA is not unique to TCF. The best characterized family of transcription factors that adopts a similar DNA binding strategy is the POU domain family. The POU domain was initially identified in the Pit-1, Oct-1 and Oct-2 and UNC-86 transcription factors. It has since been found in 20 other metazoan proteins involved in various aspects of transcriptional regulation (Herr and Cleary, 1995). POU domain proteins contain two highly conserved structurally independent DNA binding domains: an N terminus POU-specific domain (POU_S) and a C-terminus POU-type homeodomain (POU_{HD}) (Phillips and Luisi, 2000). Both the POU_S domain and POU_{HD} can bind DNA independently and specifically (Verrijzer et al., 1992). The POU domain protein Oct-1 recognizes an octamer DNA motif ATGCAAAT in the histone H2B promoter. Structural studies of an Oct-1 POU domain- octamer DNA complex revealed that the POU_S domain made direct contacts with the 5' ATGC sequence while the POU_{HD} directly bound the 3' AAAT sequence (Klemm et al., 1994).

In addition to POU domain proteins, two other groups of transcription factors are known to contain dual DNA binding domains involved in bipartite DNA recognition. The first is the Pax family of proteins that is characterized by the presence of a highly conserved DNA binding called the paired domain (PD). Several Pax proteins contain an additional DNA binding domain: the paired-class homeodomain (HD), C-terminal to the PD (Strachan and Read, 1994; Lang et al., 2007). Both the PD and HD were found to be required for Paired (Prd) (the *Drosophila* PAX protein) function in vivo, as single amino acid mutations in either domains failed to rescue activation of Prd target genes, such as *engrailed* and *gooseberry*, in a *prd* mutant background (Bertuccioli et al., 1996). While the domains can each individually bind specific DNA sequences (Wilson et al., 1995; Xu et al, 1995), they were also found to simultaneously occupy DNA motifs in two different *even-skipped* promoters. These motifs comprised two juxtaposed half-sites, one bound by the PD and the other by the HD (Treisman et al., 1991; Fujioka et al., 1996).

The multi-zinc finger transcriptional repressors Smad interacting protein (SIP1) and δEF1 are the second family of transcription factors that utilize bipartite DNA recognition. Both proteins contain two clusters of CCHH zinc fingers (ZF): the N terminal zinc finger (NZF) and the C terminal zinc finger (CZF) cluster (Remacle et al., 1999; Sekido et al., 1997; Ikeda and Kawakami, 1995). SIP1 was found to bind a bipartite motif consisting of CACCT and CACCTG half-sites in several promoters, including those of Xenopus *Xbra*, and human α4-integrin and E-cadherin. Both the NZF and CZF clusters in SIP1 were not only required for this high affinity binding, but also functionally important for optimal repressor activity in cell culture (Remacle et al., 1999).

Cooperative interactions between TCF and other transcription factors

Another mechanism that can enhance TCF specificity involves synergistic interactions between TCF and both local and signal-regulated transcription factors (Barolo and Posakony, 2002). For example, TCFs have been found to associate cooperatively with Smads at the *Msx2* promoter (Hussein et al., 2003), with AP-1 at the *c-jun* promoter (Nateri et al., 2005) and with Cdx1 for *Cdx1* autoregulation (Beland et al., 2004) (reviewed in Bhambhani and Cadigan, 2014).

A number of recent genome-wide studies have revealed that TCFs co-localize with multiple transcription factors (Trompouki et al., 2011; Junion et al., 2012; Frietze et al., 2012). These 'transcription factor collectives' show cell-specific patterns. In hematopoietic lineages, TCF4 along with SMAD1 show lineage specific co-occupancy with GATA1 and GATA2 in erythroid cells, with C/EBPa in myeloid cells and with GATA1 in progenitor cells (Trompouki et al., 2011). Many of these co-occupied regions correspond to enhancers of actively transcribed lineage-specific genes. Additionally, ectopic expression of GATA1 in myeloid cells or C/EBPa in erythroid cells, re-directed TCF4 and SMAD1 genomic binding patterns. Together, this supports a mechanism where lineage specific regulators (such as GATA1 and C/EBPa) direct TCF and SMAD proteins to tissue-specific enhancers (Trompouki et al., 2011).

Another study carried out chromatin immunoprecipitation (ChIP) analysis of TCF and four other transcription factors: pMad, Pannier (Pnr), Dorsocross (Doc) and Tinman (Tin) in *Drosophila* embryos during dorsal mesoderm specification (Junion et al., 2012). The transcription factors were found to show high levels of enrichment at shared regions in a mesoderm-specific context. A majority of these regions were occupied by all five

proteins, suggesting that the five transcription factors are co-recruited in a concerted manner. Twenty-two of twenty-four of these regions tested corresponded to enhancers that regulated expression in mesoderm lineages, verifying that the transcription collective was being recruited to functional mesoderm-specific enhancers (Junion et al., 2012).

Interestingly, the Junion et al., 2012 study found that many areas co-occupied by TCF and the other four factors were devoid of HMG binding sites, suggesting protein-protein interactions in addition to protein-DNA interactions can also influence TCF occupancy. A similar observation was made in another study, where GATA3 co-localized with TCF4 at multiple enhancer regions in MCF7 breast cancer cells (Frietze et al., 2012). Sites bound by GATA3 and TCF4 were enriched for the GATA3 binding motif, but not HMG sites. In addition, depletion of GATA3 using siRNA resulted in a reduction of TCF 4 binding at these regions. Taken together, this suggests that GATA3 is required for TCF recruitment to certain regulatory regions (Frietze et al., 2012).

RATIONALE AND SPECIFIC AIMS

The Wnt/β-cat signaling pathway controls diverse developmental and homeostatic processes in metazoans through transcriptional regulation of various downstream target genes. TCF is a major transcription factor in the pathway, with the important function of accurately recognizing target genes. The primary focus of my dissertation is to explore mechanisms used by TCF to achieve this specificity in target gene selection.

Chapter II: To explore the structural and functional properties of the C-clamp in TCF/Pan (the *Drosophila* TCF)

TCFs have been known to recognize target genes by binding specific DNA sequences in Wnt/β-cat regulated Cis Regulatory Modules (W-CRMs) using their High Mobility Group (HMG) domain. These DNA motifs, called HMG sites, display high levels of degeneracy, suggesting additional mechanisms that improve TCF's DNA specificity may be required (Archbold et al., 2012). Invertebrate TCFs and certain vertebrate TCFs have an additional domain called the C-clamp, C-terminal to the HMG domain, that allows TCF to recognize another DNA motif, called Helper sites (Chang et al., 2008). Helper sites were previously found to be required for Wnt activation of several fly and human W-CRMs (Chang et al., 2008; Hoverter et al., 2012). While the C-clamp has been shown to be important for regulating several Wnt reporter genes in cell culture (Chang et al., 2008), very little is known about this domain.

In Chapter II, I explore the structural and functional properties of the C-clamp in TCF/Pan. I found that the C-clamp is a zinc-binding domain that is sufficient for binding the Helper site. In addition to this DNA binding role, the C-clamp also inhibits the HMG domain from binding DNA. TCF mutants that were compromised for either the DNA binding activity or the "inhibitory effect" activity were isolated and tested for functional relevance in cell culture and in vivo in *Drosophila.* While the DNA-binding activity was found to be critical for TCF/Pan function in both cell culture and in vivo, the "inhibitory effect" mutation had a partial effect on TCF/Pan function in cell culture but no effect in vivo.

This work has been published in PLOS ONE (Ravindranath and Cadigan, 2014).

Chapter III: To explore the importance of C-clamp-Helper site interactions for POP-1 (the *C.elegans* TCF) function and TCF oligomerization for TCF/Pan (the *Drosophila* TCF) function

The functional contribution of C-clamp-Helper site interactions has been studied in the context of *Drosophila*, but its role in POP-1 mediated Wnt/β-cat signaling in *C.elegans* was unknown. Similarly, it was observed that POP-1 formed oligomers (Bhambhani and Cadigan, unpublished results), but whether TCF/Pan was also capable of self-associating remained to be established. Chapter III explores how conserved these two mechanisms of improving TCF specificity are across *Drosophila* and *C.elegans.*

In *C.elegans,* both HMG and Helper sites were found to be critical for activation of several W-CRMs. In addition, POP-1 required the C-clamp for enhanced DNA binding in vitro. These findings suggest that similar to *Drosophila* TCF/Pan, POP-1 is also dependent on C-clamp-Helper site interactions for its regulatory function. The contribution of Helper sites was further established with the finding that a synthetic reporter containing multimerized HMG-Helper sites displayed a unique POP-1 and BAR-1 (a *C.elegans* β-cat) dependent expression pattern in the int9 intestinal cells.

POP-1 oligomerization was previously found to be dependent on a C-terminal region (Bhambhani and Cadigan, unpublished results). The presence of a motif in this region that was conserved across the *C.elegans* POP-1 and *Drosophila* TCF/Pan, suggested that TCF/Pan may also be capable of self-associating. Surprisingly, TCF/Pan deletion mutants missing this putative oligomerization motif remained functional in cell culture.

Part of this work has been published in PLOS Genetics (Bhambhani et al., 2014). I am the second author on this paper. My contribution is detailed at the end of Chapter III.

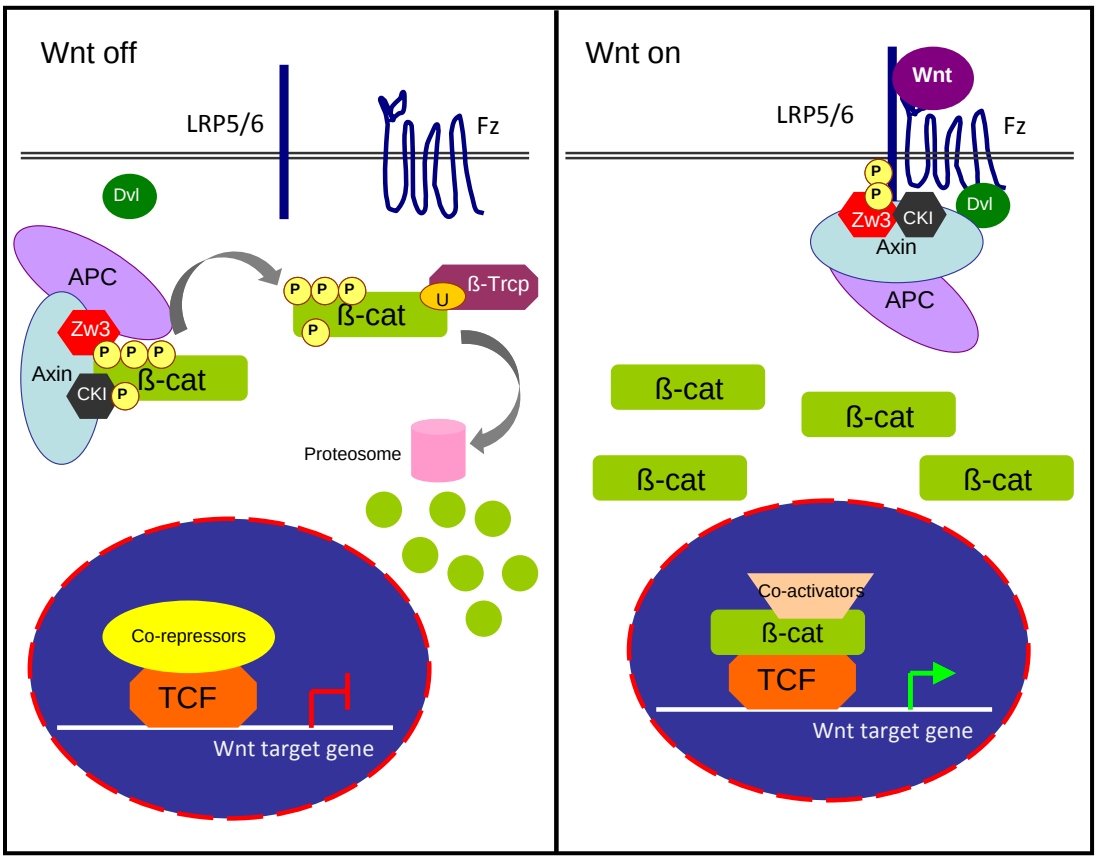

Figure 1.1. Simplified model of Wnt/β-cat signaling in the absence (Wnt off) or presence (Wnt on) of Wnt. In the Wnt off condition, β-cat is degraded in the cytosol. Under this condition, in certain contexts, TCF represses target gene activation within the nucleus. In the Wnt on condition, β-cat levels increase in the cytosol. β-cat translocates into the nucleus to bind TCF and activate transcription of target genes.

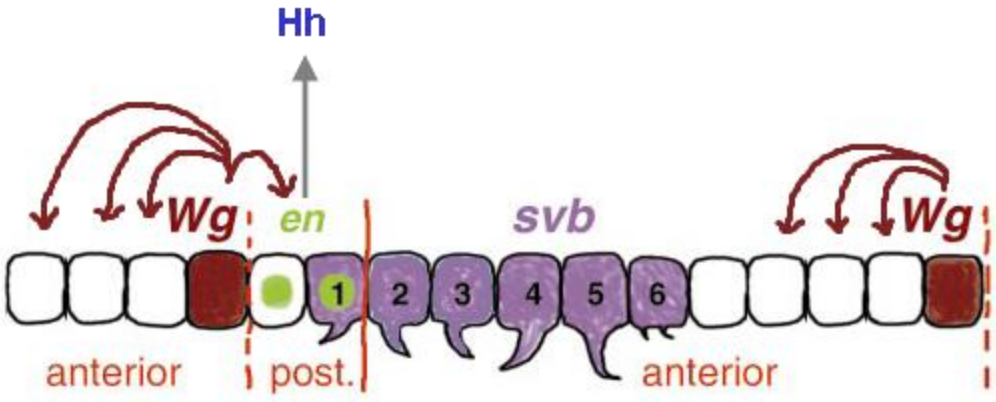

Figure 1.2. Role of Wnt/Wg signaling in *Drosophil*a epidermal patterning (Modified from Payre et al., 1999). Schematic diagram of ventral epidermis showing expression pattern of *wg* (brown), *en* (green) and *svb* (purple). Wg represses expression of *svb* in anterior cells and one posterior cell, producing naked cuticle. Cells expressing *svb* produce denticles. Wg drives expression of *en*, which in turn activates *Hh* transcription.

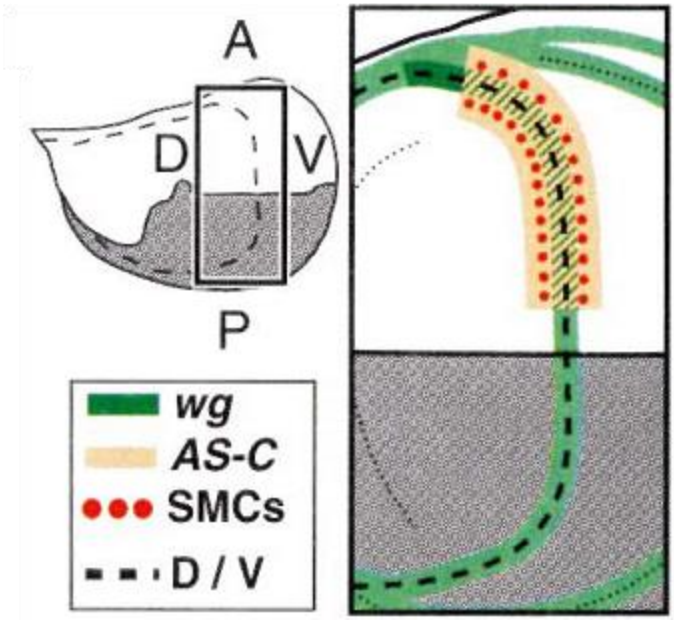

Figure 1.3. Role of Wnt/Wg signaling in *Drosophil*a wing margin specification (From Rulifson et al., 1996). Schematic diagram of late third instar wing disc with box depicting wing margin. *wg* is expressed along dorsoventral (D/V) boundary and drives expression of *achaete* (part of the achaete-scute complex (AS-C)). This establishes the pronueral region within which sensory mother cells (SMCs) give rise to chemosensory margin bristles. A: anterior, P: posterior, D: dorsal, V: ventral.

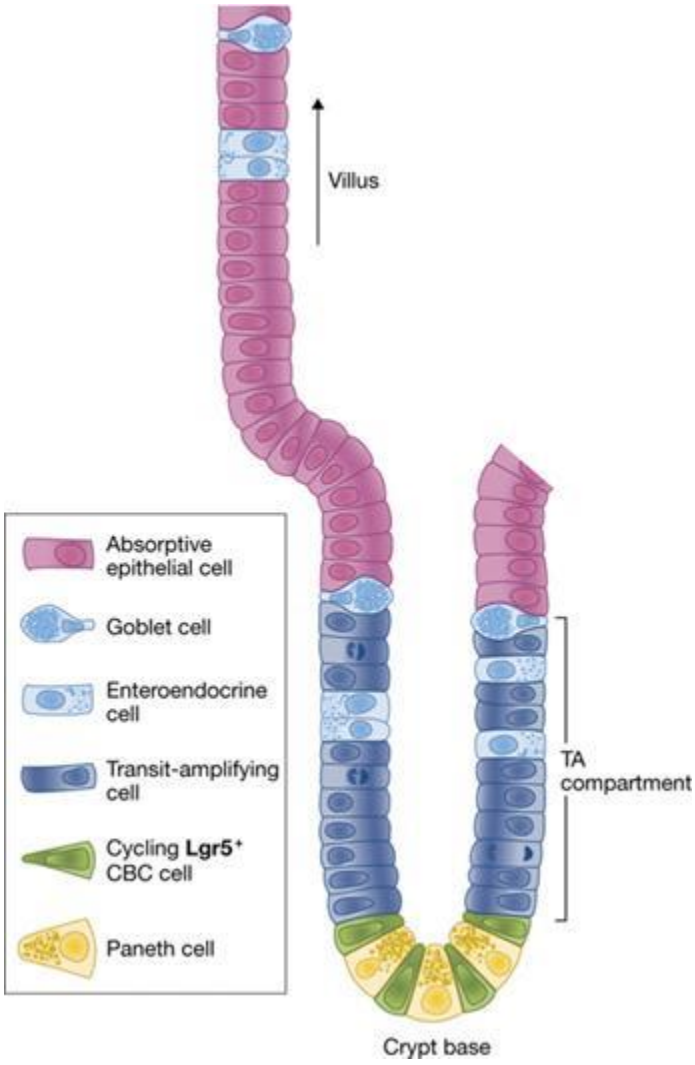

Figure 1.4. Model depicting cell types in stem cell compartment of small intestine (From Schuijers and Clevers, 2012). Stem cells reside in the bottom of the crypt, give rise to proliferating transit-amplifying (TA) cells that undergo differentiation into four cell types: enterocytes, goblet cells, enteroendocrine cells and Paneth cells. The first three types of cells migrate upwards into the villi, while Paneth cells settle down at the crypt bottom.

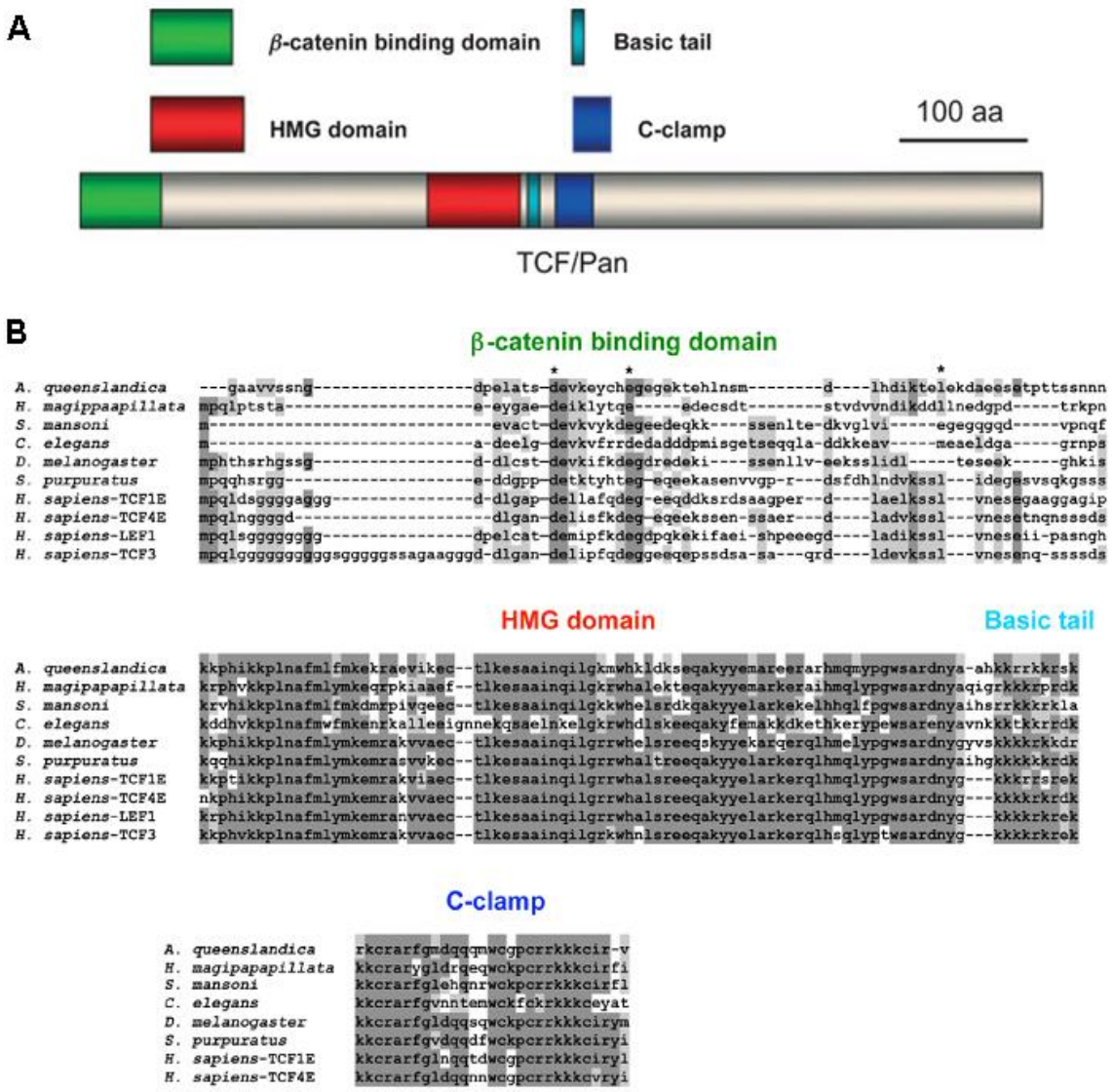

Figure 1.5. Conserved domains of TCF. (From Archbold et al., 2012; Cadigan and Waterman, 2012) **(**A) Schematic of TCF domains, showing the β-catenin binding domain (green), the HMG domain (red), the basic tail (aqua) and the C-clamp (blue). The specific TCF shown is the *Drosophila* TCF/Pangolin (B) Alignment of the β-catenin binding domains, HMG domains, basic tail and C-clamps across metazoan TCFs. Sequences from six invertebrate TCFs and the four human TCFs are included.

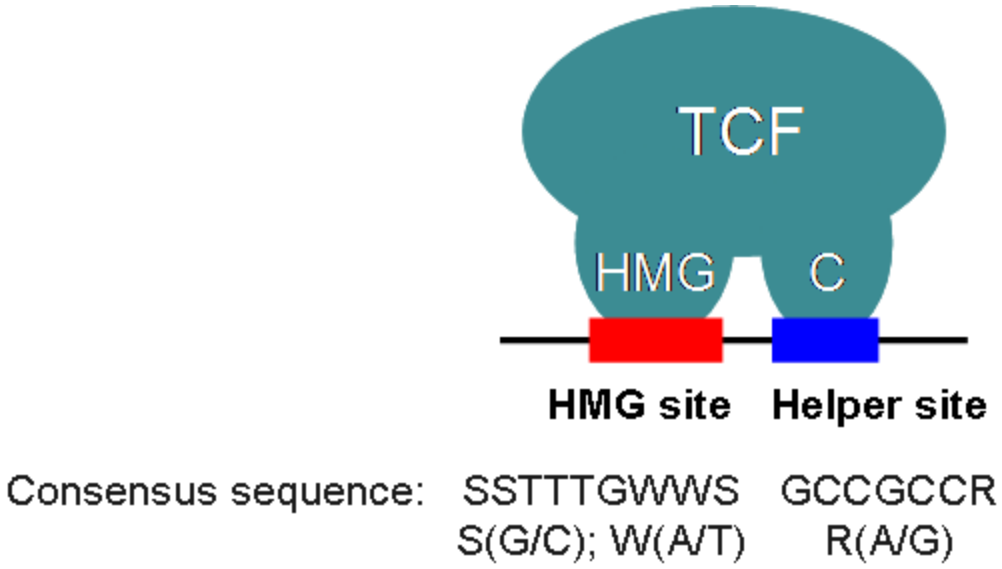

Figure 1.6. Bipartite DNA recognition model for TCF. TCF recognizes DNA through simultaneous HMG domain-HMG site and C-clamp-Helper site interactions. The HMG domain and C-clamp are labeled as HMG and C, respectively, within the TCF protein. The consensus sequence for the HMG and Helper sites are also included.

Figure 1.7. Conservation of the C-clamp across four human proteins. Alignment of the amino acid sequence from the C-clamp in human TCF1E and TCF4E isoforms, Huntington's disease binding proteins 1 (HDBP1) and 2 (HDBP2), and glucocorticoid induced gene 1 (GIG1). Regions of sequence conservation are shaded in gray.

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CHAPTER II

STRUCTURE-FUNCTION ANALYSIS OF THE C-CLAMP OF TCF/PANGOLIN IN WNT/β-CATENIN SIGNALING

ABSTRACT

The evolutionarily conserved Wnt/β-catenin (Wnt/β-cat) pathway plays an important role in animal development in metazoans. Many Wnt targets are regulated by members of the TCF/LEF1 (TCF) family of transcription factors. All TCFs contain a High Mobility Group (HMG) domain that bind specific DNA sequences. Invertebrate TCFs and some vertebrate TCF isoforms also contain another domain, called the C-clamp, which allows TCFs to recognize an additional DNA motif known as the Helper site. While the C-clamp has been shown to be important for regulating several Wnt reporter genes in cell culture, its physiological role in regulating Wnt targets is less clear. In addition, little is known about this domain, except that two of the four conserved cysteines are functionally important. Here, we carried out a systematic mutagenesis and functional analysis of the C-clamp from the *Drosophila* TCF/Pangolin (TCF/Pan) protein. We found that the C-clamp is a zinc-binding domain that is sufficient for binding to the Helper site. In addition to this DNA-binding activity, the C-clamp also inhibits the HMG domain from binding its cognate DNA site. Point mutations were identified that specifically affected DNA-binding or reduced the inhibitory effect. These mutants were characterized in TCF/Pan rescue assays. The specific DNA-binding activity of the C-clamp was essential

for TCF/Pan function in cell culture and in patterning the embryonic epidermis of *Drosophila*, demonstrating the importance of this C-clamp activity in regulating Wnt target gene expression. In contrast, the inhibitory mutation had a subtle effect in cell culture and no effect on TCF/Pan activity in embryos. These results provide important information about the functional domains of the C-clamp, and highlight its importance for Wnt/β-cat signaling in *Drosophila*.

INTRODUCTION

The Wnt/β-catenin (Wnt/β-cat) pathway is a major cell-cell signaling pathway found throughout metazoans (Archbold et al., 2012). This signaling cascade plays many important roles in animal development (Archbold et al., 2012; Logan and Nusse, 2004; Niehrs, 2010; Petersen and Reddien, 2009). For example, in *Drosophila*, the pathway is important for many cell specification events, including patterning of the embryonic epidermis (Bejsovec and Wieschaus, 1993; Klingensmith and Nusse, 1994). Wnt/β-cat signaling is also critical for adult tissue homeostasis, where it often functions as a stem cell niche signal (Holland et al., 2013; Schuijers and Clevers, 2012). Misregulation of the pathway is implicated in many diseases in humans, including many cancers (Holland et al., 2013; Polakis, 2012), bone disorders (Baron and Kneissel, 2013) and type II diabetes (Liu and Habener, 2010).

A key intracellular messenger in Wnt/β-cat signaling is β-catenin, whose degradation is inhibited by Wnt stimulation (Cadigan and Peifer, 2009; Valenta et al., 2012). Stabilized β-catenin translocates from the cytoplasm to the nucleus, where it is recruited to Wnt target gene chromatin by binding to transcription factors (Archbold et al.,

2012; Valenta et al., 2012). Members of the TCF/LEF1 (TCF) family are the best-characterized nuclear mediators of Wnt/β-cat signaling (Cadigan, 2012; Cadigan and Waterman, 2012). Vertebrates contain several *TCF* genes, while *Drosophila* has only one, *TCF/Pangolin* (*TCF/Pan*) (Brunner et al., 1997; van de Wetering et al., 1997).

All TCFs contain High Mobility Group (HMG) domains, which can bind DNA with sequence specificity (Cadigan and Waterman, 2012). Consensus HMG binding sites are 9-11 bp in length, and share the sequence SCTTTGWWS (Hallikas et al., 2006; van Beest et al., 2000; van de Wetering et al., 1997). Synthetic reporters comprised of multimerized consensus HMG sites upstream of a basal promoter can be activated by Wnt/β-cat signaling (Barolo, 2006) and functional high affinity HMG sites are found in many endogenous Wnt/β-cat regulated cis-regulatory modules (W-CRMs) (Archbold et al., 2012). In addition to these high affinity HMG sites, the HMG domains of TCFs can bind numerous lower-affinity secondary sites (Badis et al., 2009), some of which have been shown to be functional (Knirr and Frasch, 2001; Lee and Frasch, 2000). Because of the degeneracy of HMG-DNA recognition, it seems unlikely that these interactions are sufficient for TCFs to locate their nuclear targets (Archbold et al., 2012).

Several TCFs increase their DNA binding specificity through a second DNA binding domain known as the C-clamp. This domain, located just C-terminal to the HMG domain, was originally discovered in "E-tail isoforms" of vertebrate TCF1 and TCF4 (Atcha et al., 2007). C-clamps are also found in nearly all invertebrate TCFs, including TCF/Pan (Archbold et al., 2012; Cadigan and Waterman, 2012). The C-clamp is required for TCFs to bind to a second DNA motif known as the Helper site, which is critical for Wnt activation of several fly and human W-CRMs (Chang et al., 2008b;

Hoverter et al., 2012). The working model is that C-clamp containing TCFs recognize DNA through a combination of HMG domain-HMG site and C-clamp-Helper site interactions (Cadigan and Waterman, 2012). The C-clamp containing TCF1E and TCF4E isoforms have been implicated in promoting colorectal cancer (Atcha et al., 2007; Hoverter et al., 2012) and regulating Wnt targets in embryonic stem cells (Wallmen et al., 2012).

In addition to the TCF1E and TCF4E isoforms, there are other C-clamp containing proteins in humans. The best characterized is known as SLC2A4 regulator (SLC2A4RG), GLUT4 enhancer factor (GLUT4EF) or Huntington's disease binding protein 1 (HDBP1), which can bind to the promoters of Huntington's disease gene (Tanaka et al., 2004) and *GLUT4* (Knight et al., 2003) and is a candidate locus for increased risk to Crohn's disease and ulcerative colitis (Anderson et al., 2011; Franke et al., 2010). The related protein HDBP2, also known as papilloma virus binding factor (PBF) or zinc finger 395 (ZNF395), represses human papillomavirus virus expression (Boeckle et al., 2002; Hasegawa et al., 2013; Sichtig et al., 2007) and promotes adipogenesis (Hasegawa et al., 2013). The third C-clamp protein, ZNF704 or glucocorticoid induced gene 1 (GIG1) can bind to a *myoD* enhancer (Yamamoto et al., 2007). There is one homolog of these genes in *Drosophila*, known as fly *Glut4EF*, which is required for proper wing position in adults (Yazdani et al., 2008).

In contrast to the HMG domain, where a structure of the domain bound to a high affinity site has been determined (Love et al., 1995), little is known about the structure of the recently discovered C-clamp. Based on sequence alignments, the C-clamp consists of 30 residues (Archbold et al., 2012; Atcha et al., 2007; Cadigan and Waterman, 2012). C-

clamps contain four conserved cysteines, and limited mutagenesis studies indicate that some are required for function (Atcha et al., 2007). The C-clamp domains of HDBP1 and HDBP2 are sufficient for specific binding to Helper site-like sequences (Tanaka et al., 2004), but for TCFs, specific binding has only been observed in conjunction with the adjacent HMG domain (Atcha et al., 2007; Chang et al., 2008b). Further investigation of how these C-clamps recognize DNA is needed to better understand their role in Wnt/β-cat signaling and other processes.

In this report, we explore the physical properties and functional relevance of the C-clamp through a combination of biochemical and genetic assays. We find that the C-clamp of TCF/Pan is sufficient for binding to DNA containing a Helper site and that the C-clamp contains a zinc ion that is essential for this DNA-binding activity. Site-directed mutagenesis demonstrated that all four cysteines and a stretch of basic residues are essential for specific DNA binding, and the ability to activate W-CRMs reporters in fly cell culture. We also found that the C-clamp can bind to the HMG domain and inhibits its ability to bind to HMG site DNA. A *TCF/Pan* gene containing a point mutation that specifically inhibited this inhibitory activity was compromised for activity in fly cell culture, but was able to rescue the embryonic patterning defect of *TCF/Pan* mutants. However, a DNA-binding mutant had no rescue activity in fly embryos. These data provide important biochemical information about the C-clamp of TCFs, and provide the first direct evidence for the importance of this domain in mediating Wnt/β-cat target gene regulation in *Drosophila* embryos.

RESULTS

The C-clamp is a zinc ion-dependent DNA binding domain

Previous studies have shown that the presence of the C-clamp allows the HMG domain of TCF proteins to bind to a bipartite site containing HMG and Helper sites (Atcha et al., 2007; Chang et al., 2008; Hoverter et al., 2012). However, the ability of the C-clamp to bind to the Helper site independently of the HMG domain has not been tested for TCF/Pan (Chang et al., 2008), while in a human TCF1E isoform, a protein fragment downstream of the HMG domain (containing the C-clamp and additional sequences) only displayed non-specific DNA binding activity (Atcha et al., 2007). To explore whether the C-clamp of TCF/Pan has an intrinsic ability to bind to the Helper site, *E. coli* was used to purify a His-tagged 45 amino acid fragment of TCF/Pan, containing the C-clamp. This protein can bind to a DNA probe containing a Helper site (Figure 2.1A, 2.1B). Mutation of two residues at the N-terminus of the C-clamp (K371A and R373E in the full length TCF/Pan) abolished binding (Figure 2.1A, 2.1B) and neither wild-type or mutant protein bound DNA lacking the Helper site (Figure 2.1C). These results demonstrate that the C-clamp of TCF/Pan is sufficient for binding to the Helper site.

One hallmark of the C-clamp is the presence of four conserved cysteines (Cadigan and Waterman, 2012). This is characteristic of a number of zinc finger domains where four cysteine residues coordinate a zinc ion (Krishna et al., 2003; Laity et al., 2001). To explore the possibility that the C-clamp requires a metal ion, a His-tagged recombinant protein containing the HMG domain and C-clamp from TCF/Pan was treated with metal chelators and subsequently tested for binding to a DNA probe containing a HMG site and a Helper site. Treatment with the metal chelator 1,10-orthophenthroline (OPA) greatly

reduced the ability of the HMG-C-clamp protein to bind to this HMG-Helper site probe (Figure 2.2A-2.2D). A similar inhibition was observed with EDTA, another metal chelator (data not shown). OPA-treatment also inhibited the ability of recombinant C-clamp protein to bind to the HMG-Helper site probe (Figure 2.2E, 2.2F), but had no effect on HMG domain DNA binding (Figure 2.2C, 2.2D). These results suggest that the C-clamp contains a metal ion that is critical for its ability to bind the Helper site.

To determine the identity of the specific metal required for C-clamp-dependent DNA binding, OPA treated protein was incubated with several metal ions prior to DNA binding. Of the six divalent metal ions tested (Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Co^{2+} & Mn^{2+}), only zinc restored high affinity binding to the HMG-Helper site probe (Figure 2.2A, 2.2B). Consistent with these data, inductively coupled plasma mass spectrometry (ICP-MS) detected zinc at near stoichiometric levels in the HMG-C-clamp protein preparation , while only background levels were found in the recombinant HMG domain (Table 2.1). These results argue strongly that the C-clamp of TCF/Pan contains one molecule of Zn^{2+}, whose presence is required for binding to the Helper site.

Structure/function analysis of the C-clamp in cultured *Drosophila* cells

To systematically explore which regions of TCF/Pan's C-clamp are required for activation of Wnt/β-cat signaling, eight mutant constructs were generated. These include the four conserved cysteines, which were converted to alanines (Mutants 1-4; Figure 2.3A). In addition, other highly conserved charged or polar amino acids were converted to alanines or oppositely charged residues (e.g. arginine to glutamic acid) to create TCF/Pan Mutants 5-8 (Figure 2.3A). These mutants were then tested for their ability to

rescue Wnt/β-cat signaling in *Drosophila* Kc167 (Kc) cells that had been depleted of endogenous TCF/Pan using RNA interference (RNAi) (Chang et al., 2008).

The readout for Wnt/β-cat signaling used in the TCF/Pan rescue assays was the *nkd-intE* reporter, which was previously shown to contain functional HMG and Helper sites and to require the C-clamp of TCF/Pan for activation by the pathway in Kc cells (Chang et al., 2008). A constitutively active form of Armadillo (the fly β-cat), referred to as Arm*, was used to activate Wnt/β-cat signaling in these cells (Blauwkamp et al., 2008; Chang et al., 2008; Fang et al., 2006; Parker et al., 2008). Almost no detectable activation of the *nkd-intE* reporter was observed in TCF/Pan depleted cells, but expression of wild-type TCF/Pan restored robust activation (Figure 2.3B). Mutant 6 had similar levels of activity as wild-type TCF/Pan, while Mutant 8 was approximately two-fold less active (Figure 2.3B). Strikingly, the other six TCF/Pan mutants had no detectable ability to mediate activation of *nkd-intE* by Arm* (Figure 2.3B).

One caveat with the aforementioned data is that the inactive mutant TCF/Pan proteins may be misfolded or unstable. To address this concern, the wild-type and mutant *TCF/Pan* genes were tagged with the V5 epitope (Chang et al., 2008), but these proteins could not be detected via Western blot, even when transfected at much higher levels than used in the functional assay and when Kc cells were cultured in the presence of the proteasome inhibitor MG132 (data not shown). Instead, untagged versions of the proteins were tested for their ability to activate a synthetic reporter containing six HMG binding sites upstream of the core promoter/luciferase cassette (6xHMG) (Chang et al., 2008). The ability of TCF/Pan to rescue 6xHMG expression served as a proxy to control for expression/activity levels among the various TCF/Pan proteins.

6xHMG was not activated in TCF/Pan depleted Kc cells, but expression of the wild-type protein restored activation (Figure 2.3D). Mutants 1-4 and 6 showed similar activation as the wild-type (Figure 2.3D). Mutants 5, 7 and 8 also activated the synthetic reporter, but at lower levels than wild-type (Figure 2.3D). Increasing amounts of expression construct were transfected and at a ratio of 8:1, Mutants 5 and 8 could activate 6xHMG at similar levels as wild-type, with Mutant 7 reaching about 60% of the control (Figure 2.3E). However, even with higher amounts of transfected DNA, Mutants 5 and 7 were unable to activate the *nkd-IntE* reporter, while Mutant 8 topped out at 60% of wild-type (Figure 2.3C). These data indicate that several residues within the C-clamp are essential for activation of a Helper site-dependent W-CRM in cultured cells but have a minimal effect on the overall stability/folding of TCF/Pan.

The C-clamp possesses two separate functions in DNA recognition

Previous reports and this study have demonstrated that the C-clamp is necessary and sufficient for binding to Helper sites (Atcha et al., 2007; Chang et al., 2008) (Figure 2.1). In addition to this role in DNA binding, there is also published data suggesting that the presence of a functional C-clamp inhibits the ability of the HMG domain to bind to HMG sites (Chang et al., 2008; Elfert et al., 2013; Hecht and Stemmler, 2003; Weise et al., 2010). This HMG domain inhibitory function (hereafter referred to as "inhibitory" function) is poorly understood. To explore the DNA-binding and inhibitory functions of the C-clamp in more detail, recombinant HMG-C-clamp fragments of wild-type TCF/Pan and the eight mutants described in Figure 2.3A were expressed and purified and subjected

to quantitative EMSA analysis using probes containing a HMG and Helper site and a probe containing only a HMG site (Figure 2.4A).

Consistent with our earlier report (Chang et al., 2008), the recombinant HMG-C-clamp protein bound a HMG-Helper site probe with higher affinity than a HMG site probe (data not shown; see Figure 2.4 legend). In agreement with previous findings (Atcha et al., 2007; Chang et al., 2008), we found that substitutions in any of the four cysteine residues (Mutants 1-4) greatly reduced ability to bind the HMG-Helper site probe (Figure 2.4B). Mutants 5 and 7 had a moderate reduction in binding the HMG-Helper site probe, while mutants 6 and 8 had comparable binding to wild-type (Figure 2.4B). When the HMG site probe was tested, several mutants had higher than wild-type binding, indicating a defect in the inhibitory function of the C-clamp, with mutant 5 exhibiting the most dramatic effect (Figure 2.4B).

Mutants 4, 5 and 8 were selected for further study, since Mutant 4 appeared to have a specific defect in DNA binding activity (i.e., reduced binding to the HMG-Helper site probe; normal binding to the HMG site probe), Mutant 8 appeared to have a specific (though partial) defect in the inhibitory activity (i.e., normal binding to the HMG-Helper site probe; elevated binding to the HMG site probe) and Mutant 5 was defective in both activities. Additional EMSA experiments confirmed these defects (Figure 2.4C, 2.4D). Note that Mutant 5 has stronger binding to the HMG-Helper site probe than Mutant 4, presumably due to the conflicting effects of loss of Helper site binding combined with loss of inhibitory activity, which increases binding of the HMG domain to the HMG site in the probe.

To investigate the mechanism of the C-clamp inhibitory function, the ability of the HMG domain to interact with the C-clamp was tested. GST-tagged HMG domain (GST-HMG) and a His-tagged C-clamp fragment (His-C-clamp) were incubated and analyzed in a GST pulldown assay. GST-HMG binding to His-C-clamp was observed, with minimal binding by the GST negative control (Figure 2.5A). In contrast, no binding was observed between GST-HMG and the Mutant 5 His-C-clamp (Figure 2.5A). The HMG-C-clamp interaction was not affected by pre-treatment with micrococcal nuclease, ruling out the possibility that DNA was acting as an adaptor between the two protein domains (Figure 2.5B). Pretreatment of the C-clamp with 1,10-orthophenanthroline (OPA) also had no effect on binding to the HMG domain, suggesting that coordination of a zinc molecule was not required (Figure 2.5B). These data suggest that the C-clamp inhibits the ability of the HMG domain to bind its cognate site by direct protein-protein interaction with the HMG domain.

The C-clamp is required for patterning of the fly embryonic epidermis

To analyze the significance of the DNA binding and inhibitory functions of the C-clamp at the organismal level, transgenic flies with various TCF/Pan cDNAs (wild-type and Mutants 4, 5 & 8) under the control of a Gal4-inducible promoter (Brand and Perrimon, 1993) were created using P-element mediated transgenesis (Rubin and Spradling, 1983). The P[UAS-TCF/Pan] transgenes and a *Daughterless*-Gal4 driver (P[*Da*-Gal4]) were crossed into genetic backgrounds containing two independent *TCF/Pan* mutant alleles (TCF^2 & TCF^3) believed to be nulls (van de Wetering et al., 1997). The *Da*-Gal4 driver is active throughout the embryonic epidermis (Wodarz et al.,

1995). *TCF/Pan* transheterozygotes expressing UAS-TCF/Pan via the *Da*-Gal4 driver were generated via standard genetic crosses (Figure 2.6A).

TCF/Pan mutants have a strong segment polarity phenotype, which can be visualized with darkfield microscopy of late embryonic cuticles (Schweizer et al., 2003; van de Wetering et al., 1997). In control embryos, the anterior portion of each segment contains a trapezoidal array of denticles on the ventral surface, with the posterior portion of the segment displaying naked cuticle lacking denticles (Figure 2.6B). In *TCF/Pan* mutants, the posterior portion of each segment contains denticles (Figure 2.6C). Expression of a wild-type *TCF* transgene provides complete or near-complete rescue of this patterning defect with 100% penetrance (Figure 2.6D; data not shown). Fly embryos expressing either *TCF/Pan* Mutants 4 or 5 had no detectable rescue (Figure 2.6E, 2.6F). Surprisingly, Mutant 8 had a level of rescue comparable with that of wild-type *TCF/Pan* (Figure 2.6G). The P[UAS-TCF/Pan] transgenes used in the rescue assay were prescreened for similar levels of expression (Figure 2.6H).

The C-clamp is required for specification of the wing margin

To extend our in vivo analysis of the C-clamp to another context we established a TCF/Pan rescue assay in the developing fly wing. Wg signaling is required for specification of the wing margin and adjacent sensory bristles, with loss of signaling resulting in notches in the wing blade (Couso et al., 1994; Phillips and Whittle, 1993). When TCF/Pan was depleted in flies containing the wing margin specific Gal4 driver C96 (Krupp et al., 2005) and a UAS-TCFRNAi construct (Dietzl et al., 2007), notches along most of the distal margin were observed with 100% penetrance (Figure 2.7C; Table

2.2). This phenotype was used to assay the ability of UAS transgenes expressing the human TCF family member LEF1 (which contains a HMG domain but no C-clamp), or LEF1 with the C-clamp of TCF/Pan (LEF1-C-clamp; see Figure 2.7A for description of LEF1 proteins), to rescue the loss of activation phenotype in C96::TCF/Pan RNAi wings. A human TCF was used in this assay because it is insensitive to the UAS-TCF/Pan RNA hairpin, which targets the ORF of endogenous *TCF/Pan* mRNA.

While the LEF1 transgene was unable to rescue the wing notch phenotype (Figure 2.7D; Table 2.2), in contrast, the LEF1-C-clamp chimera was able to rescue the phenotype (Figure 2.7E; Table 2.2). More than a dozen independent UAS-LEF1 and UAS-LEF1-C-clamp lines were generated, and the ones at the lower end of the expression spectrum were used in this rescue experiment, because higher expression of either transgenes caused wing notches in an otherwise wild-type background (data not shown). We suspect that too much of either LEF1 protein inhibits Wg signaling by titrating out fly β-catenin in the nucleus. Western blot analysis revealed that the LEF1 and LEF1-C-clamp transgenes used for the rescue were expressed at similar levels (Figure 2.7F). These data supports the model where β-catenin dependent activation requires HMG domain-HMG site and C-clamp-Helper site binding.

DISCUSSION

The C-clamp is a zinc-coordinating motif that is sufficient to bind DNA

Previous work demonstrated that the presence of a C-clamp downstream of the HMG domain allowed TCFs to bind to an extended DNA sequence containing a HMG and Helper site (Atcha et al., 2007; Chang et al., 2008; Weise et al., 2010). However, a

recombinant protein containing the C-clamp had only non-specific DNA binding activity (Atcha et al., 2007), raising some doubt about the nature of the C-clamp-Helper site interaction. Here, we demonstrate that recombinant C-clamp of TCF/Pan specifically binds Helper site DNA (Figure 2.1). We suspect the difference between our data and that presented by Atcha and coworkers is technical. For example, they used a C-terminal fragment of TCF1E containing the C-Clamp plus 95 additional residues (amino acid residues 436 to 561 of human TCF1E). Our positive results are consistent with a report where the C-clamp of GLUT4EF/HDBP1 was sufficient for binding a Helper site-like motif (Tanaka et al., 2004).

Zn-finger motifs contain four residues that coordinate a Zn ion, either all cysteines or a combination of cysteine-histidines (Krishna et al., 2003; Laity et al., 2001). Although C-clamps possess four conserved cysteine residues that are required for DNA binding (Atcha et al., 2007; Chang et al., 2008) (Figure 2.4), it is not readily apparent from the primary sequence that C-clamps are Zn-finger domains. As outlined in Figure 2.8, C2H2 Zn-fingers and Zn2/Cys6-like fingers (found in many transcription factors) and the Zn treble clef fingers that form the DNA-binding domain of nuclear receptors have a similar spacing of the coordinating residues (Grishin, 2001; Krishna et al., 2003; Turner et al., 1998). In these DNA-binding Zn domains, a stretch of amino acids (8-13 residues) separates two pairs of closely spaced cysteines or histidines (2-5 residues; Figure 2.8). In contrast, the conserved spacing of cysteines in C-clamps is distinct (Figure 2.8). Despite this difference, our results demonstrate that the C-clamp of TCF/Pan requires a Zn ion for its DNA binding activity. Recombinant C-clamp contains near stoichiometric quantities of Zn (Table 2.1) and metal chelators inhibit Helper site

binding (Figure 2.2). This inhibition was reversed by the addition of Zn^{2+} to the metal-depleted protein (Figure 2.2A, 2.2B), providing convincing evidence that the C-clamp is a new class of Zn-finger like domain.

There are two regions between the cysteines of the C-clamp that contain polar or charged amino acids (Figure 2.3A). Surprisingly, mutation of three charged/polar residues between the first two cysteines (Mutant 6) had no effect on binding to Helper site DNA in vitro (Figure 2.4B) or the ability of TCF/Pan to activate a Helper site-dependent W-CRM reporter in cultured cells (Figure 2.3B). However, substitution of three basic residues between the third and fourth cysteines (Mutant 7) greatly reduced Helper site binding in vitro (Figure 2.4B). A TCF/Pan cDNA containing this mutation was incapable of activating the Helper-dependent reporter (Figure 2.3B, 2.3C) but was partially able to activate a synthetic HMG site reporter (Figure 2.3D, 2.3E). While it is tempting to speculate that these basic residues may make direct contact with the phosphate backbone of the Helper site, structural analysis will be required to more fully understand the nature of the C-clamp-Helper site interaction.

The DNA binding ability of the C-clamp is functionally important for patterning the *Drosophila* embryo and wing margin specification in adult *Drosophila*

Helper sites have been shown to be important for activation of several W-CRMs in *Drosophila* tissues (Chang et al., 2008) and human cell culture (Hoverter et al., 2012). Given the ability of the C-clamp to bind to Helper site DNA (Atcha et al., 2007; Chang et al., 2008b; Hoverter et al., 2012; Weise et al., 2010) (Figure 2.1), the current model posits that a combination of HMG domain-HMG site and C-clamp-Helper site interactions are

required for invertebrate TCFs and mammalian TCF1E and TCF4E isoforms to locate their W-CRM targets (Cadigan and Waterman, 2012). This model is supported by RNAi rescue experiments in fly cell culture demonstrating a requirement for the C-clamp in activating W-CRM reporters (Chang et al., 2008) (Figure 2.3B, 2.3C). In addition, the ability of a dominant negative version of human TCF1E to inhibit growth of a colon cancer cell line requires the C-clamp, suggesting that C-clamp-Helper site interactions are important for Wnt/β-catenin signaling-dependent oncogenesis (Atcha et al., 2007; Hoverter et al., 2012; Weise et al., 2010). Our structure-function analysis of the C-clamp revealed the every C-clamp mutant that had reduced ability to bind to the HMG-Helper site probe (Mutants 1-5 & 7; Figure 2.4B) also had no ability to rescue *nkd IntE* reporter activation in TCF/Pan depleted Kc cells (Figure 2.3B, 2.3C). Our results provide strong evidence that the DNA binding activity of the C-clamp is essential for its ability to activate Wnt target genes.

Previously, a *TCF/Pan* allele containing a A374V mutation (the fifth amino acid in the C-clamp) had a weak defect in Wg/Wnt signaling in the *Drosophila* embryo (van de Wetering et al., 1997). To more clearly establish the biological relevance of the C-clamp in TCF/Pan function at the organismal level, we established a rescue assay in fly embryos. Null *TCF/Pan* mutants have a strong segment polarity defect (Brunner et al., 1997; van de Wetering et al., 1997) that was rescued by heterologous expression of a TCF/Pan cDNA (Figure 2.6C). Mutation of a single cysteine residue (Mutant 4) abolished the ability of the transgene to complement the *TCF/Pan* mutant phenotype (Figure 2.6F). Given our findings that Mutant 4 is defective in binding to the Helper site in vitro (Figure 2.4), its lack of rescue indicate that the DNA-binding activity of the C-clamp is

absolutely required for TCF/Pan's function in patterning the embryonic epidermis of the fly.

The requirement for the C-clamp in mediating activation of Wnt readouts in vivo was further corroborated by the TCF/Pan rescue assay in the developing wing margins. Depletion of TCF/Pan using RNAi resulted in a notched wing phenotype, which was rescued by the transgene of a LEF1-C-clamp chimera, but not by a LEF1 transgene, suggesting a requirement for C-clamp in wing margin specification.

C-clamp inhibition of HMG domain DNA binding

In our previous report characterizing TCF/Pan DNA binding, we noted that mutating the C-clamp domain resulted in increased binding to a HMG site probe (Chang et al., 2008). Similar data has also been found in studies with different TCF4 isoforms, i.e., isoforms lacking the C-clamp had elevated binding to HMG site probes (Elfert et al., 2013; Hecht and Stemmler, 2003; Weise et al., 2010). We have confirmed this "HMG inhibitory" effect using quantitative EMSA, finding that mutations in the C-clamp resulted in up to a five fold increase in binding to a HMG site probe (Figure 2.4B, 2.4D). Our finding that the HMG and C-clamp domains directly interact in vitro (Figure 2.5) provides a mechanism for the inhibitory effect: in the absence of a Helper site, the C-clamp binds to the HMG domain, interfering with its ability to bind to DNA.

The inhibitory effect is potentially interesting because it could provide an additional mechanism for increasing TCF/Pan DNA-binding specificity. Due to the degeneracy in what constitutes a HMG binding site (Badis et al., 2009), the fly genome contains a large number of potential sites that TCF/Pan could bind to via its HMG domain, which could

prevent it from locating bona fide W-CRMs (Archbold et al., 2012). The C-clamp likely helps to overcome this problem by promoting TCF/Pan binding to HMG-Helper site pairs, while the C-clamp-HMG domain interactions could also prevent binding of these domains to unpaired HMG and Helper sites (Figure 2.9).

Given that the C-clamp inhibits HMG domain-HMG site interactions, it is also possible that the HMG domain interferes with C-clamp binding to Helper site DNA (Figure 2.9). This interaction could explain why Helper site DNA is bound very poorly by recombinant HMG-C-clamp proteins (Chang et al., 2008), while binding of the Helper site by C-clamp alone is readily detectable (Figure 2.1). It should be noted that while the C-clamp is sufficient to bind to Helper sites, synthetic reporters containing up to 12 copies of a consensus Helper site are not activated by Wnt/β-cat signaling (Chang et al., 2008). This suggests that the C-clamp-Helper site interaction is not as strong as the HMG domain-HMG site binding, since high density HMG site reporters are activated by the pathway (Chang et al., 2008; Barolo, 2006) (Figure 2.3D, 2.3E).

Our mutagenesis analysis indicated that the DNA-binding and inhibitory activities of the C-clamp are separable (Figure 2.4). This is further supported by our findings that pretreatment of the C-clamp with the metal chelator 1,10-orthophenanthroline (OPA) abolishes its DNA binding activity (Figure 2.2E, 2.2F) while having no effect on binding to the HMG domain (Figure 2.5B). The ability to genetically separate the two activities allows us to test their functional relevance, which demonstrated that the C-clamp's DNA binding activity was essential for Wnt gene regulation (Figure 2.6F). Mutant 8 (Figure 2.3A) was the best candidate for a specific inhibitory mutant, since it displayed normal DNA binding activity and was compromised for inhibitory activity (Figure 2.4B – 2.4D).

Interestingly, Mutant 8 had a reduced ability to rescue W-CRM reporter gene activation in cultured cells (Figure 2.3B), even when expressed at high levels (Figure 2.3C). However, this mutant was still functional in rescuing the *TCF/Pan* mutant cuticle phenotype in fly embryos (Figure 2.6G).

The finding that a Mutant 8 TCF/Pan is still functional in the fly embryo is disappointing, but there are some important caveats to consider. Although wild-type and mutant TCF/Pan proteins are expressed at similar levels (Figure 2.6H), this might still be higher than the endogenous concentration, allowing the mutant TCF/Pan to saturate non-functional HMG sites and still leave enough mutant TCF to bind functional W-CRMs. To address this, the rescue experiments were repeated with transgenic *TCF/Pan* lines which were expressed at significantly lower levels than the ones used in Figure 2.6, but full rescue was still observed (data not shown). Another consideration is that the defect in the inhibitory activity of Mutant 8 is only partial, e.g., compared to Mutant 5 (Figure 2.4D). Additional mutations were generated, but they all resulted in partial loss of Helper site binding activity, rendering them useless for testing the specific role of the inhibitory function. It is likely that additional structural data on the C-clamp-HMG domain complex will be needed to design suitable mutations to address the biological role of the HMG inhibitory function of the C-clamp.

In sum, our findings provide valuable new information about the biochemical properties of the C-clamp, and confirm its biological importance in Wnt/β-cat signaling. Given that almost all invertebrate TCFs possess a conserved C-clamp, our data will be relevant to important family members such as POP-1 in *C. elegans* (Jackson and Eisenmann, 2012) and TCF in Hydra (Duffy et al., 2010). Likewise, the C-clamps in

TCF1E and TCF4E isoforms probably have similar properties as we have uncovered in TCF/Pan, which likely contribute to the ability of these isoforms to activate specific Wnt targets (Atcha et al., 2007; Hecht and Stemmler, 2003; Hoverter et al., 2012; Wallmen et al., 2012) and promote oncogenesis (Atcha et al., 2007; Hoverter et al., 2012). Whether C-clamps from other proteins, e.g., GLUT4EF, can bind to the HMG domain of TCFs is another interesting question that requires further investigation.

EXPERIMENTAL PROCEDURE

Plasmids

The protein expression vector for *Drosophila* cell culture, pAc5.1/TCF/Pan-V5/His (pAc-TCF/Pan), has been described previously (Chang et al., 2008). The Quikchange II mutagenesis kit (Stratagene) was used to generate the various C-clamp mutants in the pAc-TCF/Pan expression vector. pAc-Arm* and the luciferase reporters, pGL3-nkd-intE and pGL3-6xHMG, have been described previously (Chang et al., 2008; Parker et al., 2008).

The protein expression vectors for EMSA were generated by cloning the region encoding the HMG domain and the C-clamp from the pAc-TCF/Pan constructs into the XmaI and SacI restriction sites of the pET52b(+) vector (pET) (Merck Millipore). pET-HMG and pET-C-clamp were generated by cloning the respective coding regions (residues 271 to 369 for the HMG domain; residues 363 to 408 for the C-clamp) into the same sites. The pET vector encodes a C terminus 10xHis tag, which was used for protein purification.

For transgenic flies, the *TCF/Pan* ORF was cloned from the pAc-TCF/Pan constructs into the KpnI and XbaI restriction sites of the pUAST vector. This vector contains a C terminal V5 tag, which was used to detect the protein for Western blots. pUAS LEF1-V5 and pUAS LEF1-C-clamp-V5 were generated by PCR based cloning into pUAST vector. pUAS LEF1-V5 was generated by subcloning human LEF1 fragment from a LEF1 expression plasmid (kindly provided by Dr. Marian L. Waterman) into pActin5.1 to introduce a V5 tag. pUAS-Lef1C-clamp V5 plasmid was generated by insertion of the C-clamp from fly TCF/Pan into pUAS Lef1-V5.

Drosophila cell culture, RNAi knockdown and transient transfection

Kc167 cells were cultured in *Drosophila* Schneider's media with 10% FBS. For RNAi knockdown, cells were treated with 10µg/1x10^6 cells of double-stranded RNA (dsRNA) targeting the 3'UTR of wild-type TCF/Pan. After 4 days, cells were diluted to 1x10^6 cells/mL and transfected with a plasmid mix containing pGL3-nkd-intE or pGL3-6xHMG, pAc-TCF/Pan (wild-type or mutant), S-188-cc-RLuc, a Renilla luciferase reporter (Hu et al., 2003) and pAc-Arm*. The TCF/Pan constructs contain a 3'UTR different from that in endogenous TCF/Pan, preventing them from being targeted by the dsRNA. After 3 days, cells were harvested for the Dual Luciferase reporter assay (Promega).

EMSA

Gel shifts were carried out using the Lightshift Chemiluminescent EMSA kit (Pierce). IRDYE-700 tagged DNA probes and His-tagged recombinant TCF/Pan fragments, purified from *E.coli,* were used. Band signals in the gel were detected and

quantified using the LI-COR Odyssey Infrared Imaging System. After background subtraction, the percentage bound was calculated as the signal in the shifted band/total signal in that lane. Signals for all mutants were then normalized to the wild-type protein.

Metal chelator treatment and ICP-MS

Purified proteins in the EMSA binding buffer were incubated with 3.6mM of 1, 10-orthophenanthroline (OPA) for 20 minutes at room temperature. The DNA probe was then added and the mixture was incubated for 20 minutes on ice, before being loaded into the gel. In the negative control, nuclease free water was used instead of OPA. For the rescue, OPA treated proteins were incubated with 100μM of each of the salts for 10 minutes at room temperature before DNA was added.

Samples of His-HMG-C-clamp and His-HMG proteins were sent for testing metal content using inductively coupled plasma mass spectrometry (ICP-MS), which was carried out by Dr. Ted Huston (Dept. of Earth and Environmental Sciences, University of Michigan).

Transgenic flies

Transgenic fly lines were generated by BestGene Inc. using P-element mediated transformation. Crosses for rescue of the TCF/Pan embryonic phenotype were set up as indicated in Figure 2.6A. For the cuticle analysis, flies were allowed to lay eggs on grape juice plates for 6-8 hours at 25°C. Flies were then removed and plates were incubated for an additional 24-36 hours at 25°C. During this time a wet yeast paste was applied to the centre of each plate to attract hatching larvae and periodically removed leaving behind

unhatched embryos. Unhatched embryos were collected and their cuticles were prepared as described previously (Bhanot et al., 1999).

For the Western blots, flies were allowed to lay eggs for 4 hours and the embryos were incubated for an additional 6 hours, all at 25°C. Embryos were collected and dechorionated. They were then treated with hot SDS buffer and manually ground for 5 minutes. Samples were loaded into a gel. TCF-V5 was detected using mouse anti-V5 antibody (1:5000, Invitrogen). Tubulin was used as a loading control. The ECL kit (Pierce) was used to visualize the blots.

TCF/Pan mutant alleles used were TCF^2 and TCF^3. TCF^2 contains a base pair loss of ATT to AT leading to a frameshift at I106 and TCF^3 contains a CAA to TAA mutation resulting in a stop codon at Q319 in the HMG domain (van de Wetering et al., 1997).

The *Lef1* and *Lef1-C-clamp* crosses were performed at 25°C. *C96::Gal4* was kindly provided by Dr. Rolf Boldmer (Krupp et al., 2005). The *TCF/Pan RNAi* line (#25940) was obtained from Vienna *Drosophila* RNAi Center.

Wing mounting

Adult flies were stored in 70% ethanol and soaked in 100% ethanol for two hours before dissection in 100% ethanol. Dissected wings were transferred into Xylene (Fisher) and mounted on a slide with Permount (Fisher). Wing notch and bristle images were obtained using the Nikon Eclipse 800 microscope. All images were processed using Adobe Photoshop 8.0.

GST pulldown assay

2 µg of His-C-clamp and 2 µg of either GST or GST-HMG were incubated for 1 hour at 4°C with rotation in 20mM Tris-HCl (pH 7.62), 150mM NaCl and 1% Triton X-100 binding buffer. The mixture was then incubated with Glutathione Sepharose beads (GE Healthcare) for an additional 2 hours at 4°C with rotation. After 4-5 washes with binding buffer, the sample was treated with hot SDS buffer and loaded into a gel. His-C-clamp was detected using mouse anti-His antibody (1:3000, GE Healthcare). Micrococcal nuclease treatment was carried out as described previously (Nguyen et al., 2006), using 6.6 units of micrococcal nuclease (Sigma) for a 200 µL reaction volume. In addition, the binding buffer used for this treatment contained 5mM $CaCl_2$. OPA treatment was carried out by incubating His-C-clamp in 0.363 µM OPA for 20 minutes at room temperature. GST or GST-HMG was then added and the mixture was incubated for 1 hour at 4°C with rotation prior to GST pulldown. . In the negative controls for both treatments, nuclease free water was used instead of micrococcal nuclease or OPA.

ACKNOWLEDGEMENTS

Special thanks to T. Huston for performing the ICP-MS analysis, and to L. Dostal for advice on metalloproteins. Thanks also to M. V. Chang and C. Bhambhani for preliminary characterization of the TCF/Pan rescue assays. Thanks to various Cadigan lab members for helpful discussions and to H. C. Archbold and C.U. Zhang for careful reading of the manuscript. This work was supported by NSF grant 0950348 and NIH grant GM082994 to KMC.

THIS CHAPTER WAS PUBLISHED AS:

Ravindranath, A.J., and Cadigan, K.M. (2014). Structure-Function Analysis of the C-clamp of TCF/Pangolin in Wnt/ss-catenin Signaling. PLoS One *9*, e86180.

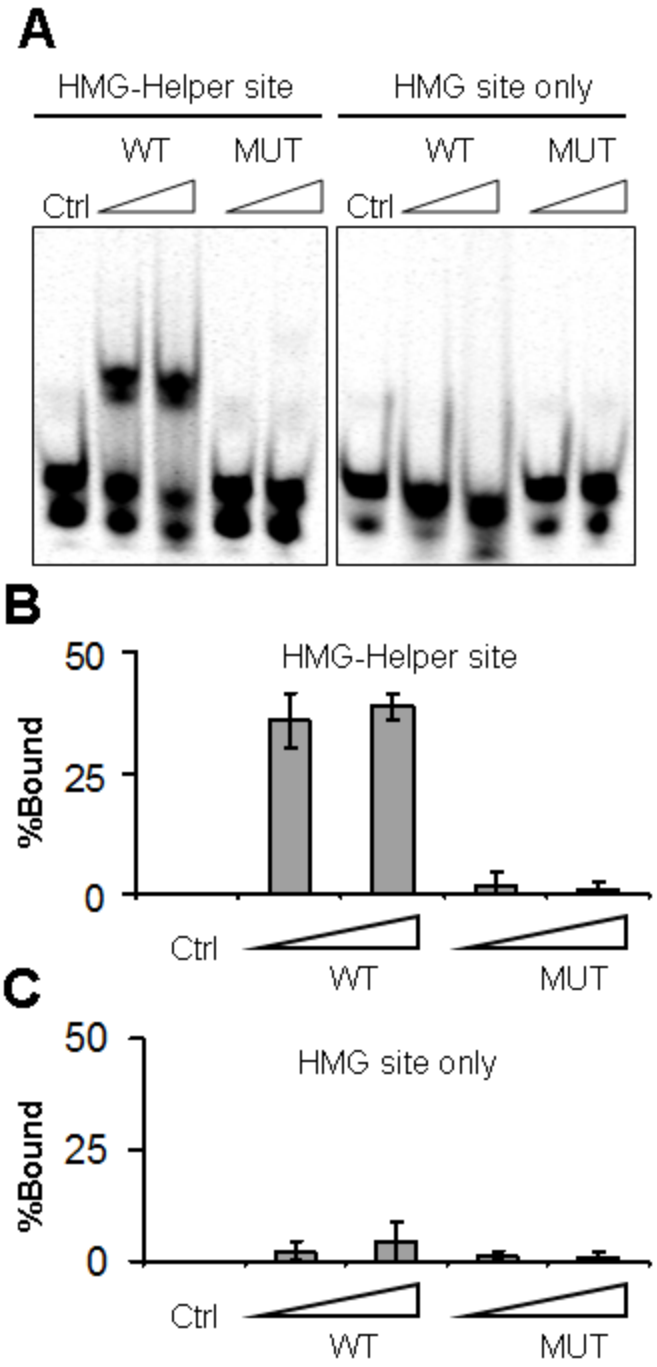


Figure 2.1. The C-clamp domain is sufficient for binding to the Helper site. (A) EMSA showing that a His-tagged C-clamp domain can bind to a DNA probe containing a Helper site, while a C-clamp protein containing two mutations in the domain (K2A, R4E; same as mutant 5 in Figure 2.3A) has greatly reduced binding. Neither protein bound a probe lacking a Helper site. For each binding reaction 50 and 100 pmoles of protein and 20 fmoles of oligonucleotide were used. (B,C) Quantification of the EMSA data using the Licor system. The bar graph results are the means of at least three separate binding reactions $\pm$ SD. See Experimental Procedure for details.

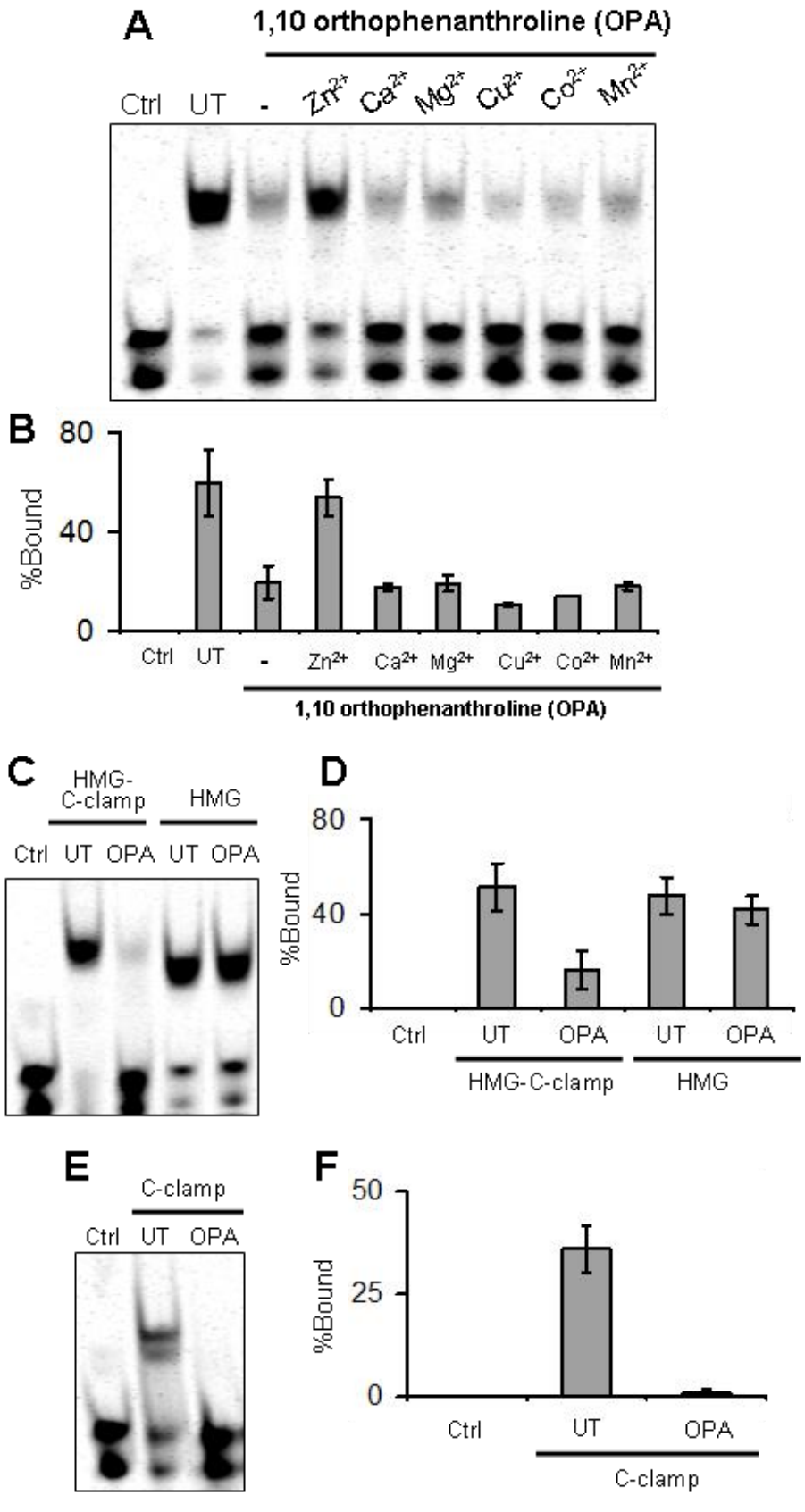

A
1,10 orthophenanthroline (OPA)
Ctrl
UT
-
Zn2+
Ca2+
Mg2+
Cu2+
Co2+
Mn2+
B
80
40
0
%Bound
Ctrl
UT
-
Zn2+
Ca2+
Mg2+
Cu2+
Co2+
Mn2+
1,10 orthophenanthroline (OPA)
C
HMG-C-clamp
HMG
Ctrl
UT
OPA
UT
OPA
D
80
40
0
%Bound
Ctrl
UT
OPA
UT
OPA
HMG-C-clamp
HMG
E
C-clamp
Ctrl
UT
OPA
F
50
25
0
%Bound
Ctrl
UT
OPA
C-clamp

Figure 2.2. The C-clamp domain requires zinc for binding to the Helper site. (A) Pretreatment of a recombinant fragment of TCF/Pan containing the HMG and C-clamp domains with the metal chelator 1,10-orthophenanthroline (OPA) inhibits its ability to bind to an oligonucleotide containing a HMG and Helper site. Binding was restored by incubation of the OPA-treated protein with zinc but not other divalent metals. Ctrl indicates a probe only lane and UT refers to protein that was untreated by OPA. For each binding reaction 9 pmoles of protein and 20 fmoles of oligonucleotide were used. (B) Licor quantification of the EMSA data. (C, D) EMSA gel and Licor quantification demonstrating that a protein fragment containing only the HMG domain was insensitive to OPA treatment. 9 (HMG-C-clamp) and 12 (HMG only) pmoles of protein and 20 fmoles of oligonucleotide were used. (E, F) EMSA and Licor quantification demonstrating that recombinant C-clamp protein is sensitive to OPA treatment. 50 pmoles of C-clamp protein and 20 fmoles of oligonucleotide were used. All experiments were carried out at least three times and the bar graph results are the means of at least triplicates $\pm$ SD.

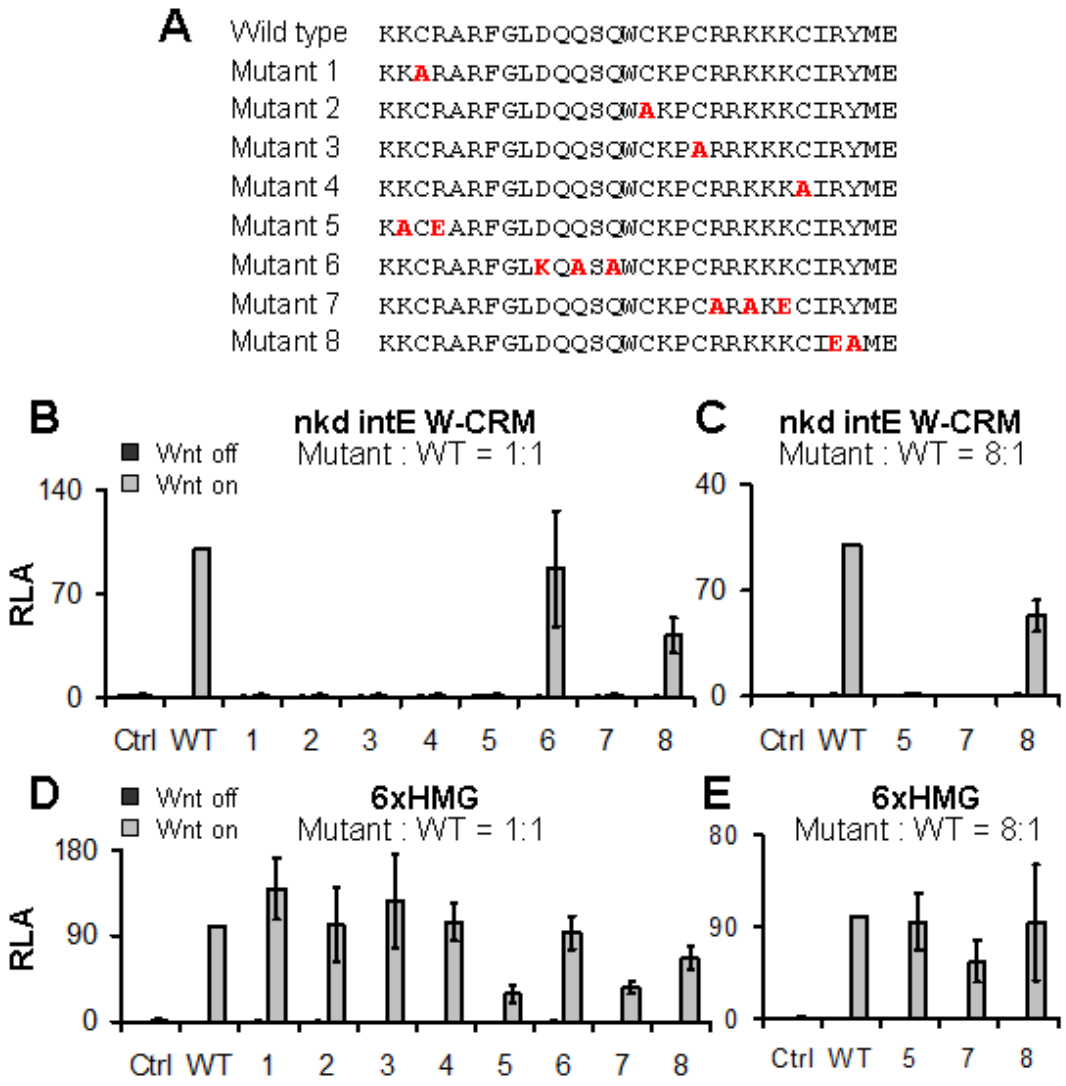


Figure 2.3. The cysteine and basic residues of the C-clamp are required for activation of a W-CRM reporter in cell culture. (A) Amino acid sequences of the C-clamp in wild-type (WT) TCF/Pan and the eight mutants. The amino acids that have been mutated are in red. **(**B-E) TCF/Pan RNAi rescue assays carried out in Kc cells. Endogenous TCF/Pan was depleted using dsRNA that targets the TCF 3' UTR, followed by transient transfection of either WT or C-clamp mutant expression constructs containing a heterologous 3'UTR that cannot be targeted by the dsRNA. The Wnt/β-cat pathway was induced (Wnt on) using Arm* (see text for further description). Ctrl and "Wnt off" refer to controls where there was transfection of an empty expression vector. The *nkd-IntE* W-CRM reporter was used as an example of a Helper site-dependent W-CRM (B, C), while the synthetic 6xHMG reporter was used as a Helper site-independent Wnt readout (D, E). For some experiments, the mutant TCF/Pan constructs were transfected at eight times the level of wild-type, to ensure that sufficient levels of mutant protein were produced (C, E). Bars are the mean of triplicate transfection ± SD. Experiments were repeated at least three times with similar results. See Experimental Procedure for additional details of the cell culture conditions.

A

HMG site Helper site

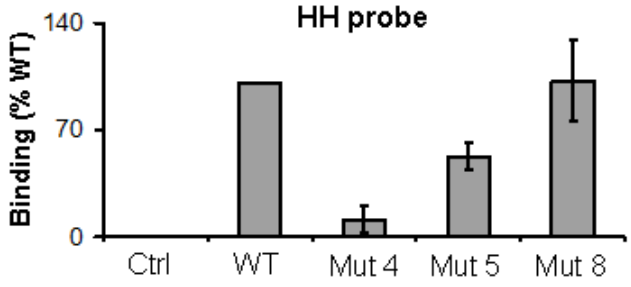
HH probe GGAAGATCAAAGGGGGTAGCCGCCAGT

HS probe GGAAGATCAAAGGGGGTATAATAACGT

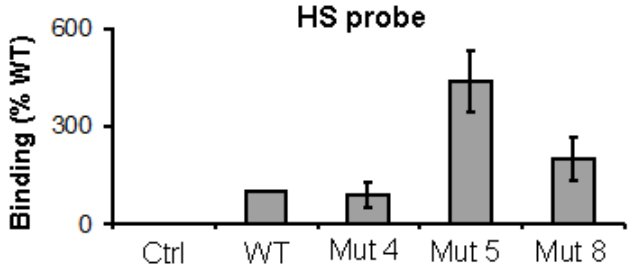
B

Protein	HH probe	HS probe
Ctrl	0	0
WT	100	100
Mutant 1	16	185
Mutant 2	7	33
Mutant 3	14	37
Mutant 4	**11**	**91**
Mutant 5	**53**	**439**
Mutant 6	101	85
Mutant 7	35	279
Mutant 8	**102**	**202**

C



D



E

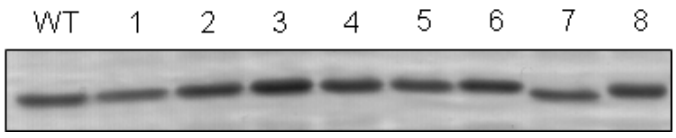


Figure 2.4. Characterization of the DNA-binding and inhibitory functions of the C-clamp. (A) Sequence of the HMG-Helper site probe and the HMG site oligonucleotide probes used to characterize the ability of C-clamp mutants to bind DNA. (B) Protein fragments containing the HMG domain and wild type (WT) or mutant C-clamps were tested for their ability to bind the HMG-Helper site and the HMG site probes using the Licor EMSA assay described in Figure 1. Ctrl indicates probe only lane. For both probes, WT bound signal was normalized to 100. All mutants were tested at least twice and the averages are reported. Mutants 4, 5 and 8 are in bold, denoting their use in follow up experiments. (C, D) EMSA experiments characterizing the defects in Mutants 4, 5 and 8 in binding to the HMG-Helper site (C) and HMG site (D) probes. For each binding reaction, 20 fmol of DNA probe and 9 pmol of protein was used. At these conditions, wild-type protein bound 7-12 times as much HMG-Helper site probe as HMG site probe (data not shown). Data represents means of triplicates $\pm$ SD. These experiments were repeated three times with similar results. (E) Commassie stained gel of purified WT and C-clamp mutant proteins, demonstrating that each preparation used contained similar amounts of TCF/Pan.

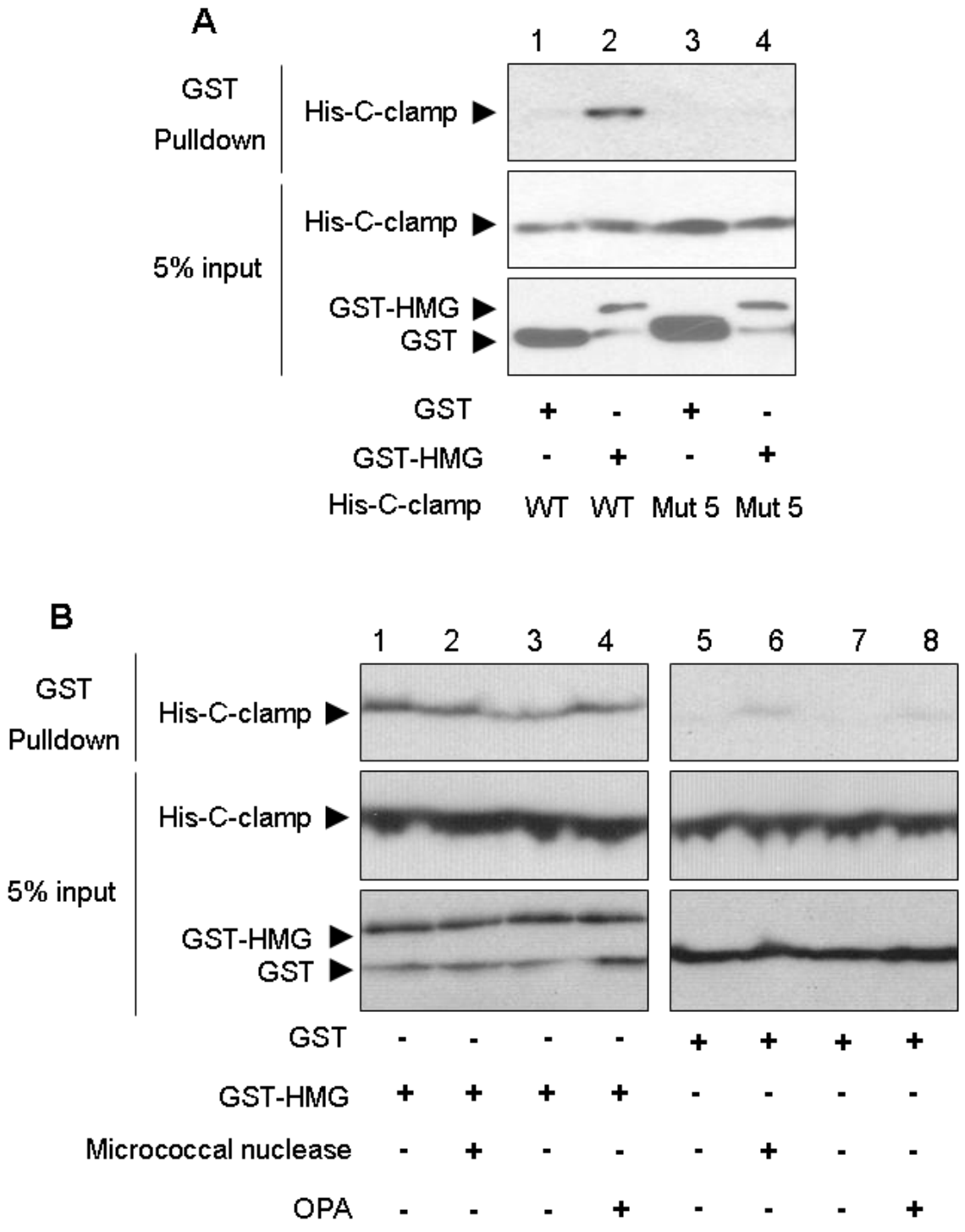


Figure 2.5. Direct binding of the C-clamp domain to the HMG domain. Western blots using anti-His tag and anti-GST tag antibodies. (A) Pulldown using GST-HMG or the GST control incubated with wild-type (WT) or Mutant 5 His-C-clamp (see Experimental Procedure for details of the binding reaction). The upper blot shows an interaction between GST-HMG and the wild-type C-clamp. The middle and lower blots are 5% input of the total reaction mixture. (B) Pulldowns using GST-HMG (lanes 1-4) or

GST control (lanes 5-8) and WT His-C-clamp. The proteins were pretreated with micrococcal nuclease (lanes 2 & 6) or 1,10-orthophenanthroline (OPA) (lanes 4 & 8). The negative controls (lanes 1 & 5 for micrococcal nuclease, and lanes 3 & 7 for OPA treatments) were subjected to the same treatment conditions with nuclease free water being used instead of micrococcal nuclease or OPA. The upper blots show the amount of His-C-clamp pulled down and the middle and lower blots are 5% input of the total reaction. All experiments were repeated three times with similar results.

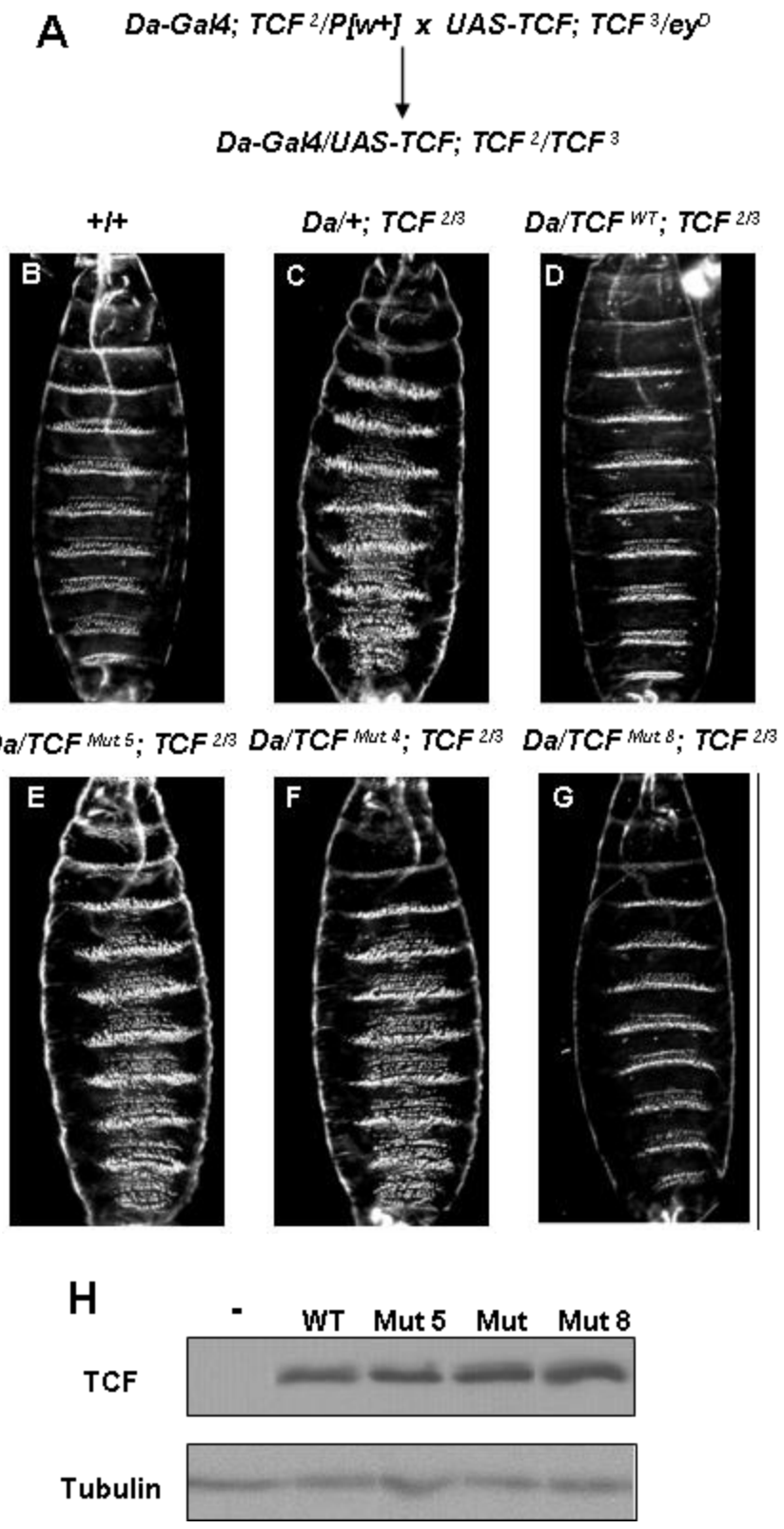

A
Da-Gal4; TCF 2/P[w+] x UAS-TCF; TCF 3/eyD
Da-Gal4/UAS-TCF; TCF 2/TCF 3
+/+
Da/+; TCF 2/3
Da/TCF WT; TCF 2/3
B
C
D
Da/TCF Mut 5; TCF 2/3
Da/TCF Mut 4; TCF 2/3
Da/TCF Mut 8; TCF 2/3
E
F
G
H
-
WT
Mut 5
Mut
Mut 8
TCF
Tubulin

Figure 2.6. The C-clamp is required for patterning of the *Drosophila* embryonic epidermis. (A) Crossing scheme used to generate embryos containing a P[*Da*-Gal4] driver and P[UAS-TCF/Pan] transgene in a TCF^2/TCF^3 transheterozygous mutant background. UAS-TCF/Pan (UAS-TCF) encodes for either wild type or a mutant *TCF/Pan*. (B-C) Darkfield micrographs of the ventral cuticle control (B) or P[*Da*-Gal4]/+; TCF^2/TCF^3 embryos showing the normal and *TCF/Pan* mutant phenotypes, respectively. (D-G) Cuticles of TCF^2/TCF^3 mutants expressing wild-type (D), mutant 5 (E), mutant 4 (F) or mutant 8 (G) *TCF/Pan* cDNAs. (H) Western blots showing comparable levels of TCF/Pan (upper blot) expression for WT and various C-clamp mutants in P[*Da*-Gal4]; P[UAS-TCF/Pan] embryos with Tubulin used as the loading control (lower blot).

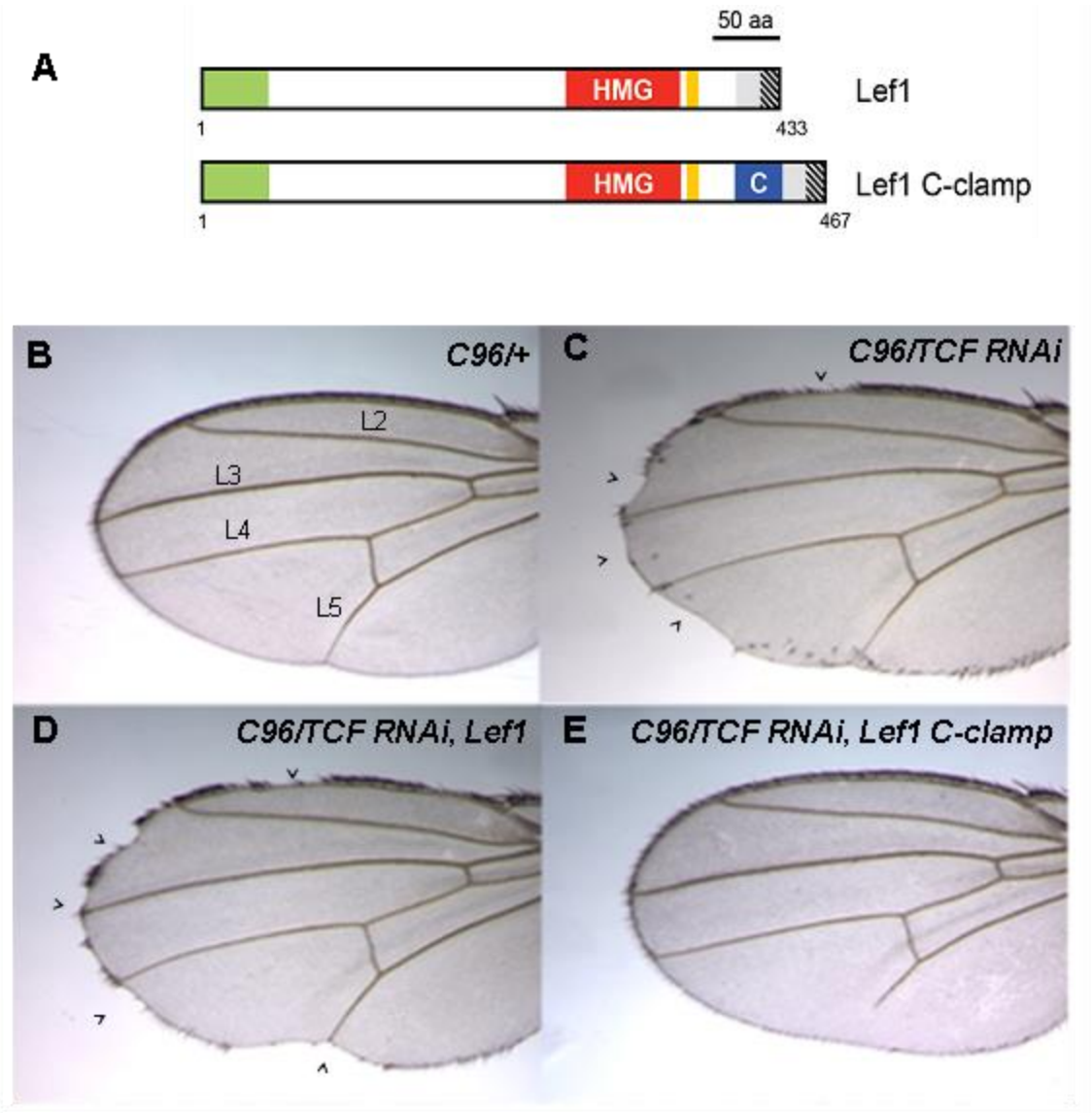

A
50 aa
HMG
Lef1
1
433
HMG
C
Lef1 C-clamp
1
467
B
C96/+
L2
L3
L4
L5
C
C96/TCF RNAi
D
C96/TCF RNAi, Lef1
E
C96/TCF RNAi, Lef1 C-clamp
F
C96 Gal4 X
+
LEF1 B
LEF1 A
LEF1 C-clamp A
LEF1 C-clamp B
Anti-V5
**
*
Anti-tubulin

Figure 2.7. The C-clamp is required for specification of the wing margin in *Drosophila*. (A) Cartoon of human LEF1 and LEF1-C-clamp fusion showing the β-catenin binding domain (green), the HMG domain (red), the basic tail (orange), the C-clamp (blue), a linker (gray) and the V5 epitope (hatched box). (B-E). Images of adult wings containing the wing driver C96-Gal4 crossed to wildtype (WT) (B), UAS-TCF/Pan RNAi (C) or UAS-TCF/Pan RNAi plus UAS-LEF1 (D) or UAS-LEF1 plus the C-clamp of TCF/Pan (E). Knockdown of TCF/Pan leads to notches (arrowheads) along the periphery of the wing (where C96-Gal4 is active; C). Expression of the human LEF1 transgene does not rescue the notches (D). Expression of a LEF1-C-clamp chimera rescues the wing margin defects (E). Details about the penetrance of these phenotypes are listed in Table 2.2. (F) Expression of human Lef1 and Lef1-C-clamp chimera in wing imaginal discs. Immunoblot showing the expression levels in dissected wing discs from two lines (A and B) of V5 tagged Lef1 (*) or the Lef1-C-clamp chimera (**).

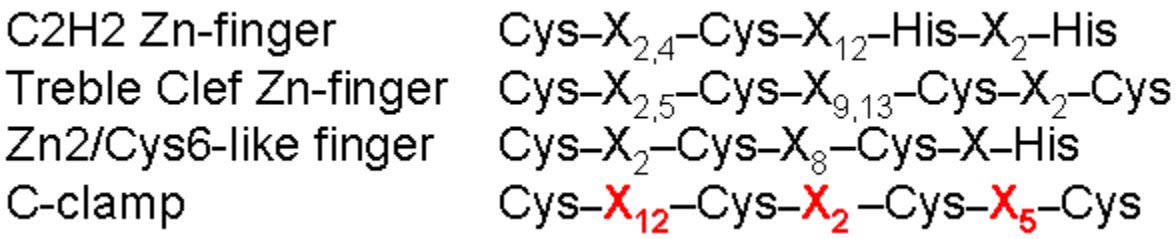


Figure 2.8. The spacing of cysteine residues in the C-clamp is distinct from other Zn-finger motifs. In a typical C2H2 Zn-finger, the two cysteine and two histidine pairs that coordinate the Zn ion are separated by a stretch of 12 amino acids (Krishna et al., 2003). In the treble clef Zn-fingers found in the estrogen and glucocorticoid receptors, two pairs of cysteines are separated by 9 or 13 residues (Grishin, 2001; Krishna et al., 2003). A similar organization is found in the Zn2/Cys6-like finger of the yeast copper-regulated transcription factor (Turner et al., 1998). In contrast, the second and third cysteines of C-clamps are closely paired, with the longest (12 residue) spacing found between the first two cysteines.

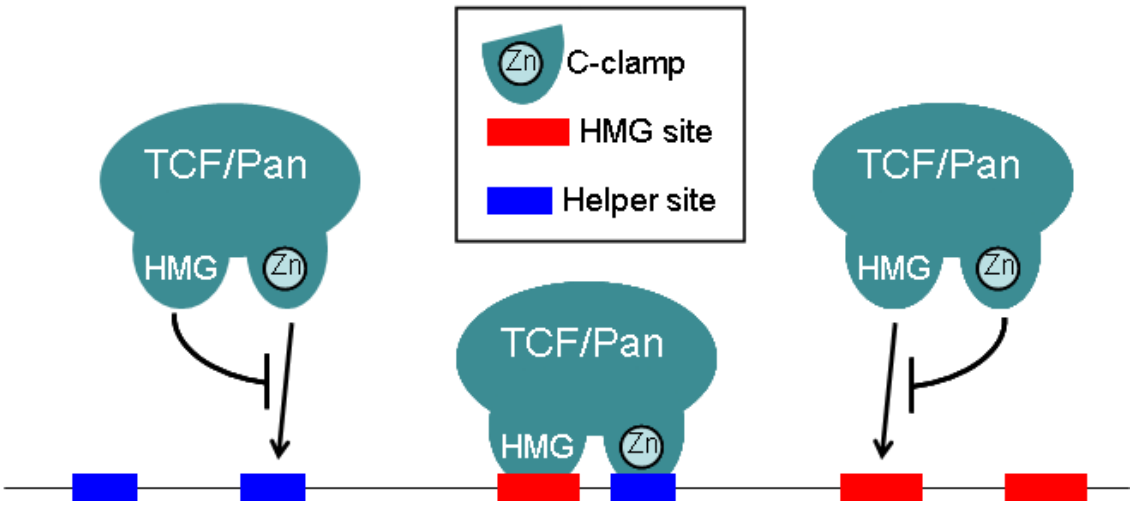


Figure 2.9. Model depicting a dual role for the C-clamp in enhancing the DNA-binding specificity of TCF/Pan. The presence of the HMG and C-clamp domains allows TCF/Pan to bind to HMG-Helper site pairs (middle). In addition, the C-clamp may inhibit TCF/Pan from binding to unpaired HMG sites (right). Conversely, the HMG domain may inhibit the C-clamp from binding unpaired Helper sites (left).

Table 2.1. Recombinant HMG-C-clamp fragment contains near stoichiometric amounts of Zinc. Recombinant HMG-C-clamp and HMG domain proteins were purified from *E. coli* and subjected to ICP-MS. See Experimental Procedure for details of ICP-MS analysis.

Protein	Protein concentration (μM)	Zn^{2+} ion concentration (μM)
HMG-C-clamp	42.1	32.0 $\pm$ 1.6
HMG	35.1	< 0.4

Table 2.2. The C-clamp is required for specification of the wing margin in a TCF/Pan rescue assay. Two independent lines of UAS-Lef1 and UAS-Lef1-C-clamp with similar expression levels (see Figure 2.7.F) were assayed. Expression of either transgene with the *C96-Gal4* driver had little or no effect on wing development in an otherwise wild-type background. Percentages tabulated for the wing phenotypes seen upon knock down of TCF. Depletion of TCF/Pan with a UAS-driven RNAi hairpin causes mostly large notches. Expression of human Lef1 (Lef1) has little effect on the size and frequency of the wing notches. In contrast, expression of Lef1 with the C-clamp of TCF/Pan (Lef1-C-clamp) rescues the wing notch phenotype.

Differences in Lef1 versus Lef1 C-clamp mediated rescue of wing margin defect caused by depletion of TCF			
***C96-Gal4* crossed to: (n)**	**Notches (%)**		
	None	**Small**	**Large**
+ (46)	100		
Lef1 A (38)	100		
Lef1 B (43)	95.7	4.6	
Lef1 C-clamp A (38)	100		
Lef-1 C-clamp B (39)	100		
TCF/Pan-RNAi (46)		2.2	97.8
Lef1 A; TCF/Pan-RNAi (60)		8.3	91.7
Lef1 B; TCF/Pan-RNAi (52)		7.7	92.3
Lef1 C-clamp A; TCF/Pan-RNAi (46)	71.7	26.0	2.3
Lef-1 C-clamp B; TCF/Pan-RNAi (38)	100		

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CHAPTER III

CONSERVATION OF MECHANISMS FOR TARGET GENE SELECTION BY TCF ACROSS DROSOPHILA AND C.ELEGANS

ABSTRACT

T-cell factors (TCFs) are transcription factors in the Wnt/β-catenin (Wnt/β-cat) pathway, which control numerous cell fate specification events in metazoans. TCF-mediated transcriptional regulation is dependent on accurate target gene selection by TCF. TCFs utilize several different mechanisms to achieve specific target recognition. TCF/Pangolin (TCF/Pan) (the fly TCF) recognizes DNA through two distinct domains, a High Mobility Group (HMG) domain and a C-clamp, which bind DNA motifs known as HMG and Helper sites, respectively. POP-1 (the *C. elegans* TCF) is capable of forming oligomers, which could provide a potential mechanism to improve TCF's flexibility in binding a greater variety of DNA sites. However, the importance of C-clamp-Helper site interactions and TCF oligomerization in POP-1 and TCF/Pan regulation, respectively, remains to be established. Here, we demonstrate that POP-1 also activates target genes through HMG and Helper site interactions, and requires the C-clamp for enhanced in vitro DNA binding. Helper sites greatly improved the ability of a synthetic enhancer to detect Wnt/β-cat signaling in several tissues. Specifically, it revealed a novel POP-1 and BAR-1 (a *C. elegans* β-catenin) dependent pattern in the int9 cells of the larval intestine.

Conversely, the relevance of TCF/Pan oligomerization was not successfully established in *Drosophila*, with TCF/Pan mutants missing a putative oligomerization motif remaining functional in cultured *Drosophila* cells.

INTRODUCTION

Wnt/β-catenin (Wnt/β-cat) signaling is an evolutionarily conserved pathway across metazoans, controlling numerous cell fate decisions during development. The T-cell factor (TCF) family of transcription factors are major nuclear mediators of Wnt/β-cat signaling. Wnt signaling promotes the stabilization and nuclear accumulation of β-cat (Cadigan and Peifer, 2009; MacDonald et al., 2009). In the nucleus, β-cat is recruited to Wnt Cis Regulatory Modules (W-CRMs), the cis response elements that control Wnt target gene transcription, through direct binding to TCFs (Cadigan, 2012; Valenta et al., 2012). TCFs activate transcription of target genes when bound by β-cat (Archbold et al., 2012; Cadigan, 2012; Valenta et al., 2012).

In *C.elegans*, Wnt/β-cat signaling is thought to act through at least two different pathways: the canonical pathway and Wnt/β-cat asymmetry (WβA) pathway. The canonical pathway is mediated by BAR-1, which is one of the four worm β-cat homologs. The interactions between BAR-1 and the worm TCF, POP-1, in this pathway are analogous to that of β-cat and TCF in *Drosophila* and vertebrates, with BAR-1 acting as a co-activator of POP-1. The WβA pathway is regulated by two other worm β-cat homologs: SYS-1 and WRM-1. In this pathway, Wnt signaling promotes nuclear influx of SYS-1, while also promoting nuclear efflux of POP-1 (Jackson and Eisenmann, 2012; Phillips and Kimble, 2009; Sawa, 2012). This efflux of POP-1 requires the transforming-

growth-factor-β-activated kinase MOM-4, the Nemo-like kinase LIT-1 and the β-cat homolog WRM-1 (Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). WRM-1 acts with LIT-1 to phosphorylate POP-1, causing its nuclear export (Lo et al., 2004).

Target selection by TCF is dependent on its ability to recognize specific DNA sequences using its DNA binding domains. All TCFs contain a HMG domain, which recognizes a 9 bp consensus of SCTTTGATS (S = G/C) with high affinity (Cadigan and Waterman, 2012; Hallikas et al., 2006; van de Wetering et al., 1997). In addition, most invertebrate TCFs and E box isoforms of vertebrate TCF1 and TCF4 contain a second DNA binding domain just downstream of the HMG domain called the C-clamp (Atcha et al., 2007; Cadigan and Waterman, 2012). This domain enables TCFs to recognize a second DNA motif, termed the Helper site, which has a consensus of GCSGS (Cadigan and Waterman, 2012).

The contribution of the C-clamp and Helper sites has been studied extensively in *Drosophila*. Functional Helper sites were found in close proximity (<10 bp) to HMG sites in several fly W-CRMs and were essential for Wnt responsiveness in vivo (Atcha et al., 2007; Chang et al., 2008; Hoverter et al., 2012). In addition, the C-clamp enhanced the DNA binding ability of fly TCF/Pangolin (TCF/Pan) in vitro and was required for TCF/Pan function in vivo (Chang et al., 2008; Figures 2.3 and 2.4). These findings suggest that TCF/Pan utilizes a bipartite DNA recognition mechanism through simultaneous HMG domain-HMG site and C-clamp-Helper site interactions (Chang et al., 2008). The presence of a conserved C-clamp domain in the *C.elegans* TCF, POP-1, suggests that it may also employ a similar bipartite recognition mechanism.

Another mechanism that has been proposed to improve TCF's DNA specificity is TCF oligomerization. Oligomerization has been observed in multiple transcription factors and is thought to increase the amount of sequence information the transcription factor can bind to compared to as a monomer. A classic example is the tetramerization of the tumor suppressor transcription factor, p53, which binds two DNA half-sites as a tetramer (Kitayner et al., 2006; Stenger et al., 1994; Weinberg et al., 2004). The variability in the orientation and spacing of HMG-Helper site pairs observed across W-CRMs (Archbold and Cadigan, unpublished results) makes it tempting to propose oligomerization as a mechanism for TCF to better accommodate these variations and bind a larger pool of sites. The *C.elegans* TCF, POP-1, was previously found to self-associate (Bhambhani and Cadigan, unpublished results). The presence of two putative oligomerization motifs that are highly conserved across *C.elegans* and *Drosophila*, suggests that this oligomerization capacity may also be conserved across these two organisms.

The conservation of common domains and motifs across the TCFs of *Drosophila* and *C.elegans* suggests they share common mechanisms of target selection. In this study, we analyzed the conservation of bipartite DNA recognition and TCF oligomerization across these two organisms. First, we extended the analysis of the C-clamp and Helper sites previously carried out in *Drosophila* to *C.elegans*. Three WβA pathway regulated W-CRMs, from the *ceh-22*, *psa-3* and *K08D12.3* loci, were found to contain Helper site motifs near functional HMG sites. These Helper sites were crucial for expression of W-CRM reporters in transgenic worms. In addition, the presence of both Helper sites and the C-clamp dramatically increased binding of POP-1 to DNA in vitro. A synthetic reporter containing concatamerized HMG and Helper sites displayed a novel POP-1 and BAR-1

dependent pattern in the int9 cells of the larval intestine. Second, the conservation of TCF oligomerization in *Drosophila* was explored. The contribution of conserved putative oligomerization motifs was analyzed using TCF/Pan deletion mutants in cultured *Drosophila* cells. Surprisingly, almost 30-40% of the TCF/Pan protein (including the putative oligomerization motifs) was found to be dispensable for TCF/Pan function in the context of the cell culture assay.

RESULTS

Helper sites are essential for POP-1 regulation of the *ceh-22b*, *psa-3* and *K08D12.3* W-CRMs

Like TCF/Pan and vertebrate TCF1E and TCF4E isoforms, POP-1 contains a C-clamp downstream of its HMG domain (Archbold et al., 2012; Cadigan and Waterman, 2012). Given the importance of Helper sites in fly and some mammalian W-CRMs that are regulated by C-clamp containing TCFs (Atcha et al., 2007; Chang et al., 2008; Hoverter et al., 2012), the possibility that similar DNA motifs were present in *C. elegans* W-CRMs was tested. Two targets that have previously been shown to be directly regulated by the WβA pathway, *ceh-22* (Lam et al., 2006) and *psa-3* (Arata et al., 2006) were examined.

ceh-22 encodes a homeodomain TF that is required for specification of the distal tip cells (DTCs) (Lam et al., 2006). The DTCs play an essential role in gonadal arm elongation during development and in maintenance of the gonadal stem cell niche (Kimble, 1981). The *ceh-22* locus produces three isoforms, but the *ceh-22b/c* isoforms (termed *ceh-22b*) (Figure 3.1A) are sufficient to rescue gonadal defects in *ceh-22* mutants

(Lam et al., 2006). In hermaphrodites, a ~1.2 kb transcriptional fusion upstream of the *ceh-22b* isoform (*ceh22b::VENUS*) is expressed in the somatic gonadal precursors (SGPs) and their descendants Z1.a and Z4.p, the distal daughters of which become the DTCs (Lam et al., 2006). This expression is dependent on SYS-1 and POP-1, and two HMG sites in the *ceh22b::VENUS* reporter (Lam et al., 2006). An examination of the sequences surrounding these functional HMG sites (termed HMG1 and HMG2) revealed the presence of two motifs (Helper1 and Helper2) similar to the Helper sites found in fly and mammalian systems (Figure 3.1A).

Mutagenesis of the HMG and Helper sites in the *ceh22b::VENUS* reporter revealed that they all contribute to expression in SGP descendants. Transgenic worms expressing stably integrated versions of the wild type (WT) or mutant *ceh22b::VENUS* reporter were generated. In addition to the SGP descendants during larval stages, *ceh-22b::VENUS* is also expressed in the pharynx (Figure 3.2A') and this latter pattern is not dependent on SYS-1 and POP-1 (Lam et al., 2006). Therefore, pharyngeal expression was used as an internal control for transgene copy number, and lines with similar expression (Figure 3.2A'-2G') were selected to test the functionality of HMG and Helper sites. The WT reporter recapitulated the previously reported pattern of this W-CRM in the Z1.a and Z4.p daughters in L1 hermaphrodites (Figure 3.2A) and subsequent stages (Lam et al., 2006). Mutation of HMG1 or Helper 2 abolished the gonadal expression of this reporter **(**Figure 3.2C, 2D, 2H). Mutation of HMG2 or Helper 1 resulted in a slightly less severe reduction (Figure 3.2H) with some animals having VENUS expression in the Z1.a or Z4.p daughters (Figure 3.2B, 2E). Simultaneous mutation of the two HMG or both Helper sites abolished gonadal expression (Figure 3.2F, 2G, 2H). These results indicated

that Helper sites are required for the WβA pathway-dependent activation of the *ceh22b::VENUS* reporter in the Z1.a and Z4.p daughters.

To extend the analysis of Helper site function to another WβA pathway target, the Meis-related factor *psa-3* was examined. In hermaphrodites, POP-1 regulates the expression of *psa-3* in the posterior T-cell descendants, which give rise to the phasmid socket cells (Arata et al., 2006). A translational *psa-3* fusion (Figure 3.1B), which can rescue a strong *psa-3* allele (Arata et al., 2006) was used as the starting point to examine the functionality of a Helper site located near a HMG site (Figure 3.1B). This HMG site, located upstream of the *psa-3* Translational Start Site (TSS; Figure 3.1B) was previously shown to be essential for expression of the translational reporter in the posterior T cell granddaughters T.pa and T.pp during the mid-L1 stage (Arata et al., 2006)(Figure 3.3A). Wild-type (WT) and Helper mutant reporters were co-injected with the *myo-2::RFP* reporter, whose pharyngeal expression was used as an internal control in the stable transgenic lines that were established (Figure 3.3A'', 2B''). Mutation of the Helper site abolished detectable expression of the *psa-3::GFP* reporter in the posterior granddaughters T.pa and T.pp in the majority of transgenic larvae examined (Figure 3.3B). These data demonstrate that a Helper site near the functional HMG site plays a crucial role in activation of the WβA pathway target *psa-3*.

A 571 bp W-CRM upstream of the annotated *K08D12.3/ZNF9* gene was also found to contain three Helper-HMG clusters. This W-CRM upstream of a VENUS reporter is expressed in the dorsal and ventral head muscles, pharyngeal muscles, intestine and the body wall muscles, throughout the larval stages and adulthood (Figure 3.3C, 3B''; data not shown). This pattern is similar (but less intense) than a previously reported pattern of

a ~2.3 kb *K08D12.3::GFP* transcriptional fusion (Dupuy et al., 2007; Hunt-Newbury et al., 2007). The requirement of the HMG and Helper sites for reporter expression was determined in late L4 hermaphrodites. At this stage, the WT reporter showed strongest expression in the head muscles, pharyngeal muscles and hindgut (Figure 3.3C, 3B'', arrowheads), with moderate expression in the foregut, midgut and body wall muscles (arrows). Larvae containing wild-type reporter, an HMG mutant or triple Helper mutant were scored based on VENUS expression in the hindgut, midgut and head and pharyngeal muscles, and characterized as strong, intermediate or weak expressers (Figure 3.3C-E). As with the *psa-3* reporter, *myo-2::RFP* was used as an internal control (Figure 3.3C'-3E'). Mutation of the HMG site and Helper sites significantly affected VENUS expression in the head muscles, midgut, and foregut with less reduction in hindgut expression (Figure 3.3C-E). The Helper site mutant lines had less expression than the HMG site mutants, most notably in the pharyngeal muscles (Figure 3.3C-E).

In analogy with fly and mammalian W-CRMs (Atcha et al., 2007; Chang et al., 2008; Hoverter et al., 2012), the Helper sites in the *ceh-22b*, *psa-3* and *K08D12.3* reporters may enhance WβA signaling by increasing POP-1 binding. To test this, Electrophoretic Mobility Shift Assays (EMSAs) were performed with recombinant POP-1 and DNA probes containing Helper 1, HMG2 and Helper 2 from the *ceh-22b* reporter (Figure 3.1A), and the functional HMG and Helper sites from psa-3::GFP (Figure 3.1B) and the *K08D12.3* reporter (Figure 3.1C). All probes showed robust binding when incubated with increasing amounts of POP-1 and mutation of the HMG or Helper sites dramatically reduced POP-1 binding (data not shown). These results are consistent with

a model where both HMG and Helper sites are required for high affinity binding of POP-1 to the *ceh-22b*, *psa-3* and *K08D12.3* W-CRMs.

The C-clamp of POP-1 is required for enhanced binding to HMG-Helper Site clusters

The finding that Helper sites are important for high affinity POP-1 binding suggests that the C-clamp of POP-1 is required for this binding. To test this, recombinant POP-1 containing two substitutions in the C-clamp (K365A & R367E) was expressed and purified. The corresponding mutations in TCF/Pan abolish DNA binding to Helper site DNA (Figure 2.4C, Mutant 5). This C-clamp mutant displayed a dramatic reduction in binding to a *ceh-22b* HMG-Helper site probe (Figure 3.4A). Mutation of the C-clamp also dramatically reduced affinity of POP-1 for HMG-Helper site containing probes from the *psa-3* and *K08D12.3* loci (Figure 3.4B, 4C). These data demonstrate that high affinity binding of POP-1 to functionally important W-CRM DNA requires the C-clamp domain. However, POP-1 can still recognize HMG site DNA in the absence of Helper motifs or a functional C-clamp, albeit at lower affinity. Under conditions allowing the detection of weaker binding, i.e., increased probe concentration and longer exposure times, wild-type and C-clamp mutant POP-1 showed comparable binding to a *ceh-22b* probe where the Helper sites are mutated (Figure 3.4D; see also Figure 3.5, which uses conditions that only detect higher affinity binding). These data demonstrate that the HMG domain of POP-1 can recognize HMG site DNA, but higher affinity binding required a bipartite mechanism similar to what we have previously reported for TCF/Pan (Chang et al., 2008).

A synthetic reporter containing HMG-Helper sequences reveals a POP-1 and BAR-1 dependent pattern in int9 intestinal cells

Synthetic reporters containing concatemerized high-affinity HMG sites have been used as Wnt/β-cat signaling readouts in several systems (Barolo, 2006), e.g, TOPFLASH in mammalian cell culture (Korinek et al., 1997) and TOPGAL in transgenic mice (DasGupta and Fuchs, 1999). Similar HMG site reporters do not work well in *Drosophila* (Chang et al., 2008), but multiple HMG-Helper site pairs provide a much more sensitive indicator of Wnt/β-catenin signaling (Chang et al., 2008). In *C. elegans,* a reporter known as POPTOP (POP-1and TCF Optimal Promoter) contains seven copies of a high affinity HMG site, and displays a wide range of expression including several cells where Wnt/POP-1 signaling is known to occur (Green et al., 2008). To test whether Helper sites can improve the sensitivity or selectivity of HMG sites in this synthetic context, a reporter containing six HMG-Helper site pairs called POPHHOP (POP-1 and HMG-Helper Optimal Promoter) was constructed, and tested for expression in *C. elegans*.

Similar to POPTOP, stably integrated POPHHOP was expressed in several cells where Wnt/β-cat signaling is known to be active (Figure 3.6D). In general, the POPHHOP pattern was more specific than POPTOP, e.g., in the DTCs of early L3 animals (compare Figure 3.6D and 6E). The POPHHOP reporter was also active in cells not previously known to receive Wnt signaling, e.g., unidentified tail neurons and posterior cells in the ventral nerve cord during the early L1 stage (Figure 3.6A). Expression was also seen in seam cell nuclei, muscle nuclei along the anterior/posterior axis and the QL.d nuclei (data not shown). The most prominent novel pattern observed with POPHHOP was in the posterior most intestinal cells known as ‘int9’, during the

early L1 stage (Figure 3.6A, 6C, 6G) onwards to adults (data not shown). In sum, the inclusion of Helper sites in a HMG site synthetic reporter altered the specificity of reporter expression.

In addition to being unique from the POPTOP expression pattern, POPHHOP expression in the intestinal 'int9' cells was interesting because a previous study had reported that POP-1 was expressed in these cells during larval development (Lin et al., 1998). The POPHHOP pattern was lost in a genetic background homozygous for the hypomorphic allele *POP-1(hu-9)*, confirming that POPHHOP was indeed a POP-1 target. This prompted us to investigate whether POPHHOP was being regulated by other signaling components in the Wnt pathway. Using RNA interference (RNAi) (Timmons et al., 2001), we knocked down POP-1 as well as other upstream Wnt pathway regulators (listed in Table 3.1) and analyzed its effect on POPHHOP expression in int9 cells. Compared to the control (Figure 3.7A), *pop-1* RNAi resulted in a decrease in POPHHOP expression (Figure 3.7B, 7D), which was expected considering previous data involving the *pop-1(hu-9)* mutant background. *bar-1* RNAi, however, resulted in a more dramatic decrease in POPHHOP expression (Figure 3.7C, 7D) than *pop-1* RNAi. RNAi treatments for all other Wnt pathway components tested did not show any significant difference in POPHHOP expression levels compared to the control (Table 3.1).

C-terminal truncation of TCF/Pan deleting putative oligomerization motifs is functional in cultured fly cells

In *Drosophila*, TCF/Pan has been found to recognize W-CRMs through bipartite binding of the HMG domain and the C-clamp to HMG and Helper sites, respectively

(Chang et al., 2008). However, fly W-CRMs show a high degree of variation in the spacing and orientation of these HMG-Helper site pairs (Archbold and Cadigan, unpublished results). Oligomerization between TCF/Pan molecules could serve as a potential mechanism that confers TCF/Pan flexibility in accommodating these variations, and remains to be tested.

Unpublished data (Bhambhani and Cadigan) suggests that the *C.elegans* TCF, POP-1, is capable of forming oligomers. A Flag-tagged POP-1 associated with a HA-tagged POP-1 when co-expressed in *Drosophila* cells (data not shown). In addition, full length POP-1 formed a higher order complex when bound in vitro to a HMG-Helper site containing DNA probe, derived from the *C.elegans ceh-22* W-CRM (Figure 3.8B). Interestingly, this oligomer complex was absent when ~100 amino acids in the C-terminus, but not in the N-terminus, of POP-1 were deleted (Figure 3.8B, constructs described in Figure 3.8A), suggesting POP-1 utilizes a region in the C-terminus to self-associate. Further examination of the C-terminal region, with the C-terminal amino acids of POP-1 aligned against those of TCF/Pan, human TCF4 and TCF3, revealed two sets of conserved motifs in the region (Figure 3.9).

The presence of conserved motifs in TCF/Pan prompted an investigation of the functional role of this region using *Drosophila* cell culture. Three C-terminal truncation mutants of TCF/Pan were created, that were 523, 471 and 398 amino acids long (compared to the 751 amino acid long full length TCF/Pan). The 523 amino acid mutant contained both conserved motifs, while the 471 and 398 amino acid mutants were missing these motifs (Figure 3.10). The mutants were tested for their ability to rescue Wnt/β-cat signaling in *Drosophila* Kc167 (Kc) cells that had been depleted of

endogenous TCF/Pan using RNA interference (RNAi) (Chang et al., 2008). The readout for Wnt/β-cat signaling used in the TCF/Pan rescue assays were the *nkd-intE* and *notum-UpEB`* reporters, both of which have been previously shown to be activated by the pathway in Kc cells (Chang et al., 2008). A synthetic reporter containing six HMG binding sites upstream of the core promoter/luciferase cassette (6xHMG) was used as a proxy to control for expression/activity levels among the various TCF/Pan proteins, since these proteins were not detectable via Western blot. A constitutively active form of Armadillo (the fly β-cat), referred to as Arm*, was used to activate Wnt/β-cat signaling in these cells (Blauwkamp et al., 2008; Chang et al., 2008; Fang et al., 2006; Parker et al., 2008).

The *nkd-intE* reporter displayed almost no detectable activation, while the *notum-UpEB* reporter showed a low level of activation when TCF/Pan was depleted in the cells. Expression of wild-type TCF/Pan restored robust activation in both reporters (Figure 3.11A, 11B). Surprisingly, all three C-terminal truncation mutants were able to rescue both reporters as well as wildtype TCF/Pan, suggesting all three mutants retained the capacity to be functional in the context of this assay (Figure 3.11A, 11B). The 6xHMG reporter displayed reduced activity in TCF/Pan RNAi treated cells, but this expression was restored by wild-type as well as all three mutant proteins (Figure 3.11C).

DISCUSSION

Conserved role of Helper sites and the C-clamp in bipartite binding by TCF

Analysis of three *C. elegans* W-CRMs indicate that Helper sites are just as important as HMG sites for their activity in vivo. In addition, both Helper sites and the C-

clamp were found to enhance POP-1's ability to bind DNA with high affinity. Thus, *C. elegans* is similar to *Drosophila*, where C-clamp-Helper site interactions have been found to be critical for TCF/Pan mediated Wnt/β-cat regulation. The working model in *Drosophila* suggests that TCF/Pan recognizes DNA through bipartite HMG domain-HMG site and C-clamp-Helper site interactions. Since functional Helper sites have been found in every fly and worm W-CRM (a total of ten) that has been rigorously characterized by the Cadigan lab, it is tempting to suggest that TCF recognizes many, perhaps most W-CRMs in these organisms through this bipartite mechanism. Considering other invertebrate phyla, such as porifera, cnidarians and echinoderms have a single TCF gene that contains a C-clamp (Adamska et al., 2010; Duffy et al., 2010; Huang et al., 2000), it is likely that their TCFs also utilize a similar bipartite recognition mechanism to perform many of their essential functions. This model may also apply to some mammalian W-CRMs, where Helper sites have been found to be important for activation by the TCF1 and TCF4 isoforms containing a C-clamp (Hoverter et al., 2012).

In addition to the DNA binding role of the C-clamp, there is published data suggesting the presence of a functional C-clamp inhibits the ability of the HMG domain to bind HMG sites. In these in vitro experiments, mutating the C-clamp resulted in elevated binding to HMG site probes (Figure 2.4D; Chang et al., 2008; Elfert et al., 2013; Hecht and Stemmler, 2003; Weise et al., 2010). Interestingly, this HMG domain inhibitory effect was not observed for POP-1, where both the wild type and C-clamp mutant POP-1 bound equally well to an HMG site probe in vitro (Figure 3.4D). This altered quality of the POP-1 C-clamp is not entirely surprising considering the C-clamp domain of POP-1 is the most diverged of all the sequenced metazoan TCFs (Archbold et

al., 2012). For example, the C-clamps of POP-1 and TCF/Pan are 59% identical/72% conserved, while TCF/Pan and human TCF4E are 83% identical/90% conserved. These differences in the worm C-clamp from the metazoan consensus may explain its altered ability in the context of the HMG domain inhibitory effect.

HMG-Helper site synthetic reporter as a tool to identify Wnt readouts in *C.elegans*

The synthetic POPHHOP fluorescent reporter displays strong expression in 'int9' cells, the posterior most intestinal cells in the larval and adult stages (Figure 3.6A). This expression pattern is POP-1 dependent (Figure 3.6H), and it has been reported that POP-1 is expressed in these cells during larval development (Lin et al., 1998). The POPHHOP reporter expression pattern was also found to be dependent on BAR-1 (Figure 3.7C). This is of particular interest as there are four β-cat homologs in *C.elegans.* In addition, Wnt/β-cat signaling functions through two known pathways in *C.elegans*: the canonical pathway and the Wnt/β-cat asymmetry (WβA) pathway, each of which is mediated by different β-cat homologs. BAR-1 regulates the canonical pathway, while β-cat homologs WRM-1 and SYS-1 regulate the WβA pathway (Phillips and Kimble, 2009). Depletion of *bar-1*, but not of *wrm-1* or *sys-1*, using RNA interference (RNAi) reduced POPHHOP reporter expression in int9 cells (Table 3.1). Despite the limitations of RNAi, this result provides evidence for a target that is regulated by the canonical pathway and is dependent on Helper sites. In contrast, Helper sites were previously found to be important in the regulation of W-CRMs of *ceh-22* and *psa-3*, both of which are well-known WβA pathway target genes. Together, this data suggests that C-clamp-Helper site mediated

regulation may be a universal mechanism used by POP-1 across different Wnt/β-cat pathways in *C.elegans*.

Although the involvement of other Wnt pathway components in the regulation of the POPHHOP reporter remains to be established, the reporter's dependence on both POP-1 and BAR-1 strongly suggest that it regulated by the Wnt/β-cat pathway. The POPHHOP reporter could hence serve as a useful tool to study Wnt readouts in *C.elegans*. An example of the POPHHOP reporter's utility is the study of the role of POP-1 in the *C.elegans* gut (Bhambhani et al., 2014). POPHHOP reporter expression in the 'int9' cells, which are known to play an important role in the defecation behavior of *C. elegans* (Teramoto and Iwasaki, 2006), suggested the potential involvement of the Wnt/β-cat pathway in this physiological process. Careful analysis found that POP-1 indeed regulated the frequency and regularity of the defecation cycle, hence revealing a previously unknown role for POP-1 in the post-embryonic gut (Bhambhani et al., 2014). Further study could help elucidate the role of Wnt/β-cat signaling and identify its downstream targets important in the defecation cycle, and hence *C.elegans* gut physiology.

Conservation of TCF oligomerization across *C.elegans* and *Drosophila*

Previous data found that the *C.elegans* POP-1 was capable of oligomerizing and that it required its C-terminus to do so (Figure 3.8). The identification of two C-terminal motifs that were conserved across *C.elegans* and *Drosophila* (Figure 3.9) suggested that the *Drosophila* TCF/Pan may also self-associate. This was particularly interesting since oligomerization could serve as a potential mechanism to confer more flexibility to

TCF/Pan to bind a larger pool of HMG-Helper site pairs with variable spacing and orientation, than a single TCF/Pan molecule.

Surprisingly, TCF/Pan mutants lacking extensive C-terminal regions, including the putative oligomerization motifs, retained the capacity to activate W-CRM reporters in cell culture (Figure 3.11). This suggests that either this region does not contribute to oligomerization (and/or other TCF/Pan function) or that it does but the assay or W-CRM reporters used are not sensitive to its functional contribution.

To address these possibilities, it would be important to first confirm whether TCF/Pan is capable of oligomerizing. Co-immunoprecipitation (CO-IP) (Bhambhani et al., 2011) could be used to analyze its protein-protein binding capacity. If found to co-immunoprecipiate, TCF/Pan with mutations in the putative motif could also be tested. It is possible that TCF/Pan oligomerizes, but uses other mechanisms, not involving the putative oligomerization motif, to do so. Previous data revealed direct HMG domain-HMG domain (data not shown) and HMG domain-C-clamp (Figure 2.5A) interactions, either or both of which could serve as oligomerization mechanisms. Both possibilities can be tested using the CO-IP set up.

The finding that 30-50% of the TCF/Pan sequence is dispensable for its function in cell culture is surprising. However, this result may be specific to the context of cell culture. The wild-type and mutant TCF/Pan proteins activated a 6xHMG reporter to similar levels (Figure 3.11C), suggesting these proteins were expressed at similar levels as well. However, this level of expression might still be higher than endogenous TCF/Pan concentrations, allowing TCF/Pan with compromised abilities to retain the capacity to activate reporters. A transgenic *Drosophila* setup may be more sensitive to alterations

made to the TCF/Pan protein. Specific mutations targeting regions found to contribute to TCF/Pan oligomerization in vitro could be tested in a *Drosophila* TCF/Pan rescue assay (described in Chapter II).

Alternately, the W-CRM reporters (*nkd intE* and *notum UpEB*` reporters) used in the cell culture assay may represent W-CRMs that are capable of being activated by TCF/Pan monomers. Testing a wider range of W-CRM reporters could help identify W-CRMs whose activity is dependent on TCF oligomers.

EXPERIMENTAL PROCEDURE

Plasmids

Site directed mutagenesis for all constructs was performed using the Quickchange II Kit (Stratagene). The *ceh-22b::Venus* plasmid (Lam et al., 2006) (pJK1082, kindly provided by Dr. Judith Kimble) and the *psa-3::GFP* plasmid (Arata et al., 2006) (kindly provided by Dr. Hitoshi Sawa) have been described previously. The *K08D12.3::Venus* plasmid was generated using the *ceh-22b::Venus* plasmid. The *POPHHOP* plasmid was generated by cloning 6XHMG-Helper (6X GAAGATCAAAGGGGGTAGCCGCCAGT) (Chang et al., 2008) upstream of a NLS-GFP in *pPD107.94* (also known as L3135) vector. 6xHistagged full length POP-1 plasmid (*pRSETA-POP-1*) was kindly provided by Dr. David M. Eisenmann. The protein expression vector for *Drosophila* cell culture, *pAc5.1/TCF/Pan-V5/His* (*pAc-TCF/Pan*), has been described previously (Chang et al., 2008). *pAc-Arm** and the luciferase reporters, *pGL3-nkd-intE, pGL3-notum-UpE* and *pGL3-6xHMG*, have been described previously (Chang et al., 2008; Parker et al., 2008).

C. elegans transgenics and genetics

Worm strains were derived from the wild-type *C.elegans* N2 Bristol strain and cultured using standard protocols. Transgenic strains with extrachromosomal arrays were generated by injecting WT or mutant versions of *ceh-22b::Venus (*100 ng/µl), *psa-3::GFP* (50 ng/ µl), *end-1::GFP::H2B* (100ng/microl) or *k08d12.3::Venus* (150 ng/ µl) plasmid into N2 worms, along with coinjection marker *myo2::RFP* plasmid (3ng/ µl) and *pActin5.1* (upto a total of 200 ng/µl). Stable integrants were generated by UV irradiation using a Stratalinker (Stratagene) at power 325. *POPHHOP* plasmid (1 ng/microl) was injected along with a *dpy-20(+)* plasmid (50ng/µl) and *pBluescript* (100 ng/µl) and stable integrants generated by gamma-radiation. Animals with integrated transgenes were outcrossed at least three times. Transgenic *POPTOP* (*7X HMG::mCherry*) strain was kindly provided by Dr. Paul W. Sternberg (Green et al., 2008). *POPTOP* and *POPHHOP* were crossed into a *pop1(hu9)* background and analyzed at different stages. All reporter strains were maintained at 25°C except for *psa-3::GFP* transgenic analysis where synchronized L1s were grown at 20°C for ~9 hours only for the reporter analysis. *pop-1(q645)* strain (JK2944) was obtained from Caenorhabatitis Genetics Center (CGC).

Imaging

Methods for mounting and viewing *C.elegans* larvae and embryos by Nomarski optics have been described previously (Sulston and Horvitz, 1977; Sulston et al., 1983) *ceh-22b::Venus, psa-3::GFP, k08d12.3::Venus, myo-2::RFP* and POPHHOP (Figure 3.7) reporter expression was analyzed by fluorescence on a Olympus BX61 microscope. Images were taken using a Hamamatsu ORGA-ERCA-CCD camera, with a specific exposure time for each W-CRM reporter and multiple focal planes were merged to obtain

the representative image. Deconvolution was performed using slidebook 5.0 software and the nearest neighbors method. *POPHHOP* and *POPTOP* reporter expression (Figure 3.6) was analyzed using a Zeiss Axioscope microscope equipped with a Zeiss Axiocam digital camera. All images were processed using Adobe Photoshop 8.0.

EMSA

Full-length 6XHis-POP-1 was expressed in *E.Coli* and purified using nickel beads (Sigma). ESMA was performed as described previously (Chang et al., 2008) using 6% native gels. POP-1(300-900 ng) in 10% glycerol and the biotin labeled DNA probe (4-8 picomoles) were incubated with 50ug/ml poly (dI-dC), 0.05% NP-40, 5 mMMgCl2 and 2μl of 50% glycerol in the presence of binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM DTT) for 5 min on ice and 20 min at room temperature.

Feeding RNAi treatment

Feeder RNAi plates were prepared by seeding NGM plates containing 1mM IPTG and 25ug/ml Ampicillin with RNAi bacteria strains (kindly provided by Dr. Gyorgyi Csankovszki) cultured for 6-12 hours at 37°C, and were allowed to dry overnight. POPHOP transgenic adults were bleached/washed using standard protocols to obtain embryos, which were left to hatch in M9 buffer overnight. The synchronized L1s were plated onto the RNAi feeder plates and incubated at 20°C. After 3-4 days at the L4 stage, worms were anesthetized using 1mM levimasole and placed on an agar pad for imaging.

***Drosophila* cell culture, RNAi knockdown and transient transfection**

Kc167 cells were cultured in *Drosophila* Schneider's media with 10% FBS. For RNAi knockdown, cells were treated with 10µg/1x10^6 cells of double-stranded RNA (dsRNA) targeting the 3'UTR of wild-type TCF/Pan. After 4 days, cells were diluted to 1x10^6 cells/mL and transfected with a plasmid mix containing pGL3-nkd-intE or pGL3-6xHMG, pAc-TCF/Pan (wild-type or mutant), S-188-cc-RLuc, a Renilla luciferase reporter (Hu et al., 2003) and pAc-Arm*. The TCF/Pan constructs contain a 3'UTR different from that in endogenous TCF/Pan, preventing them from being targeted by the dsRNA. After 3 days, cells were harvested for the Dual Luciferase reporter assay (Promega).

ACKNOWLEDGEMENTS

We thank J. Kimble, H. Sawa, R. Lin, D. M. Eisenmann, P. W. Sternberg, I. A. Hope, M. L. Waterman, R. Bodmer, Vienna *Drosophila* RNAi center and Bloomington Stock Center for providing reagents. Some strains were provided by the CGC which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Special thanks to J. K. Kim, S. Surana and V. Khivansara for invaluable help in teaching C. B. transgenesis in *C. elegans*. Special thanks to G. Csanskovszki for the deconvolution software and other *C. elegans* related facilities, as well as many helpful discussions. We also thank O. Bossinger for providing the OLB11 strain for intestine-specific RNAi. We thank M. A. Freeberg for providing the genomic sequences used for the computational search, and L. Buttitta for helpful comments. Thanks to J. D. Coolon for help with the statistical analysis and the boxplot. We thank E. J. O'Rourke for the initial suggestion to examine the connection between POP-1 and the defecation cycle. Thanks also to P. J. Hu

for nematode related equipment, K. Thompson for initial work with the TCF/Pan RNAi rescue assay, and to P. Burby and H. C. Archbold for careful reading of the manuscript.

A PART OF THIS CHAPTER WAS PUBLISHED AS:

Bhambhani, C., **Ravindranath, A.J.**, Mentink, R.A., Chang, M.V., Betist, M.C., Yang, Y.X., Koushika, S.P., Korswagen, H.C., and Cadigan, K.M. (2014). Distinct DNA Binding Sites Contribute to the TCF Transcriptional Switch in C. elegans and Drosophila. PLoS Genet *10*, e1004133.

In this chapter, I generated the data as below.

Figure 3.4

Figure 3.5

Figure 3.7

Figure 3.10

Figure 3.11

Table 3.1

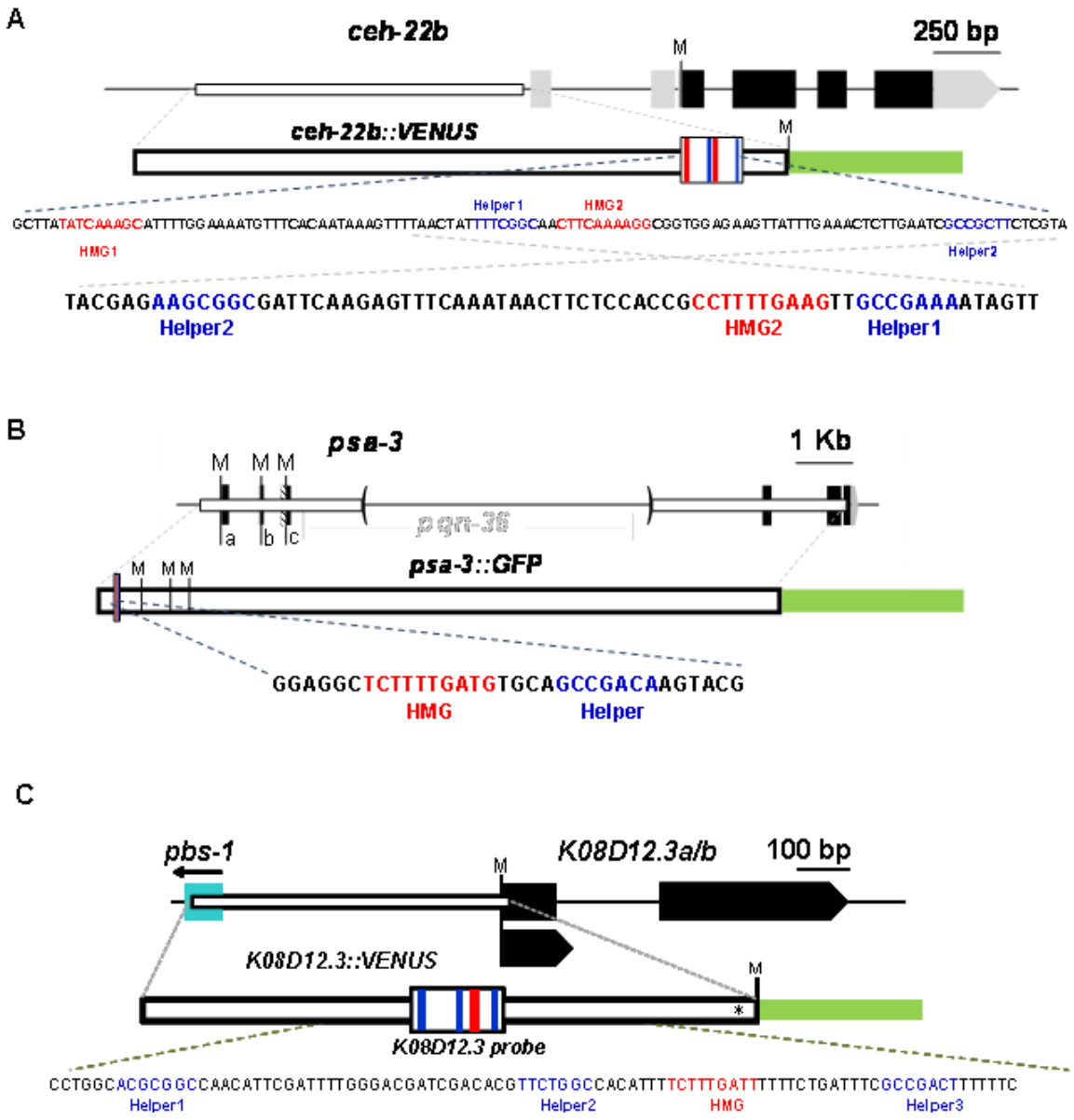

Figure 3.1. Schematics of the *ceh-22, psa-3* and *K08D12.3* loci. For each locus, black boxes represent exons and gray boxes untranslated regions (UTRs). Start codons representing the Translation Start Site (TSS) for each isoform are marked by 'M'. White boxes represent the genomic region used to construct the W-CRM reporters and the green box the GFP variant used White boxes in the W-CRM reporter show the location of the HMG (red lines) and Helper sites (blue lines). Below each schematic are the genomic sequences highlighting the putative Helper sites (blue) and functional HMG sites (red) which were targeted for mutagenesis. (A) For the *ceh-22* gene, a transcriptional fusion of the *ceh-22*b gene called *ceh-22b::VENUS* (46) was used for reporter analysis. (B) For *psa-3*, a translational fusion (psa-3::GFP) including the first exons of the a, b & c isoforms was used, where the *pqn-36* gene, located in the third intron, was deleted, as indicated by the parentheses (Arata et al., 2006). (C) For *K08D12.3*, the blue box

represents the flanking gene *pbs-1* and the asterisk indicates where the *K08D12.3* start codon was mutated to allow GFP to be read in the correct frame.

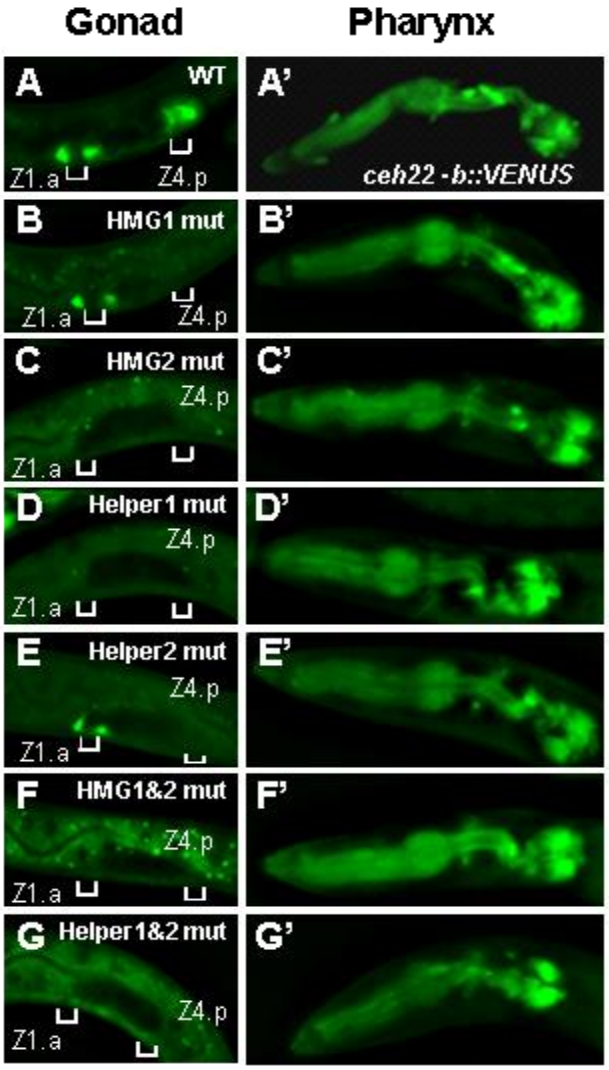

Figure 3.2. Helper sites are required for regulation of the *ceh-22* W-CRM reporter. Deconvolved images of fixed L1 larvae showing the expression of stably integrated *ceh-22b::VENUS* post-division of distal SGP daughters Z1.a and Z1.p (A-G) and expression in the pharynx (A'-G'). Venus expression in (A) Wildtype (WT), (B) HMG1 mutant, (C) HMG2 mutant, (D) HMG 1 & HMG2 mutant, (E) Helper1 mutant, (F) Helper2 mutant, and (G) Helper 1& Helper 2 mutant were analyzed. Three independent lines were used for each construct, except for HMG1 & HMG2 double mutant where two lines were examined.

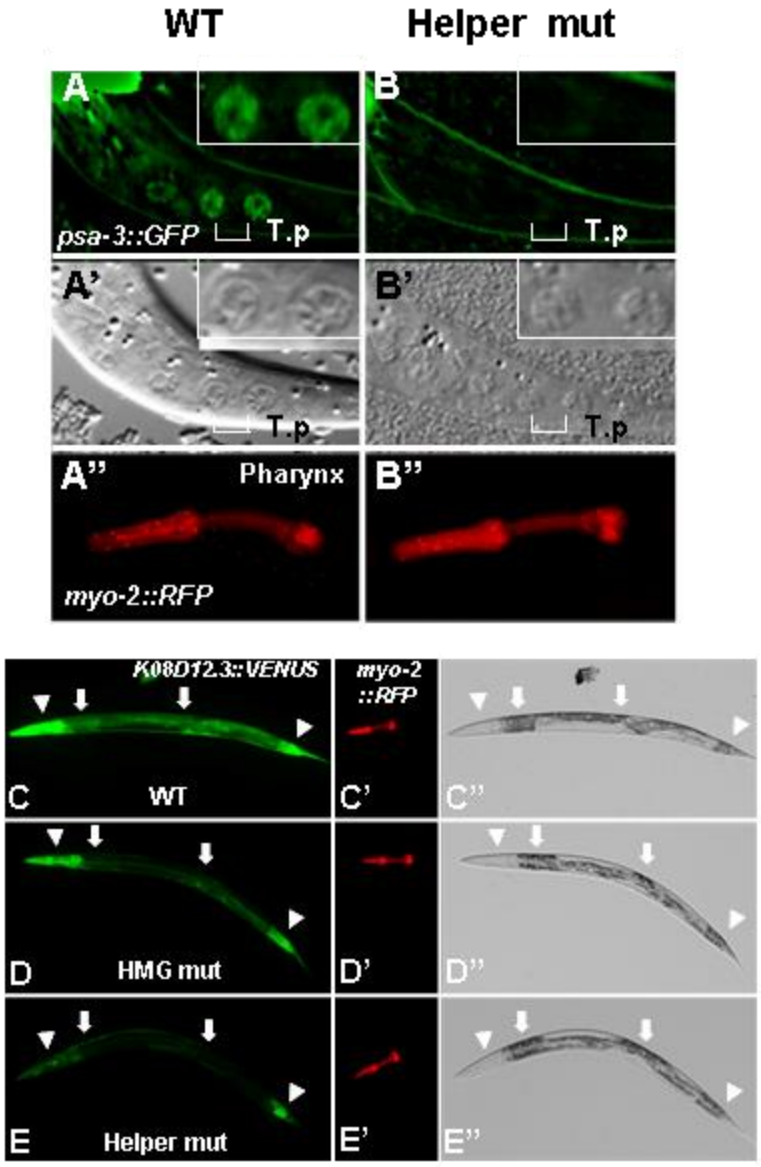

Figure 3.3. Helper sites are required for regulation of *psa-3* and *K08D12.3* W-CRM reporters. Deconvolved (A & B) and Nomarski images (A' & B') of live L1 larvae with (A) Wildtype (WT) and (B) Helper mutant *psa-3::GFP* reporter. GFP expression was scored in the T cell granddaughters T.pa and T.pp which have the granular nuclear morphology of a neuroblast (A' and B', inset) (Herman and Horvitz, 1994). *myo-2::RFP* was used as a coinjection marker. WT (A'') and Helper mut (B'') animals with a similar RFP expression level in the pharynx were analyzed. Three independent lines were used for each construct. Fluorescence (C-E) and Brightfield (C''-E'') images of live late L4 larvae extrachromosomally expressing the *K08D12.3::Venus* reporter. Strong Expression was seen in the head muscles, pharyngeal muscles and hindgut (arrowheads) and a moderate expression in the midgut (arrows). (C) Wildtype, (D) HMG mutant and (E) Helper mutants worms were scored based on the VENUS expression in the head and pharyngeal muscles and intestine. Three independent lines were used for each construct.

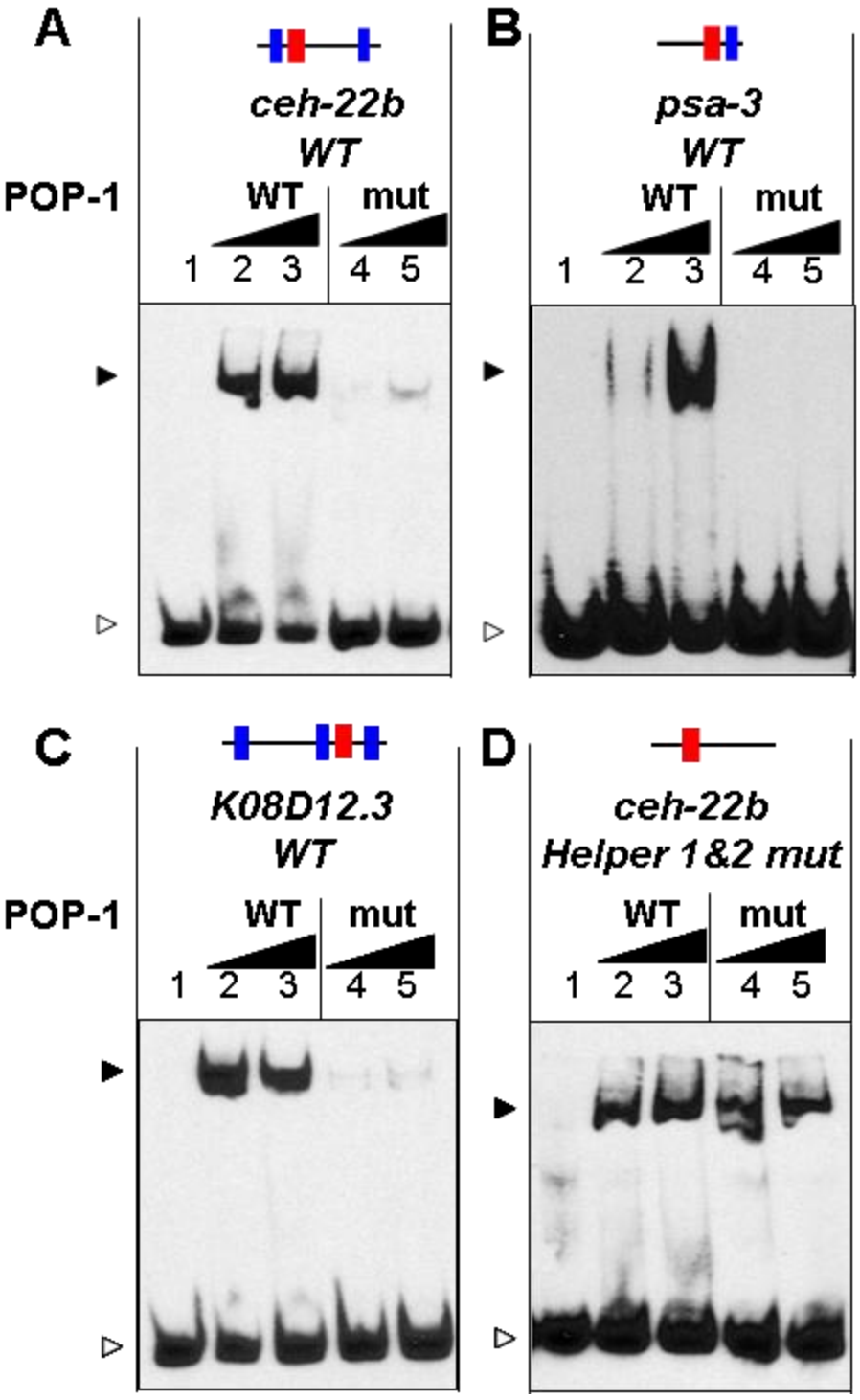

Figure 3.4. The C-clamp of POP-1 facilitates binding to DNA containing Helper sites. (A-D) EMSAs showing binding of wild-type recombinant POP-1 and a POP-1 C-clamp mutant to the *ceh-22b* W-CRM probe (1.5 femtomoles/reaction) described in Figure 3.1A (A), the *psa*-3 probe (3 femtomoles/reaction) described in Figure 3.1B, the *K08D12.3* probe (4 femtomoles/reaction) described in Figure 3.2.1C (C) and the *ceh-22b* probe (3

femtomoles/reaction) with both Helper sites mutated (D). The *ceh-22b*, *psa-3* and *K08D12.3* WT probes show strong binding with increasing amounts (0.4 and 0.8 μg/reaction) of POP-1 WT protein (lanes 2 and lane 3 respectively) but not with the POP-1 C-clamp mutant (lane 4 and lane 5 respectively). Under conditions designed to detect lower affinity binding (0.75 and 1.5 μg of POP-1; 3 femtomoles of probe and longer exposure times), binding to the *ceh-22b* probe lacking Helper sites (containing only the HMG2 site) was similar with WT and mutant POP-1. The data are representative of three independent experiments.

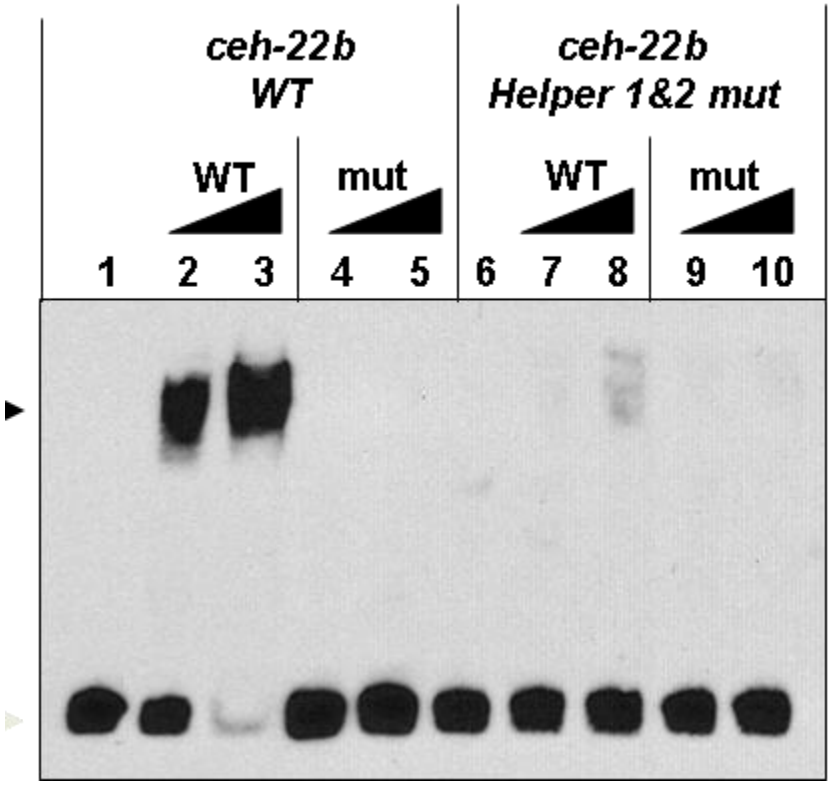

Figure 3.5. Helper sites are important for binding of wild-type and C-clamp mutant POP-1 to *ceh-22b* W-CRM probes. EMSA analysis of the *ceh-22b* W-CRM probe containing two Helper sites (lanes 1-5) and a probe where these motifs are mutated (lanes 6-10). Recombinant wildtype or C-clamp mutant POP-1 (400 & 800 ng/reaction) was added where indicated. Either probe was used at 1.5 femtomoles/reaction. A dramatic reduction in binding was observed in the C-clamp mutants (lanes 4, 5, 9 &10) or when wild-type POP-1 was incubated with the Helper site mutant probe (lanes 7 & 8).

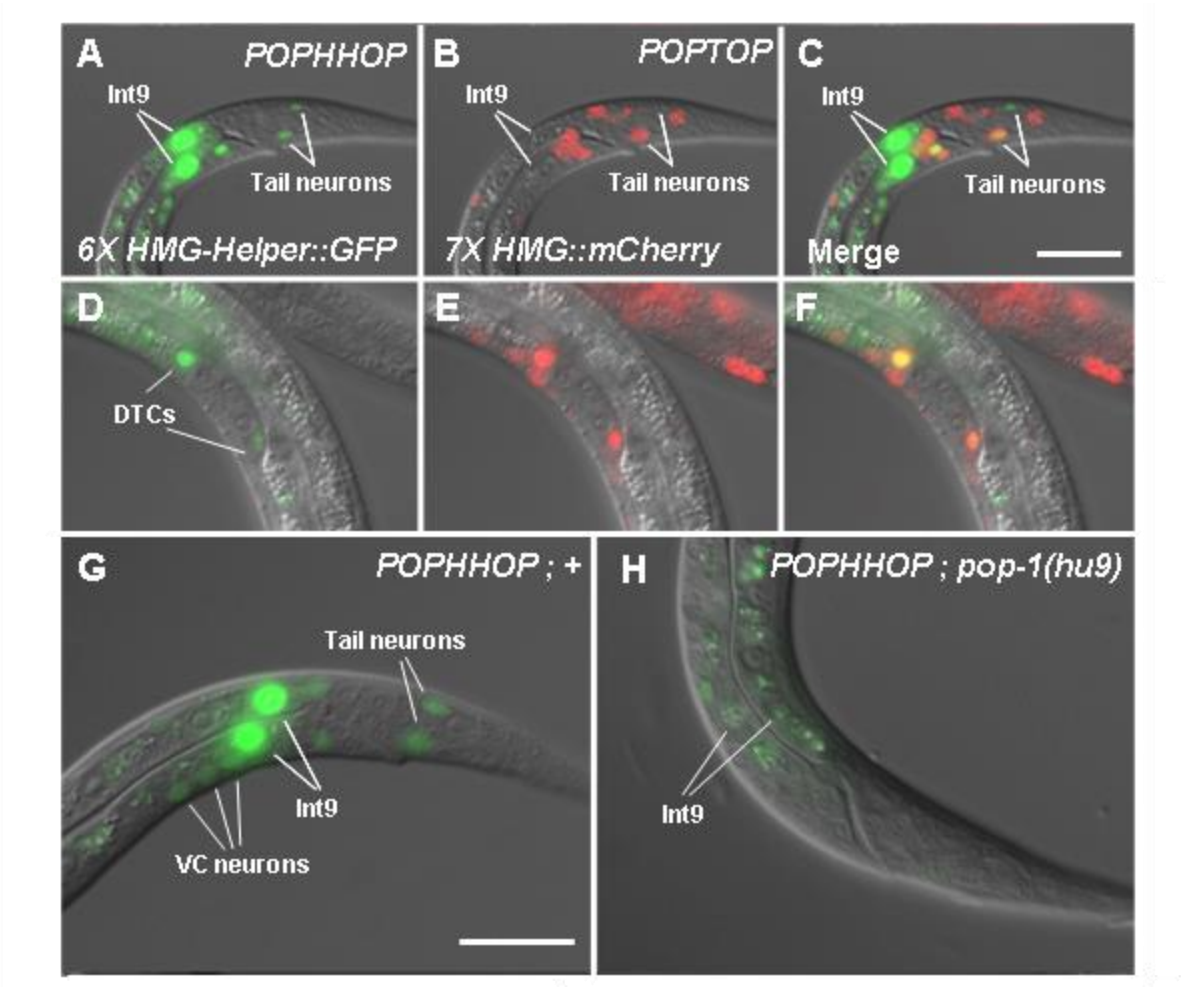

Figure 3.6. A synthetic HMG-Helper site reporter reveals a novel expression pattern and is POP-1 dependent. (A-F) Nomarski images of animals with stably integrated *POPHHOP* (*6X HMG-Helper::GFP*) and *POPTOP* (*7X HMG::mCherry*) reporters showing GFP (A, D) and mCherry (B, E) fluorescence. Live L1 larvae express both GFP and mCherry in some tail neurons (A-C) and live L3 larvae display overlapping DTC expression (D-F). In addition, POPHHOP displayed strong expression in the int9 intestinal cells of L1 Larvae (A) onward through adulthood (not shown). (G-H) Stably integrated *POPHHOP* animals in a wild-type (G) or *pop-1(hu9)* background (H). The reporter expression seen in the int9 cells, tail neurons, and occasionally in the VC neurons is low or undetectable in the *pop-1* mutants. Scale bars = 10 μm.

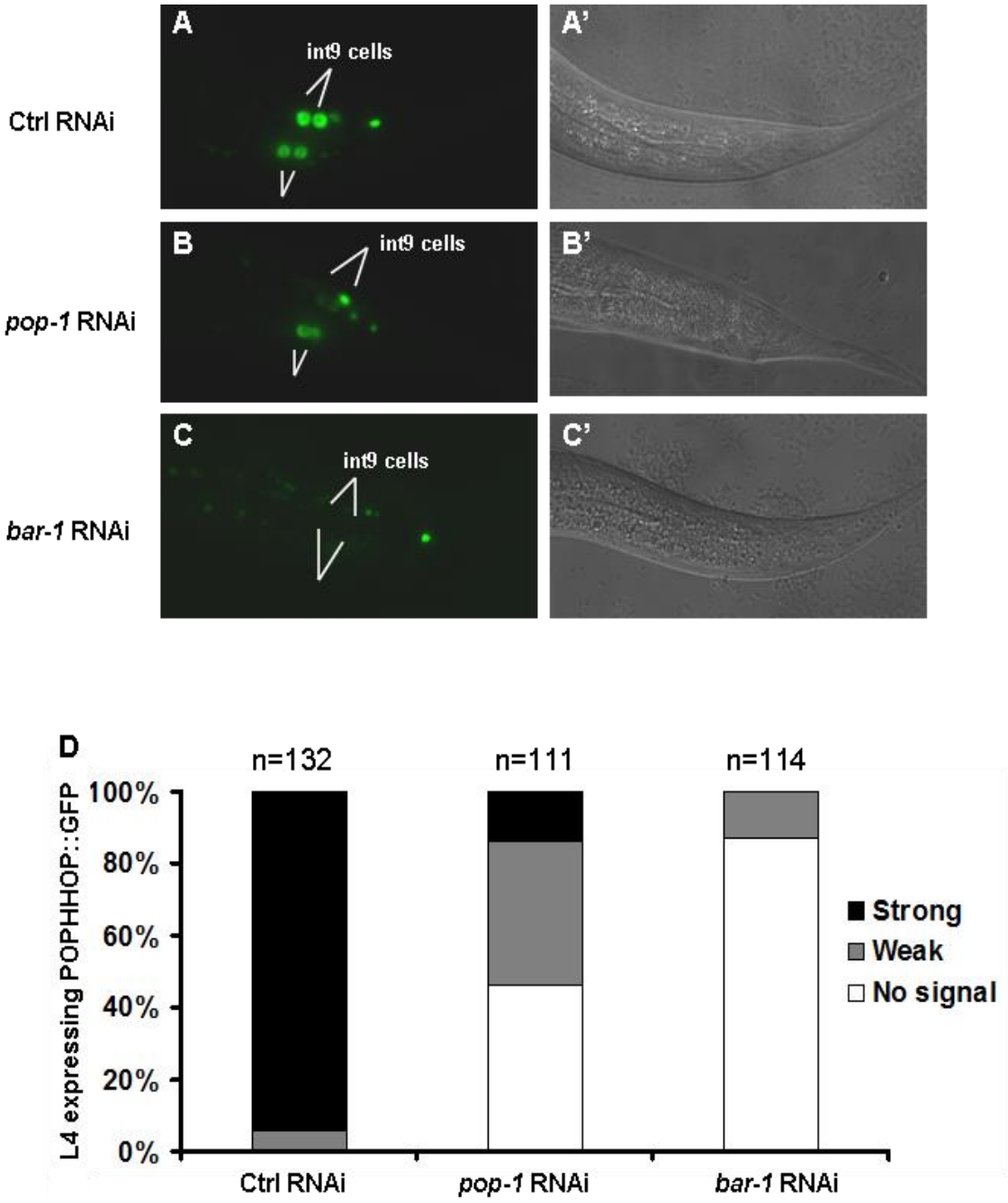

Figure 3.7. The expression of the synthetic HMG-Helper site reporter in int9 intestinal cells is POP-1 and BAR-1 dependent. Fluorescence (A-C) and brightfield (A'-C') images of live L4 larvae showing the expression of the stably integrated *POPHHOP* (*6X HMG-Helper site::GFP*) reporter in the int9 intestinal cells, after feeding RNAi treatment. Reporter expression was reduced in L4 larvae fed dsRNA targeting *pop-1* (B), but showed a more dramatic reduction in larvae fed with dsRNA targeting *bar-1* (C), compared to non-specific dsRNA (Control (Ctrl) RNAi) (A). (D) Semi-quantification of expression patterns across three RNAi treatments, grouped by whether

individuals displayed strong, weak or no signal. The data is representative of three independent experiments.

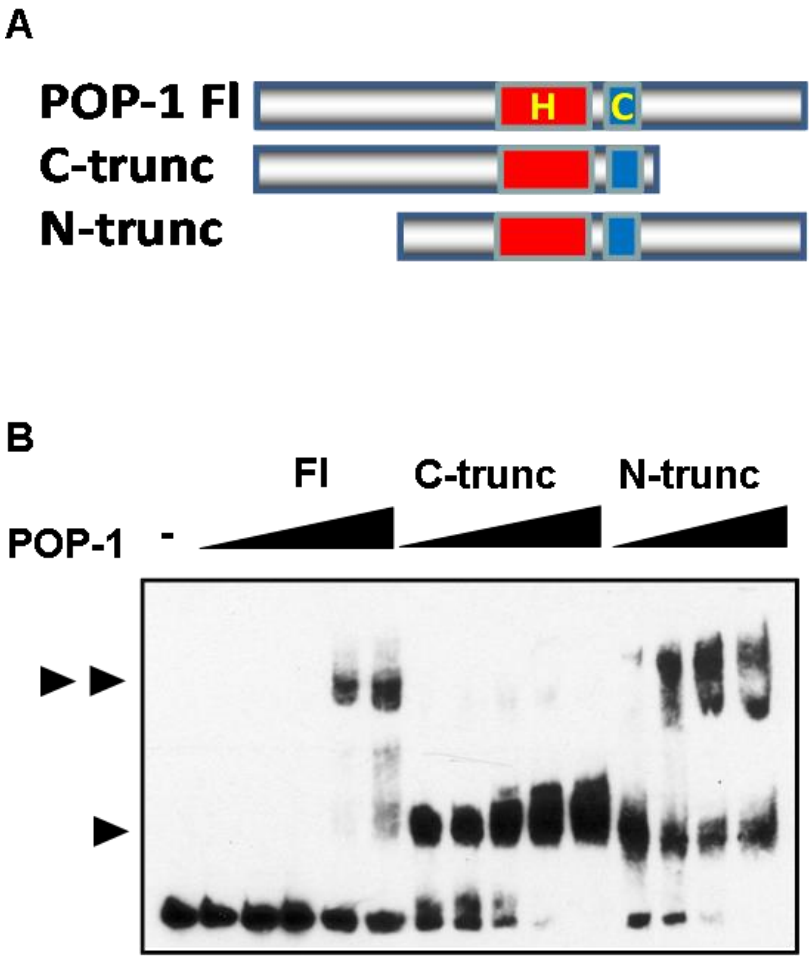

Figure 3.8. POP-1 forms a higher order complex when bound to DNA and requires its C-terminal region to do so (From Bhambhani and Cadigan, unpublished results). (A) Cartoon representation of full length (Fl) POP-1 protein and mutants with N-terminal (N-trunc) and C-terminal truncations (C-trunc). The red box represents the HMG domain (H) and the blue box the C-clamp (C). N-trunc and C-trunc are missing 100 amino acids in their N and C-terminals, respectively. (B) EMSA showing formation of higher order complex (double arrowhead) when POP-1 Fl is bound to DNA (lanes 2-6). Truncation of the C-terminus (lanes 7-11), but not the N-terminus (lanes 12-15), causes a loss of formation of the higher order complex (single arrowhead). DNA probe used was derived from the *ceh-22* W-CRM, and is described in Figure 3.1A.

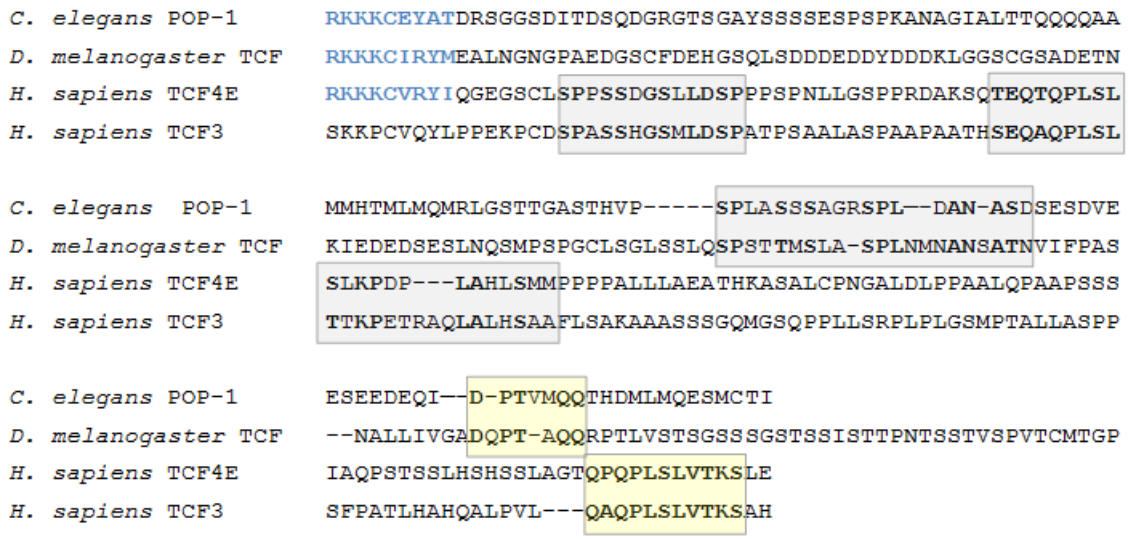

Figure 3.9. Putative oligomerization motifs in the C-terminal regions of several metazoan TCFs (From Bhambhani and Cadigan, unpublished results). Alignment of C-terminal regions of *C.elegans* POP-1, *Drosophila* TCF/Pan and human TCF4E and TCF3. Two conserved regions across the four TCFs are marked as putative oligomerization motifs (boxed regions). Amino acids marked in blue represent the C-terminal end of the C-clamp.

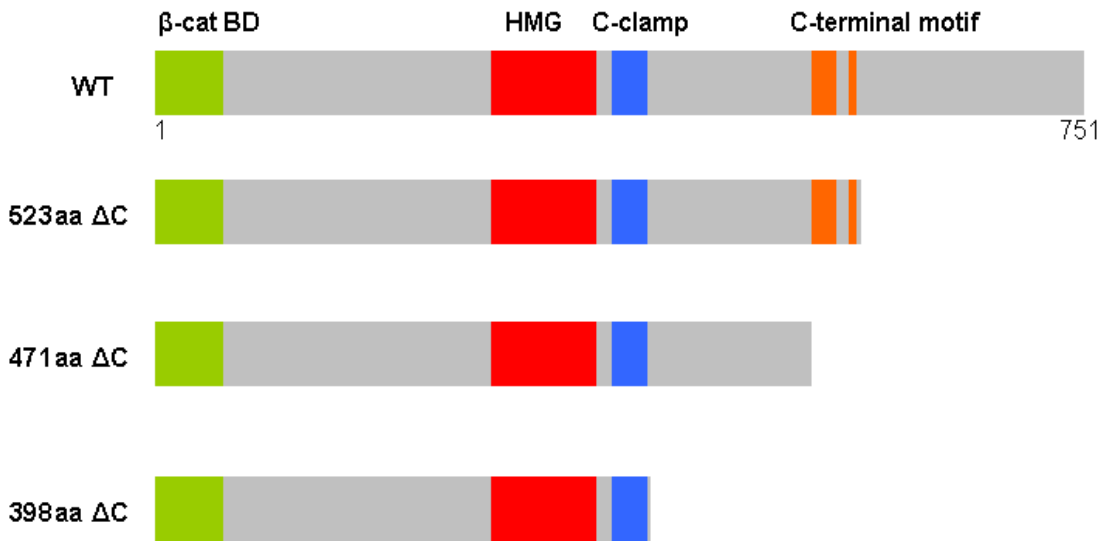

Figure 3.10. C-terminal deletion constructs of *Drosophila* TCF/Pan. Cartoon depicting full-length wild-type (WT) TCF/Pan and the three C-terminal deletion constructs (ΔC) used in the *Drosophila* cell culture rescue assay. The deletion constructs were 523, 471 and 398 amino acids long. The green box represents the β-cat binding domain (β-cat BD), the red box the HMG domain, the blue box the C-clamp and the orange boxes the putative oligomerization motifs. The putative motif is present in the 523 aa ΔC construct, but is deleted in the 471 and 398 aa ΔC constructs.

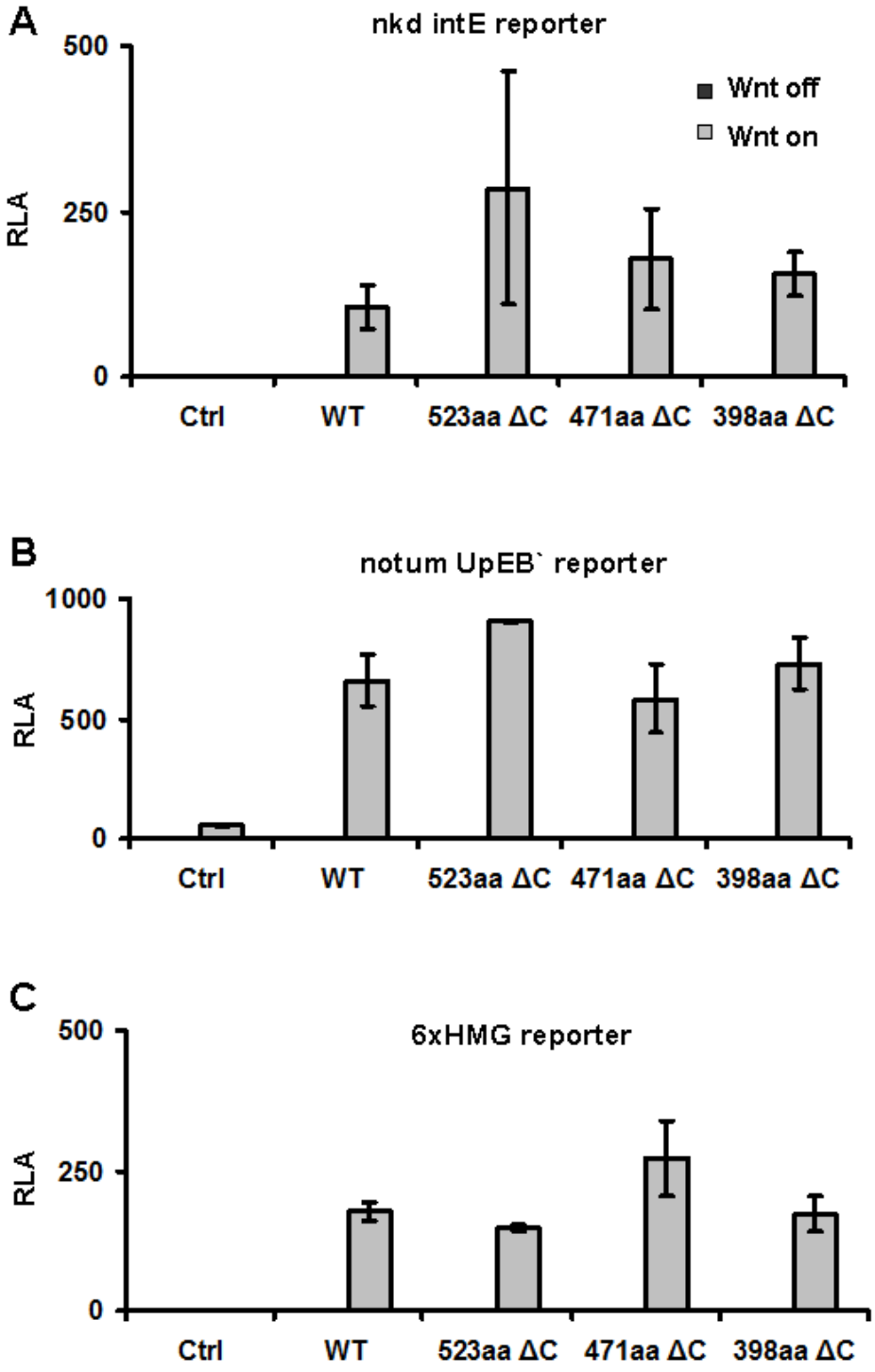

Figure 3.11. All C-terminal truncation constructs of TCF/Pan are able to activate W-CRM reporters in cell culture. Endogenous TCF/Pan was depleted using dsRNA that targets the TCF 3' UTR, followed by transient transfection of either WT or C-terminal deletion mutant expression constructs containing a heterologous 3'UTR that

cannot be targeted by the dsRNA. The Wnt/β-cat pathway was induced (Wnt on) using Arm* (see text for further description). Ctrl and "Wnt off" refer to controls where there was transfection of an empty expression vector. The *nkd-IntE* and *notum-UpEB*` W-CRM reporters were used as Wnt readouts (A, B), while the synthetic 6xHMG reporter was used as a control (C). Bars are the mean of triplicate transfection ± SD. Experiments were repeated at least three times with similar results. See Experimental Procedure for additional details of the cell culture conditions.

Table 3.1. List of genes targeted by feeding RNAi in transgenic POPHHOP worms. Number of worms that showed strong, weak or no signal in intestinal 'int9' cells after RNAi treatment have been categorized for each gene targeted.

Target Gene	Strong Signal	Weak Signal	No Signal
Control	124	8	0
pop-1	14	41	56
bar-1	0	13	101
egl-20	42	0	0
cwn-2	45	0	0
lin-44	44	0	0
mom-2	45	11	11
mom-5	45	2	0
dsh-1	51	0	0
mig-5	83	4	0
pry-1	89	0	0
apr-1	58	0	0
gsk-3	40	2	0
gsk-3A	30	1	0
wrm-1	36	4	0
sys-1	44	8	8
lit-1	45	3	3
hmg1.2	62	0	0

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CHAPTER IV

FUTURE DIRECTIONS

Exploring the functional importance of the C-clamp in vivo in *C.elegans* and mice

The finding that the C-clamp is required for both embryo epidermal patterning (Figure 2.6) and wing margin specification (Figure 2.7) in *Drosophila* is the first piece of evidence establishing the functional importance of the domain in vivo. Considering the C-clamp is highly conserved across metazoans (Archbold et al., 2012), it would be interesting to extend the in vivo analysis of this domain to other organisms.

C.elegans and mice would be two good candidate model systems to study the C-clamp in vivo. *C.elegans*, like most other invertebrates, has only one TCF which contains a C-clamp, suggesting a conserved function for this domain (Archbold et al., 2012). Moreover, functional Helper sites have been discovered in several worm W-CRMs (Figure 3.1) and the C-clamp in POP-1 (the worm TCF) is required for high affinity in vitro DNA binding (Figure 3.4). On the other hand, the mouse, like most other vertebrates, has multiple TCFs, with only certain isoforms of two TCFs, TCF1 and TCF4, containing the C-clamp (Archbold et al., 2012). Helper sites have been found in a handful of mammalian W-CRMs and the C-clamp was required for activation of reporters of these W-CRMs in cultured mammalian cells (Atcha et al., 2007; Hoverter et al., 2012), suggesting a role for the C-clamp in vertebrate systems. However, considering the high

level of functional divergence seen among vertebrate TCFs (Archbold et al., 2012; Cadigan and Waterman, 2012), there is a possibility that the C-clamp is only required for the regulation of a handful of Wnt targets, and is hence more dispensable. Mice would be a good system to test the in vivo functional contribution of this domain in the context of vertebrates.

To test the functional relevance of the C-clamp in vivo in *C.elegans*, I propose introducing C-clamp mutant genomic constructs into a *pop-1* mutant background (eg. *pop-1 (zu189)* or *pop-1(q645)* mutant worms (Lin et al., 1998; Siegfried and Kimble, 2002)). However, it is worth noting that genomic fragments have not been previously used to rescue *pop-1* mutant phenotypes, with most experiments involving transgenes with heterologous promoters (Gay et al., 2003; Robertson et al., 2011; Shetty et al., 2005). This may be indicative of some technical issues associated with creating transgenes with *pop-1* genomic fragments. Considering this, the recently developed genome-engineering technology of CRISPR/Cas (Chiu et al., 2013; Lo et al., 2013) may provide a more straightforward system to introduce targeted mutations directly into the C-clamp in the *C.elegans* genome.

POP-1 is important for several developmental and physiological processes. The *pop-1* mutant, *pop-1 (zu189)*, is embryonic lethal (Lin et al., 1998). Other *pop-1* mutants, such as *pop-1 (hu9)* and *pop-1(q645)*, which contain mutations in the β-cat binding domain, display defects in axis formation during gonadogenesis (Siegfried and Kimble, 2002), show arrhythmicity in defecation cycle behavior (Bhambhani et al., 2014) and are unable to activate W-CRM reporter constructs derived from endoderm lineage-specific genes, such as *sdz-23*, *sdz-26* and *end-1*, in E-cells in vivo (Shetty et al., 2005). It is

tempting to predict that similar phenotypes would also be observed in POP-1 C-clamp mutants, considering the presence of only a single C-clamp containing TCF in *C.elegans*. In addition, W-CRMs associated with some of these phenotypes, such as that of *end-1* (Bhambhani et al., 2014), contain functional Helper sites, further establishing a potential link with the C-clamp

To study the functional contribution of the C-clamp in mice, I propose creating C-clamp mutant mice by specifically disrupting the exons encoding the C-clamp using conventional gene targeting (Gregorieff et al., 2004; Korinek et al., 1998). Since both the isoforms TCF1E and TCF4E contain a C-clamp, a double TCF1E and TCF4E C-clamp mutant would be required, to confidently rule out potential redundancy between the two C-clamps. An alternative to conventional gene targeting would be the CRISPR/Cas system (Wang et al., 2013b; Yang et al., 2013) which could be used to make more targeted mutations (versus deletions using conventional gene targeting) within the C-clamp in the mouse genome. Mutations that disrupt the C-clamp (Mutant 5, Figure 2.3A) or affect either of its functions (DNA binding Mutant 4, Figure 2.3A; stronger inhibitory mutants yet to be identified) could be introduced using this system.

Previous studies that analyzed TCF1/TCF4 double mutant mice (where the HMG domain was specifically disrupted) noted multiple developmental defects including lack of hind limbs, posterior body and tail, duplication of the neural tube and absence of parts of the hindgut in embryos (Gregorieff et al., 2004). It would be interesting to analyze whether there is any overlap between any of these phenotypes and those observed in TCF1/TCF4 double C-clamp mutants.

Inhibition of HMG DNA binding by the C-clamp

Previous in vitro DNA binding experiments using either fly TCF/Pan or TCF4 isoforms found that mutating the C-clamp resulted in increased binding to a DNA probe containing only a HMG site (Chang et al., 2008; Hecht and Stemmler, 2003; Weise et al., 2010). I further characterized this "inhibitory effect" of the C-clamp on the HMG domain's DNA binding ability, by identifying a C-clamp mutation that compromised the C-clamp's inhibitory activity in vitro (Mutant 8, Figure 2.4) and reduced TCF's ability to rescue W-CRM reporter activation in cell culture (Mutant 8, Figure 2.3). This mutation did not affect the C-clamp's DNA binding affinity to Helper sites, suggesting it was specifically an inhibitory effect mutant. In addition, I found the C-clamp capable of directly binding the HMG domain (Figure 2.5), providing a possible mechanism for the inhibitory effect.

Unfortunately, this inhibitory effect mutant was found to be still functional in vivo in *Drosophila* embryos (Mutant 8, Figure 2.6). While this result was disappointing, it could be reconciled by the possibility that the mutation only partially affected the inhibitory activity and that the partial mutant retained sufficient capacity to function in vivo. Going forward, a stronger inhibitory effect mutant needs to be identified to further explore the functional relevance of this C-clamp activity. A more thorough mutational analysis across all 30 amino acids of the C-clamp would be required to isolate an effective mutant. This could however be challenging if the residues involved in the inhibitory activity are multiple and spaced widely across the domain. Information from structural analysis of HMG domain-C-clamp interactions would be invaluable in this case as it could help narrow down on residues in the C-clamp that directly bind the HMG

domain. It should be mentioned that while there is hope in the possibility of identifying a stronger inhibitory effect mutant, one cannot rule out the possibility that such an ideal mutant (one which only displays a strong loss in inhibitory activity and not in DNA binding function) may not be successfully isolated.

The ultimate goal is to analyze the in vivo functional contribution of the C-clamp's inhibitory activity. If identified, a strong inhibitory effect mutant could be tested in vivo in the *Drosophila* embryo patterning and wing specification rescue assays (described in Chapter II). In addition, this mutation could be introduced into TCFs in other organisms, such as *C.elegans* and mice, to better understand the role of the "inhibitory effect" in TCF function more broadly across metazoans.

The C-clamp's inhibitory effect is interesting because it could provide an additional mechanism for improving the DNA binding specificity of TCF. Considering the high level of degeneracy seen in HMG sites, clusters of degenerate non-functional HMG sites in the genome could potentially draw and bind TCF molecules. By inhibiting the HMG domain in the absence of Helper sites, the C-clamp could prevent TCF from being sequestered by these degenerate HMG sites. In addition, the C-clamp's affinity for Helper sites could further direct TCF to HMG-Helper site pairs in functional W-CRMs (Model described in Figure 4.1 A-B).

Inhibitory effect mutants that are successful in compromising TCF function in vivo in *Drosophila* could be used to further verify and elaborate on the above mentioned model. Chromatin immunoprecipitation (ChIP) of wild-type versus the "inhibitory effect" mutant TCF (using antibody described in Parker et al., 2008) could be used to compare the recruitment of both TCFs to W-CRM reporters in vivo in *Drosophila* embryos. I

propose using the *notum UpEB*` and 6xHMG site reporters. The *notum UpEB*` reporter has been previously shown to recruit wildtype TCF (Parker et al., 2008), while the 6xHMG site reporter, which displays reduced activity in *Drosophila* embryos (Chang et al., 2008), would be expected to show low wildtype TCF recruitment. I hypothesize that if the "inhibitory effect" mutant TCF is indeed inefficient in locating its targets, it would get sequestered by HMG sites in non-functional genomic regions. Hence, the mutant would be expected to show reduced recruitment to the *notum UpEB*` reporter. Conversely, the HMG sites in 6xHMG site reporter could attract the compromised mutant TCF and display increased recruitment of mutant TCF compared to wildtype TCF. A potential crossing scheme to generate *Drosophila* embryos for this ChIP experiment is described in Figure 4.1C.

HMG-Helper site synthetic reporter as a tool to understand Wnt biology and identify direct Wnt targets in *C.elegans*

The synthetic POPHHOP reporter was found to display a unique expression pattern in the int9 cells, the most posterior intestinal cells in the *C.elegans* gut (Figure 3.6). This finding was interesting as int9 cells are known to play a central role in regulating the defecation cycle in *C.elegans* (Branicky and Hekimi, 2006). In adult worms, the cycle starts with contractions in the posterior body wall muscles (pBoc) at 40-45 second intervals (Thomas, 1990). Each pBoc is followed by anterior body wall contractions (aBoc) after ~3 seconds and an immediate expulsion (Exp) of the fecal content completing a defecation cycle (Thomas, 1990). The intestine acts as a pacemaker where calcium oscillations set the period of each cycle (Dal Santo et al., 1999; Espelt et

al., 2005; Teramoto and Iwasaki, 2006; Wang et al., 2013a). Calcium spikes originate in the int9 cells, which initiate the pBoc step (Teramoto and Iwasaki, 2006).

The dependence of the POPHHOP reporter expression in int9 cells on POP-1 and BAR-1 (Figure 3.7) suggested that the Wnt/β-cat pathway may also play a role in regulating rhythmic defecation in *C. elegans*. Bhambhani et al., 2014 examined the role of POP-1 in the *C. elegans* defecation cycle and found that lack of functional POP-1 affected both the length between pBocs (Figure 4.2.B and D) and rhythmicity of the cycle (Figure 4.2.A and C). POP-1 mutants tested contained point mutations in the β-catenin binding domain, making it is very likely that POP-1 is working with Wnt/β-cat signaling to control this rhythmic behavior. In addition, the *C.elegans* Wnt genes *egl-20*, *cwn-1* and *lin-44* are expressed close to or overlapping the int9 cells (Jackson and Eisenmann, 2012; Sawa, 2012), suggesting that they could be contributing to the non-neuronal pacemaker function of the int9 cells.

The first step towards establishing the dependence of the defecation cycle on the Wnt/β-cat pathway would be by assaying the cycle in worms mutant for *bar-1* and other upstream regulators of the pathway. In addition, it would be interesting to further elucidate Wnt biology in the gut by investigating how Wnt/β-cat signaling affects the calcium fluxes that are associated with coordinating the periodicity of the defecation cycle (Dal Santo et al., 1999; Espelt et al., 2005; Teramoto and Iwasaki, 2006). Previously established assays using calcium imaging (Teramoto and Iwasaki, 2006) could be used to track the intestinal calcium oscillations in worms mutant for or treated with RNAi targeting various components in the Wnt pathway.

The dependence of the defecation cycle on POP-1 also suggests the presence of direct downstream targets of Wnt/β-cat signaling that are important for the defecation cycle. An interesting candidate is the phosholipase Cβ, *egl-8*. Similar to *pop-1* mutants, *egl-8* mutants affect defection cycle length and rhythmicity, though more dramatically (Espelt et al., 2005; Lackner et al,1999; Thomas, 1990). In addition, an EGL-8::GFP transcriptional fusion construct containing an *egl-8* regulatory region displayed a posterior intestine specific expression pattern (Lackner et al., 1999). Interestingly, two HMG-Helper site clusters have been found in this *egl-8* regulatory region (Figure 4.3). It would be interesting to analyze whether this *egl-8* reporter construct is responsive to the Wnt/β-cat pathway, by testing its dependence on Wnt signaling components using RNAi or a mutant gene background. In addition, mutational analysis could be used to study whether some or all of the HMG-Helper sites identified are functional.

Oligomerization in *Drosophila* TCF/Pangolin

The *C.elegans* POP-1 is capable of forming oligomers and this activity is dependent on a C-terminal region. The presence of a motif in this region that is conserved across both *C.elegans* POP-1 and *Drosophila* TCF/Pan, suggested that TCF/Pan could also potentially oliogmerize. To test this possibility and the functional importance of the putative oligomerization motif, C-terminal truncations of TCF/Pan were generated and tested in cultured *Drosophila* cells (Figure 3.10). Surprisingly, TCF/Pan mutants with 30-40% of their sequence missing, including the putative oligomerization motif, retained their functional capacity (Figure 3.11). This negative result, however, may be specific to the context of cell culture where overexpression of TCF/Pan could negate any functional

deficiencies in the protein. Alternately, the W-CRM reporters used in this assay may represent those that do not require TCF/Pan oligomers for activation.

A more careful analysis of TCF/Pan's oligomerization capacity is required before it is ruled out as a possibility. Firstly, whether TCF/Pan can self-associate needs to be established. Co-immunoprecipitation (Co-IP) (Bhambhani et al., 2011) of differently tagged TCF/Pan molecules could help determine this. If a positive result is obtained, point mutations could be introduced into the putative oligomerization motif and tested for its contribution in vitro. There is a possibility that TCF/Pan is capable of self-associating, but the putative motif does not contribute to this function. It was previously found that the HMG domain was capable of directly binding both itself (Ravindranath and Cadigan, unpublished results) and the C-clamp (Figure 2.5A)(Ravindranath and Cadigan, 2014), offering some alternate mechanisms for oligomerization.

If the putative oligomerization motif is found to contribute to TCF/Pan self-association, a more robust and sensitive setup to test the functional relevance of this motif would be in vivo in *Drosophila* embryos using the previously established rescue assay (described in Chapter II). TCF/Pan with point mutations in the motif that disrupt in vitro binding could be tested for their ability to rescue the TCF/Pan mutant cuticle phenotype.

Lastly, although the putative oligomerization motif was originally identified in the C-terminus of the *C.elegans* POP-1, this motif remains to be tested in the context of POP-1. Even if oligomerization is not observed in the *Drosophila* TCF/Pan, it would be interesting to explore the mechanism of self-association in POP-1. POP-1 containing mutations in the putative motif could be tested for its ability to form oligomers, using Co-

IP (Bhambhani et al., 2011), as well as its ability to form higher order structures when bound to DNA, using in vitro DNA binding EMSA assays (Chang et al., 2008).

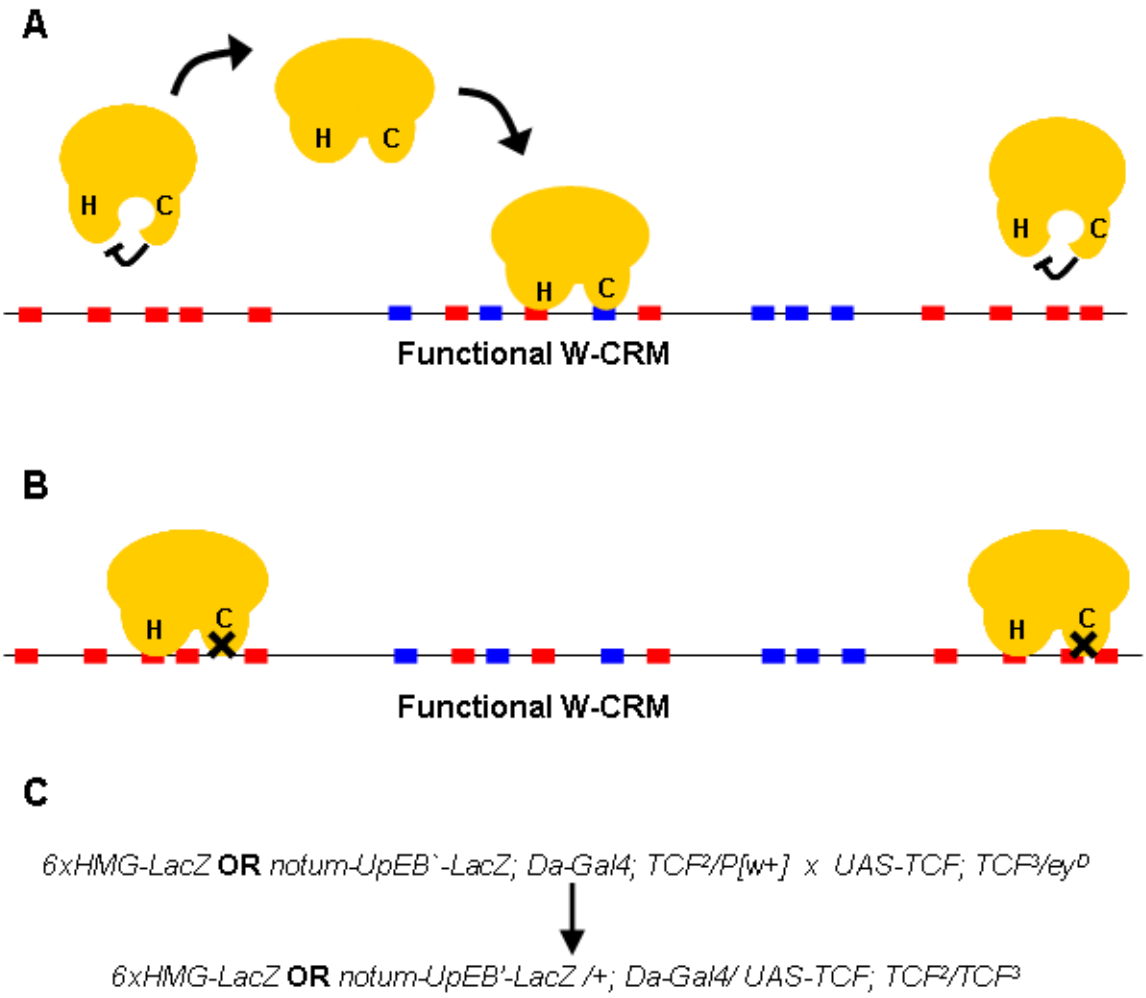

Figure 4.1. The "inhibitory effect" of the C-clamp and its effect on TCF specificity. (A,B) Model depicting how the C-clamp inhibitory activity could contribute to improving TCF specificity. (A) In wild-type TCF, the C-clamp inhibits the HMG domain from binding degenerate non-functional HMG sites (red boxes). However, at a functional W-CRM containing both HMG and Helper sites (blue boxes), TCF displays enhanced bipartite binding through simultaneous HMG domain-HMG site and C-clamp-Helper site interactions. (B) TCF specificity is compromised in the C-clamp "inhibitory effect" mutant (mutation indicated with cross). TCF is distracted by the non-functional HMG sites. Based on in vitro DNA binding data that suggests TCF binds very poorly to Helper sites alone (Chang et al., 2008), it is proposed that conversely the HMG domain also inhibits the C-clamp from binding Helper sites in the absence of HMG sites. This mechanism would prevent the C-clamp of TCF being distracted by non-functional degenerate Helper sites in the genome. (C) Potential crossing scheme to generate embryos for ChIP. These embryos would contain a P[*Da*-Gal4] driver and P[UAS-TCF/Pan] transgene in a TCF^{2}/TCF^{3} transheterozygous mutant background with either P[*6xHMG-LacZ*] or P[*notum-UpEB`-LacZ*] reporters. UAS-TCF/Pan (UAS-TCF) encodes for either wild type or C-clamp "inhibitory effect" mutant *TCF/Pan*.

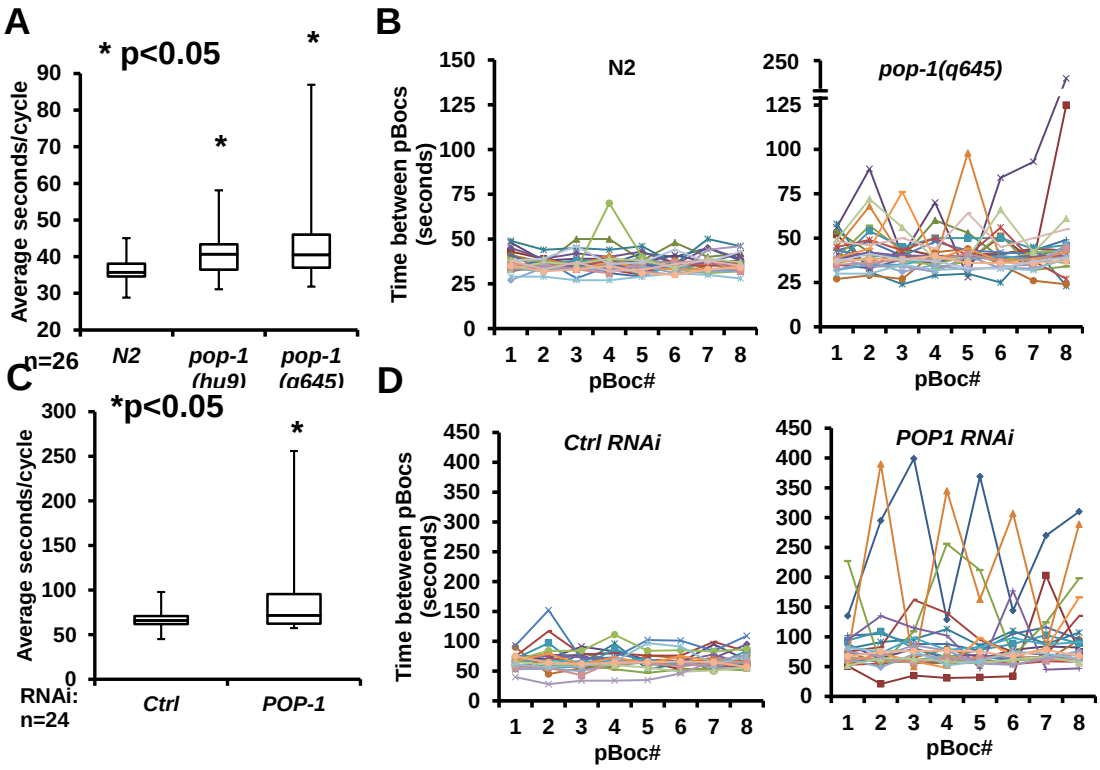

Figure 4.2. A novel POP-1 function in rhythmic defecation in *C.elegans* (From Bhambhani et al., 2014) (A) Box-whisker plot showing the median (line inside the box), third quartile (upper box), first quartile (lower box), longest pBoc cycle time (upper whisker limit) and shortest pBoc cycle time (lower whisker limit) for N2 controls and two pop-1 alleles at the L2 stage. A statistically significant increase was seen in the pBoc cycle time based on a Student's two-tailed t-est. (B) 8 individual pBocs (X-axis) were monitored (n = 26, each color representing one larva) for each genotype and plotted against time between each pBoc (y-axis). pop-1(q645) mutants have greater variability between pBocs than the wild-type N2 control. (C) Box-whisker plot showing the pBoc period of pop-1 depleted worms compared to ctrl RNAi worms using the OLB11 strain, which allows intestine-specific RNAi (McGhee et al., 2009; Pilipiuk et al., 2009). Animals were assayed at the young adult stage. A statistically significant increase was seen in the pBoc cycle time based on a Student's two-tailed t-test (D) 8 individual pBocs (X-axis) were monitored in young adults (n = 24, each color representing one adult) for each genotype and plotted against time between each pBoc (y-axis). pop-1 RNAi leads to a high variability in the cycle time inpop-1 depleted adults compared to controls.

egl-8

i)

gttcaagagctgtttgttaaattgtgagtttttcttctcaaaaataaaattactgacttt
tttgttgtaattgcagatttctgatatattggaaaatgtatttttcaaaaaaaaattgcc
ttaactaacagaaaatgtactattacaccgattattagtacttataatttttttttgaa
agttggaaaatattttaaagtttgtaaaaattctataaataatattgaatattataacgg
taaaatataatatttatagaatttttcaattttcaaaaaagtactgtttgtcatttgtt
tttgtttgttagaatacctttttaatagcggtaccgaaatctgggaaatattttcaaatga
ctccaaattttcgcctgattccgaatatctatgtgaaaaaaagtgaaaaaaaacatccct
tattttatatttcatttccaattcccaatttcatttgtgcccgcattacttttttcaaac
gcgcgcccaaataaattttcattagagcgcgtttgctttgtgtcgatttacgggagctct
ccatttacaaaaaaaattaacgctttttttaaacagtttttaagcgagtttcctacttttt
ttaatcgatttcagcggaacggtttttcttgaaatatcgtttttaaggtcaattgatatg
ataataaaatatattgttgaccttaaaacacattaaaaaaacttcgacaagaaccgacaa
aaagaagaaaaccggttgaaaacgataaagataaaacatttcgtaaatatacacaaatct
cgtaaatcgacacatggcgttttttggcgcgaaaattcggcgtttgaaaaacttttcaaaaag
tttcactgtcttatttggtaattatgtcaccgactggcgctgctccacctttaaaaata
ttttcctcttgcagatccggacagaaagagtacctaaccaaagagcgactcatcaatttt

ii)

tggctgtggataaatttaaggttagttagtgggtggcagaaacagatagcagccgatatt
atgttagtcctgcaatatatcggctgctgttttcaaaagaaggagtcgccatttttcgggtt
ggtcctgttatgtgtcgtttgctacattgaagtacttcaaaagagcagcggacactatgc
gggttagtcctggaacatgtcgactgtcaattagaagtggcagctgacatcttacgggag
agtcctgtgatgagtcggctgccaatttgtgtggcagcggacattatgggcctttgtttt
ctgctactccaaaaatcgttaacaaagttctgcaatacgtcggctgccatttaaaagtgg
cagctgacatttaacgagttagtccagcaatctgacgacagcgatttcgatgtagcagcc
gacatattgtgggttaattcggcaatgtgtcgggtgccatttcatagtggcagctgacat
ttcacgggtcagttcttcgatttatcgaatattactatcaagtagtggccggcatttttg
ctgctattcctaaaaatgcgtaaactagggtcttgcaatatgtcggctgccaaatcaaag
tagcagccgaccatccgcgttttcactacaaaaacatatcaaaacttttctctaagaccc
tggccaaactgactcgaacatttactcgaaagtcggcgtccgcttcgccagcccacccaa
aaatcgggatcggtcacccaccggctggctcagctccgaaaccttcaacatcagtcgggg
cacccgaacggcttcaggttaccccacctagaactccctctttctcaacttttttttca
aatgaatatgatcctcctgcaagtctcgtgaatgatcttatagatatccagttttttaaa
ctttccaatttcttgtgattttaaaaaccgagtaaatttttcaggttgacccaatcgaag

Figure 4.3. HMG-Helper site clusters in the putative POP-1 target *egl-8* (From Bhambhani et al., 2014). Genomic sequence of *egl-8* showing clusters i and ii with HMG (red) and Helper (blue) sites identified in the intronic regions of *egl-8*. The flanking exon sequences are highlighted in green.

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