Regulation and Function of Directly Repressed Targets of Wnt Signaling in *Drosophila*

by

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“Free your mind.”

-- Morpheus

-- The Matrix (1999)

To the amazing world

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Chapter I:

General Introduction

I. Wnt signaling

In multicellular organisms, cells communicate with each other through biochemical cascades known as signaling pathways. Wnt genes encode secreted, glycosylated, lipidated proteins that are ligands for one of these pathways (Willert and Nusse, 2012; Mikels and Nusse 2006). While Wnts are known to trigger several distinct signaling pathways (Gordon and Nusse, 2006), this thesis will focus on the so-called canonical Wnt signaling, which occurs through regulation of the intracellular levels and subcellular localization of β-catenin. Wnt/β-catenin signaling is repetitively used throughout animal development as well as in adult tissue regeneration and stem cell maintenance (Clevers et al., 2014; Logan and Nusse, 2004). Misregulation of Wnt/β-catenin signaling has been connected to several cancers (MacDonald et al., 2009), bone disease (Rudnicki and Williams, 2015; Leucht et al., 2008) and metabolic disorders (Sethi and Vidal-Puig, 2010).

While Wnts were discovered more than 30 years ago and have been intensively studied (Nusse and Varmus, 2012), many unanswered questions remain about how they influence cell fate and behavior. For example, there has been considerable progress on understanding the basic mechanisms of how Wnt/β-catenin

signaling regulates the transcription of Wnt targets (Cadigan, 2012; Cadigan and Waterman, 2012) but how this pathway regulates different genes in distinct tissues/cell types is still poorly understood (Bhambhani and Cadigan, 2014; Archbold et al., 2012).

My thesis work addressed the question of how Wnt signaling diversity is achieved by examining the Wnt-dependent regulation of *Tiggrin* (*Tig*), an essential *Drosophila* gene encoding an extracellular matrix protein. Unlike the vast majority of Wnt/β-catenin targets, which are activated by the pathway, Tig is directly repressed by Wnt stimulation. One chapter of my thesis deals with my progress towards understanding how Wnt signaling represses Tig expression. The subsequent chapter describes more recent work describing the role of Tig in influencing hematopoiesis in *Drosophila* larva.

Overview of Wnt/β -catenin signaling

Wnts proteins trigger diverse signaling pathways through binding to multiple types of cell-surface receptors, including the Frizzled (Fz) family of seven-pass transmembrane receptors and the Lipoprotein receptor-related protein 5 (LRP5) and LRP6 co-receptors, receptor tyrosine kinase Ror2, and the atypical tyrosine kinase Ryk (Green et al., 2014; van Amerongen and Nusse, 2008). Various receptors have been linked to β-catenin-dependent (canonical) β-catenin-independent (non-canonical) signaling. For example, Wnt binding to Fzs and LRP5/6 triggers stabilization and nuclear translocation of β-catenin to promote canonical signaling

(MacDonald and He, 2012).

While specific receptors are linked to canonical and non-canonical Wnt signaling, the same can not be said for Wnt ligands, though researchers often make this distinction. For example, Wnt5a is often called a non-canonical Wnt, based on its ability to activate β-catenin-independent pathways (Moon et al., 1993; Tada et al., 2002; Wallingford et al., 2001). However, Wnt5a can also induce secondary body axes in *Xenopus* embryos (He et al 1995), a commonly used readout for canonical Wnt signaling (Itoh and Sokol, 1998; McCrea et al., 1993). A clear line separating the Wnt family into two signaling groups should not be made; rather the combination of available Wnts and receptors determines the regulatory pathway used in a certain tissue (Willert and Nusse, 2012).

β-catenin shuttles between the cytosol and nucleus and plays a key role in Wnt/β-catenin signaling, in addition to being an essential factor in cadherin-based cell adhesion (Stepniak et al., 2009). Without Wnt stimulation, the cytoplasmic pool of β-catenin is phosphorylated and ubiquitinated by a complex containing Axin, Adenomatous polyposis coli (APC) protein, glycogen synthase kinase 3 (GSK3), casein kinase I (CKI) and the F-box protein β-TrCP (Cadigan and Piefer, 2009; Kennell and Cadigan, 2009). Wnt signaling compromises the activity of this "destruction complex" leading to accumulation of β-catenin (Cadigan and Piefer 2009; MacDonald et al., 2009). The Armadillo (Arm) repeats of β-catenin resemble those of β-importin, and several lines of evidence indicate that β-catenin has the intrinsic ability to translocate across the nuclear pore complex (Henderson and Fagotto, 2002;

Sharma et al., 2012). Thus, stabilized β-catenin enters the nucleus, a process that can be influenced by the concentrations of cytoplasmic tethers such as Axin and APC and transcription factors (TFs) that bind β-catenin (Tolwinski and Wieschaus, 2004; Zhang et al., 2011; Jamieson et al., 2012).

Once in the nucleus, β-catenin can bind to several TFs, the best characterized one being members of the TCF/LEF family (Behrens et al., 1996; Molenaar et al., 1996; Cadigan and Waterman, 2012). TCF/LEF is best known for its ability to activate Wnt target gene transcription, but it also represses transcription in the absence of signaling (Cavallo et al., 1998). Because of this dual regulation, TCF/LEF is often described as a transcriptional switch (Cadigan et al., 2012). Different sets of transcriptional cofactors are used at different signal levels to allow these regulations (Zhang and Cadigan, 2014). A model describing the canonical Wnt signaling is shown in Figure 1.1. The TCF-mediated gene regulation will be described in greater detail in the next section.

Wnt signaling in *Drosophila*

There are seven Wnts in the *Drosophila* genome, but only one, Wingless (Wg), seems to play a major role in development and accounts for almost all of the known Wnt signaling phenotypes. *wg* mutant embryos are lethal due to severe disruptions in embryonic patterning (Baker et al 1988), but mutations of the other Wnt genes display more subtle defects, e.g., partial lethality at the late pupal stage, male or female sterility (Kozopas et al., 1998; Cohen et al., 2002; Lim et al., 2005; Fradkin et

al., 2004; see http://web.stanford.edu/group/nusselab/cgi-bin/wnt/drosophila for a summary of mutant phenotypes). Spatial and temporal loss of Wg activity reveals that Wg participates broadly throughout development (Baker et al., 1988; Bejosvec and Martinez Arias 1991; Couso et al 1993). Many *wg* mutant phenotypes are also seen when *armadillo* (*arm*, the fly b-catenin) is mutated (Peifer et al., 1991; Brunner et al., 1997; Lai et al., 1997; Cadigan et al., 1998; Bejsovec, 2006), indicating that Wg signaling through the canonical pathway. I will hereafter refer to Wnt/β-catenin and Wg signaling as "Wnt signaling" for simplicity. Compared to the situation in mammals, where there are 19 known Wnts with overlapping and non-overlapping roles in development (Logan and Nusse, 2004; Chien and Moon, 2007), the study of Wnt signaling in *Drosophila* is simplified by the dominant role of the wg gene in activating the pathway.

Wg has been long been assumed to act as a morphogen, since a gradient of Wg protein is observed in tissues (Siegfried and Perrimon, 1994; Zecca et al., 1996; Neumann and Cohen, 1997). Evidence of transportation of Wg protein across epithelium cells have been described (Strigini and Cohen 2000; Kicheva et al., 2007; Gallet et al 2008; Yamazaki et al 2016). This gradient has been proposed to contribute to output diversity, as activation of Wg targets have been connected to different levels of Wg expression (Zecca et al 1996; Neumann and Cohen, 1997; Tomlinson, 2003). However, the biological importance of the gradient has been controversial (Sampedo et al., 1993; Pfeiffer et al., 2000). A group recently showed

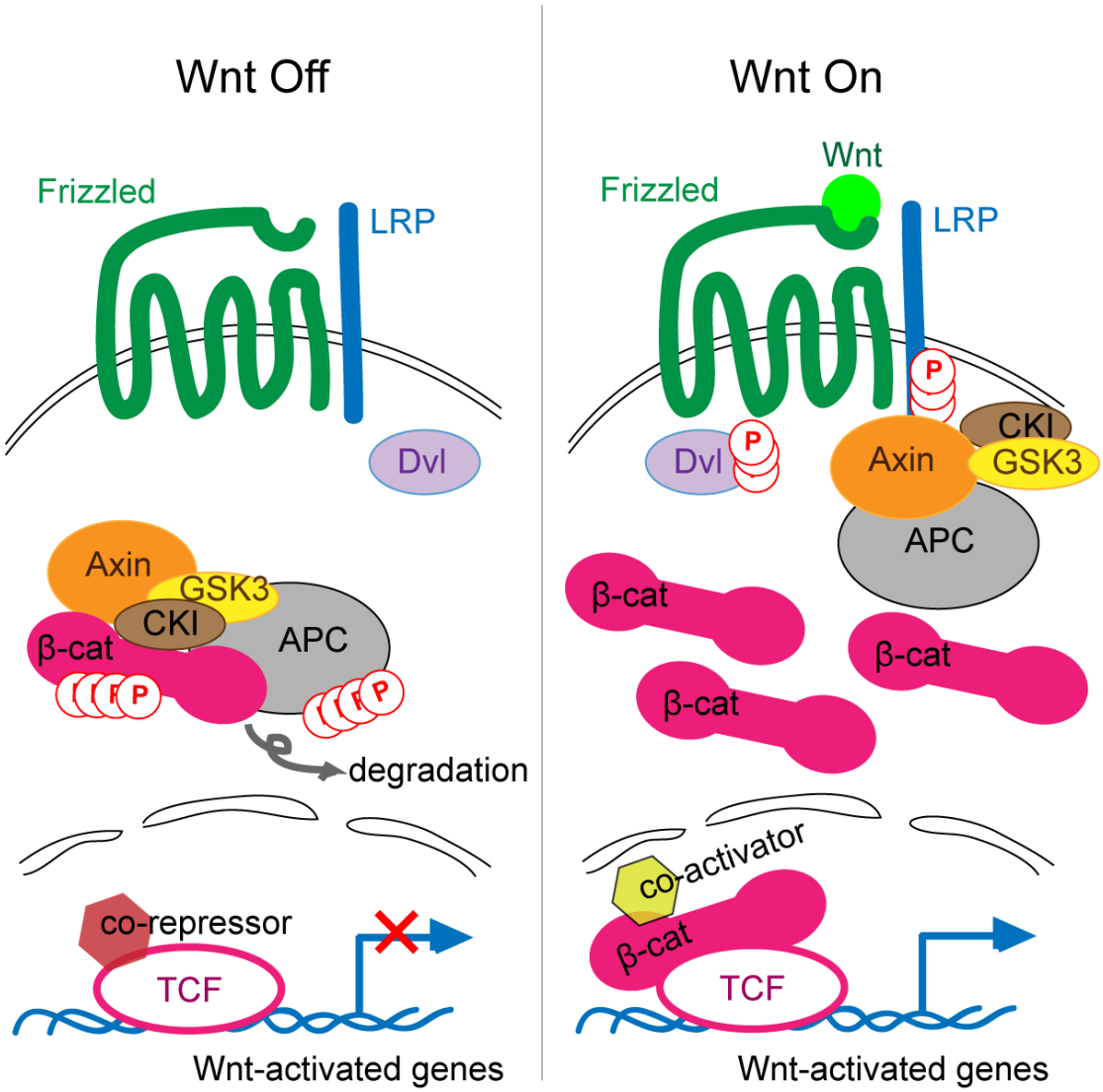

Figure 1.1. An oversimplified outline of the Wnt/β-catenin signaling pathway. (A) In the absence of Wnt ligand, a"destruction complex" containing APC, Axin, and GSK3 and CKI kinases targets β-catenin for proteosomal degradation. In the nucleus, TCF recruits co-repressors to the chromatin and inhibits target gene transcription. (B) Upon Wnt ligand binding, the destruction complex is inactivated, resulting in the stabilization and increased nuclear translocation of β-catenin. In the nucleus, β-catenin binds TCF and recruits transcriptional co-activators to the DNA controlling Wnt-activated genes. See text for more details. APC, adenomatous polyposis coli; β-cat: β-catenin; CKI, casein kinase I; Dvl, Dishevelled; GSK3, glycogen synthase kinase 3; LRP, lipoprotein receptor related protein; TCF, T-cell factor 1.

that replacing the endogenous Wg gene with one encoding a membrane-tethered protein via genome editing only caused slight developmental delay in flies, and target genes that are activated by the pathway several cell diameters away from Wg expressing cells was only partially reduced (Alexandre et al., 2014). One possible reason is that Wg exhibits a broader expression pattern at earlier developmental stages, which might contribute to the memory of target gene expression at the later observed stages (Baena-Lopez et al., 2009). The results of Alexandre and coworkers suggest that diffusion/transport of Wg away from its site of synthesis is likely not be a major factor in Wnt patterning and growth in *Drosophila* development.

In contrast to mammals, which have four TCF/LEF family members, there is a single *TCF* gene in *Drosophila* called dTCF, TCF or *pangolin* (*TCF/pan*) that mediates Wnt signaling. *TCF*/*pan* mutant embryos exhibit a qualitatively similar defect in embryonic epidermal pattering as *wg* mutants (Brunner et al., 1997; van de Wetering et al., 1997). Moreover, over-expressing a dominant-negative form of pan in different tissues results in phenotypes consistent with loss of Wnt signaling (van de Wetering et al., 1997; Johntson and Sanders, 2003; Collins and Treisman, 2000). The dominant-negative TCF/Pan lacks the N-terminal β-catenin/Arm interaction domain, and presumably works by competing with TCF/Pan for binding to Wnt regulatory DNA (van de Wetering et al., 1997). The available phenotypic data argues that in *Drosophila*, most Wnt signaling is triggered by Wg and Wnt target genes are regulated by Arm and TCF/Pan.

II. TCF-dependent transcriptional regulation in Wnt signaling

TCFs were first discovered as sequence-specific DNA binding proteins in lymphocytes (Laudet et al., 1993). The N-terminus of TCFs can bind to β-catenin and TCFs lacking this domain are potent inhibitors of Wnt signaling (e.g., Molenaar et al., 1996; van de Wetering et al., 1997). All TCFs contain a High Mobility Group (HMG) domain followed by a stretch of basic residues that contribute to DNA binding and bending (Giese et al., 1991; Love et al., 1995) and nuclear import (Prieve et al., 1998). Synthetic reporters containing TCF binding sites are activated by the pathway in many in vitro and in vivo contexts (Barolo, 2006). Genetic analysis of TCF genes in several invertebrate and vertebrate organisms cemented their position as important physiological regulators of Wnt/β-catenin signaling (Archbold et al., 2012; Cadigan and Waterman, 2012).

TCFs work as transcriptional switches in multiple organisms

The current working model for TCFs acting as transcriptional switches was derived from several lines of evidence. In *Drosophila*, *pan* mutants show similar but less severe defect compared to *wg* mutants, for example, in embryonic epidermal patterning (Brunner et al., 1997; van de Wetering, 1997). Interestingly, *wg, pan* double mutants appear to be very similar as *pan* single mutants, in other words, *wg* mutants was partially rescued by TCF/*Pan* mutants (Cavallo et al., 1998). This can be explained by the model that TCF/*pan* works as a transcriptional switch: TCF/*pan* represses target transcription in the absence of signaling and activates transcription

in the presence of signaling. In *wg* mutants, TCF/*pan* still represses transcription, results in a large reduction of transcriptional activity and a more severe phenotype. In *pan* mutants, however, target genes get derepressed and transcriptional activity is not totally lost. A similar rescue was also observed in *wg*, *groucho* (*gro*) double mutants (Cavallo et al., 1998), and knockdown of Gro derepresses target gene expression (Fang et al., 2006; Mieszczanek et al., 2008). Gro belongs to the Transducin-like Enhancer of Split (TLE) family of co-repressors, which directly bind to TCFs (Roose et al., 1998). In addition, while mutation of TCF binding sites in reporters for Wnt-dependent cis-regulatory modules (W-CRMs, our favored term for Wnt regulated enhancers, also called Wnt responsive elements or WREs) reduces their expression in transgenic fly tissues, sometimes there is an accompanying expansion of reporter gene expression (Lee and Frasch, 2000; Yang et al., 2000). A model summarizing these data is shown in Figure 1.2. In the absence of Wnt signaling, TCF/Pan and Gro repress (along with other factors discussed in the following sections) target gene expression (Figure 1.2A). When signaling promotes nuclear accumulation of β-catenin, it binds to TCF/Pan, displacing Gro and recruiting co-activators to activate Wnt targets (Figure 1.2A').

While the classic switch model outlined above has been a useful paradigm for understanding Wnt target gene regulation, it does not universally apply to targets in all organisms. In *C. elegans*, there is abundant evidence that a distinct type of switch occurs involving POP-1 (the single worm TCF) and Sys-1 (one of the four worm β-catenins). This alternative switch is often referred to as the "Wnt/β-catenin

asymmetry pathway" due to its prevalence in regulating asymmetric cell divisions in worm development. This pathway has been reviewed in detail elsewhere (Phillips and Kimble 2009; Sawa, 2012) and is briefly summarized here. In addition to nuclear accumulation of Sys-1, this pathway also requires nuclear efflux of POP-1 for Wnt targets to be activated. This efflux is mediated by binding of the β-catenins Wrm-1 to POP-1, along with phosphorylation by Lit-1, a Nemo-like kinase (NLK) (Phillips and Kimble 2009; Sawa, 2012). This POP-1 efflux is required to enhance the formation of POP-1-Sys-1 complexes on Wnt target gene chromatin (Figure 1.2B-B'). The genetic evidence in worms indicates that this asymmetry pathway plays several important roles in *C. elegans* cell fate specification, but it is not yet clear how important this pathway is in other animals (Phillips and Kimble 2009; Cadigan, 2012).

Flies and worms have only one TCF gene each with little isoform diversity (Archbold et al., 2012; Cadigan and Waterman, 2012). This implies that a single species of TCF acts in both sides of the transcriptional switches outlined above. The situation is more complicated in vertebrates, where amphibians and mammals have four TCF genes, TCF1, LEF1, TCF3 and TCF4, also known as TCF7, LEF1, TCF7L1 and TCF7L2, respectively. Bony fish have two closely related TCF3 genes in addition to the other three TCFs (Dorsky et al., 2003). Loss of function analysis suggests that the vertebrate TCFs are more specialized for repression or activation than invertebrate TCFs. TCF3 appears to function solely as a repressor (Kim et al., 2000; Merrill et al., 2004; Liu et al., 2005) while LEF1 appears to be an activator (van

Genderen et al., 1994; Reya et al., 2000; Kratochwil et al., 2002). The data for TCF1 and TCF4 suggest that these TCFs retain both functions (Korinek et al., 1998; Galceran et al., 1999; Roose et al., 1999; Tang et al., 2008; Nguyen et al., 2009). However, these genes can produce truncated isoforms lacking the β-catenin binding domain, which can function as inhibitors of the pathway (Vacik and Lemke, 2011). Indeed, in colorectal tumor cells, which possess elevated Wnt/β-catenin signaling (Polakis, 2012), there is an enrichment of "full length" TCF1 isoforms, where normal tissue expresses mostly the truncated TCF1 (Najdi et al., 2009). This may explain why TCF1 behaves as an intestinal tumor suppressor in mouse knockouts (Roose et al., 1999). A morpholino specific for the dominant negative TCF4 isoform revealed a biologically important role for this truncated TCF4 in antagonizing Wnt signaling during *Xenopus* embryogenesis (Vacik et al., 2011). Development of more isoform-specific inhibitors of TCF1 and TCF4 will be needed to better understand how these TCFs regulate the Wnt pathway.

Mutagenesis of some vertebrate W-CRMs clearly suggests that they are both negatively and positively regulated by TCFs (Brannon et al., 1997; Hikasa et al., 2010). This raises the possibility that multiple TCFs are involved in a transcriptional switch. Wnt signaling stimulates the phosphorylation of TCF3 in frog embryos, which inhibits its ability to associate with target gene chromatin (Hikasa et al., 2010; Hikasa et al., 2011). This phosphorylation occurs through Homeodomain interacting kinase 2 (HIPK2). TCF1 lacks HIPK2 phosphorylation sites (Hikasa et al., 2011), supporting the "TCF exchange" model outlined in Figure 1.2C-C'.

In mouse embryonic stem cells (mESCs), TCF3 promotes differentiation by repressing pluripotency genes (Sokol, 2011, Merrill, 2012). TCF1 functions antagonistically with TCF3 in this process (Yi et al., 2011), suggesting that some Wnt targets may undergo a TCF exchange as described in frogs, through this remains to be demonstrated. TCF3 expression is also inhibited by Wnt/β-catenin signaling in mESCs (Atlasi et al., 2013; Shy et al., 2013), providing another variation on how multiple TCFs can regulate Wnt targets in vertebrate cells.

As described above, many Wnt targets and W-CRMs are both repressed and activated by TCFs in the absence and presence of Wnt signaling, respectively. However, it should be pointed out that many W-CRMs have little detectable TCF repression in the absence of signaling, based on TCF site mutagenesis (e.g., Yamaguchi et al., 1999; Galceran et al., 2004; Lam et al., 2006; Chang et al., 2008b). Most likely, these W-CRMs lack sequences for general activators, so that they have little basal activity whether TCF is bound or not, but require TCF and β-catenin for activation (Archbold et al., 2012). Wnt targets can also have multiple W-CRMs controlling their transcription, as has been found for the *naked cuticle* gene in flies (Chang et al 2008a) and *c-myc* in humans (He et al., 1997; Yochum et al., 2008; Pomerantz et al., 2009; Wright et al., 2010). Due to these complexities, the generic situations outlined in Figure 1.2 may be an oversimplification.

Co-regulators of TCF/β-catenin transcription

There are a large number of nuclear factors that negatively or positively

influence Wnt target gene regulation. The intention of the following section is to provide a brief outline of some of the mechanisms by which these co-regulators operate. The co-repressors and co-activators refer to factors that are found on W-CRMs often through binding to components of the Wnt pathway, but chromatin modifiers and remodelers which regulate transcription through a more direct mechanism are discussed separately. A list of many of these factors are shown in Tables I and II and more comprehensive reviews can be found elsewhere (Willert and Jones, 2006; Mosimann et al., 2009; Cadigan, 2012; Valenta et al., 2012).

Factors and mechanisms repressing Wnt target genes

In general, the described mechanisms for repressing Wnt target gene transcription fall into two main categories: recruitment of co-repressors and histone deacetylases (HDACs) to W-CRMs, and factors that block TCF binding to β-catenin or DNA. Examples are discussed below.

Several Wnt target gene co-repressors act by direct binding to TCFs. The best studied case is members of the Gro/TLE family, which repress Wnt target gene expression in flies, worms and vertebrate systems (reviewed in Cadigan, 2012). Gro/TLEs bind to many other TFs besides TCFs and repress transcription by recruiting HDACs to target gene chromatin (Turki-Judeh and Courey, 2012). Other TCF co-repressors include Co-repressor of Pangolin (Coop) in *Drosophila* (Song et al., 2010) and members of the Myeloid Translocation Gene (MTG) family in mammals (Moore et al., 2008; Barrett et al., 2012). Like Gro/TLE, these proteins compete with β-catenin for binding to TCFs (Daniels and Weis, 2005; Arce et al., 2009; Song et al.,

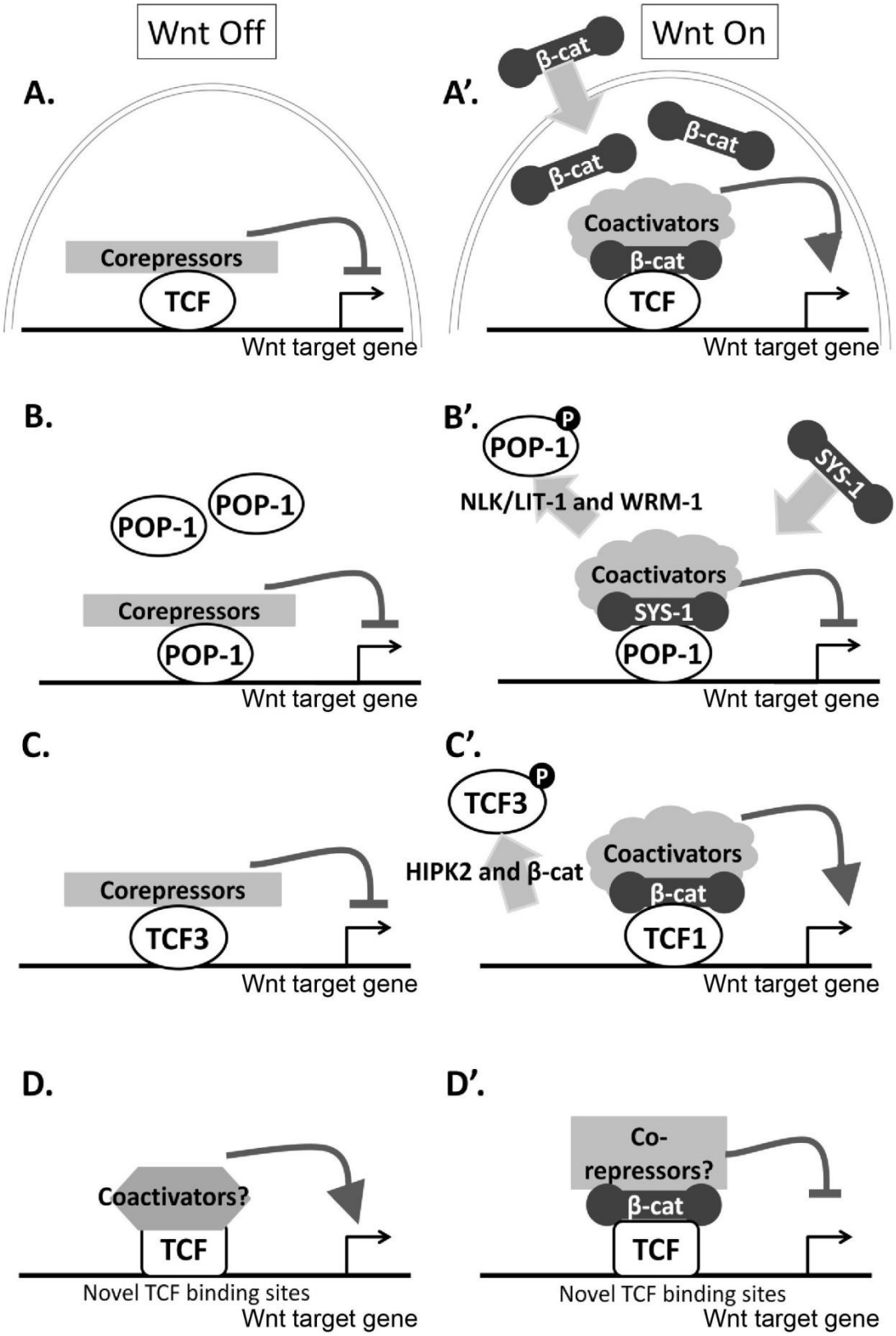
Wnt Off
A.
Corepressors
TCF
Wnt target gene
Wnt On
A'.
β-cat
β-cat
β-cat
Coactivators
β-cat
TCF
Wnt target gene
B.
POP-1
POP-1
Corepressors
POP-1
Wnt target gene
B'.
POP-1
P
NLK/LIT-1 and WRM-1
SYS-1
Coactivators
SYS-1
POP-1
Wnt target gene
C.
Corepressors
TCF3
Wnt target gene
C'.
TCF3
P
HIPK2 and β-cat
Coactivators
β-cat
TCF1
Wnt target gene
D.
Coactivators?
TCF
Novel TCF binding sites
Wnt target gene
D'.
Co-
repressors?
β-cat
TCF
Novel TCF binding sites
Wnt target gene

Figure 1.2. Different types of TCF transcriptional switches. Depiction of a Wnt target gene and surrounding nucleoplasm in the absence or presence of Wnt/β-catenin signaling. **(A-A')** Summary of the TCF transcriptional switch in *Drosophila* cells. In the absence of Wnt signaling, nuclear β-catenin (β-cat) level is low, and TCF/Pan recruits co-repressors such as Gro to the chromatin and inhibits transcription. In the presence of Wnt signaling, nuclear β-catenin level increases, which overcomes the TCF co-repressors and binds to TCF/Pan on target gene chromatin. β-catenin recruits a variety of co-activators to activate gene transcription. **(B-B')** Summary of the TCF transcriptional switch in the Wnt/β-catenin asymmetry pathway in *C. elegans*. In unstimulated cells, high levels of TCF/POP-1 in the nucleus recruits co-repressors to the chromatin and inhibit transcription. Wnt signaling increases the nuclear concentration of Sys-1, the worm β-catenin, and lowers the level of POP-1 through its phosphorylation via the Lit-1 kinase, which promotes nuclear efflux. The interaction between POP-1 and Sys-1 also shifts the equilibrium on chromatin from POP-1 to POP-1-Sys-1 complexes, resulting in transcriptional activation. **(C)** Summary of the TCF exchange between TCF3 and TCF1 on Wnt targets in *Xenopus* embryos. In cells with low nuclear β-catenin, TCF3 represses Wnt target gene transcription. Wnt signaling activates HIPK2, which acts with β-catenin to phosphorylate TCF3, removing it from target gene chromatin, where it is replaced by TCF1, which activates target gene transcription. **(D)** Summary of the reverse switch mechanism used for Wnt-mediated repression of target genes in *Drosophila*. TCF binds to untraditional TCF binding sites and activates transcription through unknown co-activators in the absence of Wnt signaling. In the presence of signaling, target transcription is inhibited by TCF and β-catenin via unknown co-repressors. **(A-D)** Wnt signaling is inactivated. **(A'-D')** Wnt signaling is activated.

2010; Moore et al., 2008). This means in addition to their role as co-repressors, they may prevent low levels of nuclear β-catenin from inappropriately activating Wnt targets.

After β-catenin recruitment to W-CRMs, co-repressors can still influence Wnt target expression. For example, Reptin/TIP49b directly binds β-catenin, and represses gene expression via its DNA helicase activity (Rottbauer et al., 2002). The Nuclear receptor co-repressor (NCoR) and Silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) co-repressors can bind to the β-catenin-TCF heterodimer and recruit HDACs to W-CRMs (Song and Gelmann, 2008). These factors could act to dampen the amplitude of target gene expression levels in cells receiving Wnt stimulation.

In addition, some co-repressors act on W-CRM chromatin in parallel with TCF. In *Drosophila* cells, C-terminal binding protein (CtBP) is recruited to Wnt targets independently of TCF/Pan, presumably through other transcriptional repressors (Fang et al., 2006). In vertebrate systems, CtBP has been reported to bind directly to TCFs (Brannon et al., 1999; Valenta et al., 2003), but other reports do not see this interaction (Hamada and Bienz, 2004; Valenta et al., 2006). In *Xenopus* embryos, Kaiso and TCF bind to W-CRMs in close proximity and repress transcription (Park et al., 2005; Park et al., 2006; Hong et al., 2010). The importance of Kaiso in regulating Wnt targets has been questioned (Ruzov et al., 2009a; Ruzov et al., 2009b) and Kaiso has also been reported to activate Wnt targets in *Xenopus* (Iioka et al., 2009). Likewise, CtBP can also directly activate Wnt targets in flies (Fang et al.,

2006). This bimodal regulation is not uncommon for Wnt regulators (see Tables I & II), though the molecular mechanism is usually unknown. In the case of CtBP, its oligomerization status detemines whether it will repress (oligomeric) or activate (monomeric) Wnt targets (Bhambhani et al., 2011).

CtBP can also inhibit Wnt signaling by diverting β-catenin away from TCF, in a complex with APC (Hamada and Bienz, 2004). APC binds β-catenin and has been proposed to promote its nuclear efflux (Brocardo and Henderson, 2008) and is thought to act on W-CRM chromatin to remove β-catenin from TCF activating complexes (Sierra et al., 2006). Other proteins that bind β-catenin and prevent it from associating with TCFs include Inhibitor of β-catenin and TCF4 (ICAT) (Tago et al., 2000; Hasegawa et al., 2007), Sry-type HMG box containing protein 9 (Sox9) (Akiyama et al., 2004; Topol et al., 2009) and Chibby (Cby; Takemaru et al., 2003; Li et al., 2008; Love et al., 2010). Depletion of Cby in *Drosophila* embryos via RNAi can partially rescue *wg* but not *arm* mutants (Takemaru et al., 2003), suggesting that even in the absence of Wnt signaling, there is some β-catenin in the nucleus with the potential for activating Wnt targets. The significance of Cby in fly development has recently been challenged, since *Cby* null mutants do not have Wg-related phenotypes (Enjolras et al., 2012). Nonetheless, it seems likely that there are several "TCF-β-catenin buffers" in the nucleoplasm that help set the threshold for how much β-catenin is required to convert TCFs to transcriptional activators (Figure 1.2A').

Factors and mechanisms activating Wnt target genes

Once β-catenin binds to TCF and displaces or otherwise overcomes the aforementioned negative regulators, it serves as a “landing platform” for a variety of transcriptional co-activators (Table II). Many of these factors can be roughly divided into four categories: 1) factors that facilitate TCF-β-catenin interaction, 2) co-activators bound to the N-terminal transactivation domain of β-catenin, 3) co-activators bound to the C-terminal transactivation domain and 4) chromatin modifying complexes recruited to W-CRMs by β-catenin. Here I briefly describe the basic features of these proteins.

Although the N-terminus of TCF is sufficient for interaction with the Arm repeats of β-catenin in vitro (van de Wetering et al., 1997; Behrens et al., 1996; Graham et al., 2000; Poy et al., 2001), there are additional factors that are necessary for association of these proteins in vivo. Transducin-like protein 1 (TBL1) and TBL1-related protein (TBLR1), which are subunits of the SMRT-NCoR co-repressor complex, have a distinct function in recruiting β-catenin to W-CRMs, with TBL1 binding to both TCFs and β-catenin (Li and Wang, 2008). TBL1 and TBLR1 are SUMOylated upon Wnt signaling, which releases them from the SMRT-NCoR complex, allowing them to promote β-catenin recruitment to Wnt targets (Choi et al., 2011). The RING Finger Protein 14 (RNF14) binds to TCFs and is required for β-catenin recruitment to several vertebrates W-CRMs (Wu et al., 2013). While TBL1, TBLR1 and RNF14 appear to be general promoters of Wnt/β-catenin signaling, the Centromere Binding Protein B (CENPB) domain protein Jerky/Earthbound 1

(Jerky/Ebd1) also functions as a β-catenin-TCF adaptor, but only in specific *Drosophila* tissues (Benchabane et al., 2011).

β-catenin contains at least two domains capable of activating transcription when fused to DNA binding domains (reviewed in Mosimann et al., 2009). Several factors that bind to the N-terminal transactivation domain have been reported (Table II) but the best characterized is known as Legless (Lgs) in flies and B cell lymphoma 9 (Bcl9) and Bcl9-2 in mammals (Mosimann et al., 2009). These proteins bind to the first Arm repeat in β-catenin (Hoffmans and Basler, 2004; Valenta et al., 2011) serving as an adaptor between β-catenin and Pygopus (Pygo) proteins (fly Pygo and mammalian Pygo1 and Pygo2) (Kramps et al., 2002). Lgs and Pygo are essential for Wnt signaling in flies (Kramps et al., 2002; Thompson et al., 2002; Parker et al., 2002; Belenkaya et al., 2002) and are also significant contributors to the pathway in mice (Schwab et al., 2007; Brack et al., 2009; Gu et al., 2009). The Pygo proteins (fly Pygo and the mammalian Pygo 1 and Pygo 2) are thought to activate transcription by interacting with subunits of the mediator complex (Carrera et al 2008), basal transcription factors (Wright and Tjian, 2009), CBP (Andrews et al., 2009) and the Mixed lineage leukemia 2 (MLL2) histone methyltransferase (Chen et al., 2010). While clearly a major mediator of N-terminal transactivation by β-catenin, mouse embryos carrying a point mutation in β-catenin (D164A), which abolishes BCL9/BCL9-2 binding, display more severe defects than BCL9/BCL9-2 double mutants (Schwab et al., 2007; Valenta et al., 2011). These results suggest that additional co-activators utilize this region of β-catenin to activate Wnt targets.

The C-terminal transactivation domain consists of the last three Arm repeats and the adjacent C-terminus of β-catenin (Willert and Jones, 2006; Mosimann et al., 2009). Several co-activators have been found to bind directly with this domain (Table II). Consistent with a role for HATs in Wnt target gene activation, Wnt/β-catenin signaling promotes an increase in acetylated histones at Wnt targets (Kioussi et al., 2002; Sierra et at., 2006; Parker et al., 2008). Since factors binding to the transactivation domain are often chromatin regulators that are general co-activators involved in the regulation of many genes (Goodman and Smolik, 2000; Sudarsanam and Winston, 2000), studying the specific contribution of these factors to Wnt gene activation is difficult. However, clonal analysis in flies and partial knockdown by RNAi have demonstrated specific role in Wg/Wnt signaling for CBP (Li et al., 2007; Parker et al., 2008). Likewise, siRNA of Brg-1 and p300 in mammalian cell culture results in loss of regulation of Wnt targets, as well as many non-Wnt targets (Mahmoudi et al., 2010). In addition, a small molecule (ICG-001) that blocks the interaction between β-catenin and CBP inhibits several Wnt/β-catenin readouts (Ma et al., 2005; Henderson et al., 2010). Emerging techniques such as engineered CRISPER/Cas-9 can direct transcriptional co-activators to specific endogenous genomic loci (Konermann et al., 2015), but it requires overexpression of co-activators and would only work with the assumption that the factor(s) being over-expressed is sufficient for gene activation.

In addition to histone acetylation, several other chromatin marks and the enzymes that catalyze them have been linked to gene activation by the Wnt pathway

(Table III). It is not clear how consistently chromatin modifications occur among different Wnt targets, e.g., for some targets, there is no change in histone acetylation upon Wnt signaling (Wohrle et al., 2007; Blythe et al., 2010). In constrast, a microarray study in HEK293T cells demonstrated that most genes that are activated by Wnt3a treatment required DOTL1 for this regulation (Mahmoudi et al., 2010).

With so many factors connected with β-catenin and TCF on either the ON or OFF side of the transcriptional switch, it is difficult to envision them all working simultaneously. This has led to suggestions of co-activator cycling on and off β-catenin (Mosimann et al., 2009; Valenta et al., 2012). Indeed there is some evidence for cycling of negative and positive regulators on the c-myc W-CRM (Sierra et al., 2006). While co-regulator dynamics is likely occurring on W-CRMs, another consideration is whether all identified factors act on every Wnt target. There are some clear examples of tissue-specific regulators, e.g., Osterix, an osteoblast-specific TF that binds to TCFs and inhibits their ability to bind DNA (Zhang et al., 2008) and Jerky/Ebd which is only required for Wg/Wnt signaling in a few fly cell types (Benchabane et al., 2011). For most Wnt co-regulators, their involvement in other pathways or possible redundancy with related proteins makes it more difficult to assess whether they are general or gene/cell specific Wnt factors.

Other TFs that mediate Wnt signaling

The genetic data in Drosophila suggests that TCF/Pan mediates most Wnt signaling in this organism, at least during embryonic and larval development (van de Wetering et al, 1997; Brunner et al., 1997). However, the overall importance of

TCFs for the pathway in vertebrate is less clear. Conditional deletion of β-catenin has revealed numerous developmental phenotypes in mice (Grigoryan et al., 2008), but only a limited number can be unambiguously linked to TCFs (e.g., van Genderan et al., 1994; Kratochwil et al., 2002; Korinek et al., 1998; Galceran et al., 1999). While this may be due to redundancy and the repressive properties of some TCFs, the other possibility is that additional TFs can also recruit β-catenin to their respective enhancers. Indeed, the list of TFs with this function is large and diverse (Table III) and has been reviewed in detail elsewhere (Beildeck et al., 2010; Archbold et al., 2012, Cadigan and Waterman, 2012; Valenta et al., 2012). In one recent report, RNAi based screens in human cancer cells with elevated Wnt signaling identified the T-box protein Tbx5 and the co-activator Yes-associated protein 1 (YAP1) as β-catenin binding proteins. When YAP1 is phosphorylated by the tyrosine kinase YES1, the YAP1-β-catenin-Tbx5 complex associated with and activated antiapoptotic genes (Rosenbluh et al., 2012). A YES1 inhibitor dramatically reduced growth of β-catenin-dependent cancer cells and tumors (Rosenbluh et al., 2012), providing a dramatic example of how β-catenin can act through non-TCFs to affect cell behavior.

In addition to recruiting β-catenin to their respective target genes, the aforementioned TFs can also divert β-catenin away from TCFs, inhibiting TCF-dependent gene expression. This appears to be a biologically important function of Hypoxia induced Factor 1 (HIF1) and Forkhead box (FOX) proteins during hypoxia and oxidative or nutritional stress (Kaidi et al., 2007; Almeida et al., 2007; Hoogeboom et al., 2008; Liu et al., 2011).

There is also a growing list of TFs that interact with TCFs on chromatin. In some cases, this appears to be a mechanism for enhancers to integrate information from Wnt and other signaling pathways, e.g., serum growth factor signaling via c-Jun-TCF interactions (Nateri et al., 2005, Yochum et al., 2008) or bone morphogenetic protein (BMP) signaling via Smad-TCF binding (Eivers et al., 2009; Itasaki and Hoppler, 2010). Recent ChIP-seq data suggests that TCF occupancy is heavily influenced in distinct cell types by co-localization with other TFs, some of which bind directly to TCFs (Bottomly et al., 2010; Trompouki et al., 2011; Junion et al., 2012; Frietze et al., 2012).

Adding to the complex nature of transcriptional responses to Wnt signaling, β-catenin is not the only transcriptional regulator whose stability is controlled by the β-catenin destruction complex. The transcriptional repressor Snail is phosphorylated by GSK3 and undergoes β-TrCP ubiquitination and proteasomal degradation (Zhou et al., 2004; Yook et al., 2005). Down-regulation of this process by Wnt leads to increased Snail levels, which can promote epithelial-mesenchymal transitions (Yook et al., 2005). More recently, Transcriptional activator with PDZ binding motif (TAZ), a relative of YAP1 and an important transcriptional co-activator in the Hippo signaling pathway that controls cell proliferation and survival (Pan, 2010), has been reported to be targeted for degradation by the β-catenin destruction complex (Azzolin et al., 2012). Wnt stimulation leads to accumulation of nuclear TAZ, and transcriptome analysis revealed that the majority of Wnt targets in a human breast cancer cell line were TAZ-dependent (Azzolin et al., 2012). These examples

make it clear that Wnt researchers have to look beyond the classic Wnt-β-catenin-TCF axis when considering how Wnts affect gene expression.

III. DNA binding by TCFs – bipartite recognition with considerable flexibility

People are still learning more about how TCF recognizes DNA sequences more than two decades after its discovery (van de Wetering et al., 1991; Travis et al., 1991; Waterman et al., 1991). Early work characterizing DNA sequences bound to a certain protein often employed methods strongly biased for high affinity binding sites, e.g. systematic evolution of ligands by exponential enrichment (SELEX) (Oliphant et al., 1989; Tuerk et al., 1990). A high affinity TCF binding site (CCTTTGATC) was discovered using such method (van de Wetering et al., 1997). Detailed investigations on single W-CRMs revealed more diversity in functional TCF binding sites (Barolo, 2006; Archbold et al., 2012). This fits with TF-DNA in general, where systematic surveys have revealed that most TFs bind to many sequences that can be significantly distinct from the high affinity consensus sequence (Badis et al., 2009; Hume et al., 2015; Narasimhan et al., 2015). The challenge for future studies is to better understand the biological relevance of a TF's numerous "secondary" binding sites.

The HMG DNA binding domain on TCF

All TCFs contain a highly conserved HMG domain that binds specific DNA sequences. Its high affinity binding site in vitro, 5'-CCTTTGATS-3' (S=C/G), termed

"HMG site" (or "TCF site" before the discovery of the Helper site, see next page) (Hallikas et al., 2006; van Beest et al., 2000; van de Wetering et al., 1997), has been found in many W-CRMs and shown to be both necessary and sufficient for Wnt-induced transcription in cell culture and model organisms (Korinek et al., 1997; DasGupta and Fuchs, 1999; Dorsky et al., 2002; Maretto et al., 2003; Nakaya et al., 2005). In addition to its DNA binding ability, HMG domain also bends DNA through contacts with specific nucleotides in the minor groove, which has been suggested to affect regulatory complex assembly on the W-CRM (Love et al., 1995; Giese et al., 1995).

High affintity TCF/HMG binding sites can be sufficient for driving Wnt signaling dependent transcriptional activation. Synthetic reporters containing multimerized HMG sites upstream of a basic promoter used for detecting the presence of Wnt signaling (Barolo, 2006). These reporters typically contain 3-12 copies of high affinity HMG sites. Although such reporters are sensitive to Wnt signaling in some contexts, they often do not faithfully respond to all endogenous Wnt signals. For example, HMG site reporters fail to reproduce the endogenous Wg expression pattern in fly embryos and larval imaginal discs (Chang et al., 2008a; Barolo, 2006). Combined with the fact that endogenous W-CRMs do not contain high density clusters of high affinity TCF/HMG binding sites (Archbold et al 2012), it seems likely that additional DNA sequences are required to confer Wnt responsiveness onto a W-CRM.

The C-clamp domain recognizes Helper sites

One mechanism that some TCFs employ to increase its DNA binding specificity is through a second sequence-specific DNA binding domain, the C-clamp domain. It is a highly conserved domain comprised by a stretch of about 30 amino acids located to the C-terminus of HMG domain, including 4 cysteine residues that coordinate a Zinc ion and are required for its DNA binding ability (Atcha et al., 2007; Ravindranath and Cadigan, 2014). Domains similar to the C-clamp in TCFs have also been noted in other proteins including HDBP1, HDBP2, and Gig1 (Tanaka et al., 2004), but their function has not been extensively studied. The C-clamp recognizes CG-rich DNA motifs called Helper sites, and the consensus is GCCGCCR (R = A/G) in *Drosophila* and GCSGS in mammals (Chang et al., 2008a; Hoverter et al., 2012). This interaction greatly augments in vitro interaction between the HMG domain and HMG sites (Atcha et al., 2007; Chang et al., 2008a), and is essential for the activity of many W-CRMs from vertebrates, *Drosophila* and *C. elegans* (Atcha et al., 2007; Hoverter et al., 2012; Chang et al., 2008a; Zhang et al., 2014; Bhambhani et al., 2014). These data supports a model where TCF recognizes DNA through bipartite HMG domain/HMG site and C-clamp/Helper site interactions.

The C-clamp has a broad impact on TCF function in *Drosophila*, as only WT but not a mutant TCF that cannot bind Helper sites rescues the patterning deficiency in TCF loss-of-function embryonic epidermis of *Drosophila* (Ravindranath and Cadigan, 2014). This is consistent with that C-clamp is present in the major TCF isoform in *Drosophila* at multiple developmental stages and in multiple tissues

(http://www.modencode.org/). The E-isoforms of two of the vertebrate TCFs, TCF1 and TCF4 also contain C-clamp (Atcha et al., 2007). Half of the target genes repressed by dominant-negative TCF1E in mammalian cell culture are no longer repressed when the DNA binding ability of C-clamp is abolished by surgical mutations, some of which are likely direct targets of TCF according to previous TCF and beta-cat ChIP-seq data (Hoverter et al., 2012; Bottomly et al., 2010). For the TCFs not containing a C-clamp domain, TFs bound to adjacent DNA sequences might help TCF to locate the W-CRM (Bhambhani and Cadigan, 2014; Archbold et al., 2014).

Helper site was named because, unlike HMG sites, synthetic reporter containing only multimerized Helper sites exhibit no detectable response to Wnt signaling (Chang et al., 2008a). But besides simply increasing DNA binding affinity and reporter sensitivity, Helper sites might also qualitatively refine the tissue specificity of Wnt response. For example, POP-1 (the worm TCF) is required for the proper function of int-9 cells in *C. elegans* which controls the worm defecation cycle, but reporter expression in int-9 cells is only observed when Helper sites are added to a TOPFLASH-style (HMG site-only) reporter (Bhambhani et al., 2014). In addition, although TCF is remarkably flexible with the spacing and orientation between HMG site and Helper site, certain combinations display tissue-specific patterns in Drosophila (Archbold et al., 2014). These data suggest that Helper sites play important role in setting the threshold for Wnt activation and tissue responsiveness.

Degeneracy in binding sites of TCF and other TFs

Although the classic HMG site consensus has been defined for decades, more and more degeneracy are found in validated functional HMG sites as individual W-CRMs are investigated in detail (Chang et al., 2008a; Knirr and Frasch, 2001; Lee and Frasch, 2000; Lam et al., 2006). In particular, with a single nucleotide different from the well accepted consensus (CCTTTCATG, underlined is the different nucleotide), an HMG site in a distant W-CRM of *c-myc* gene shows considerable affinity to TCF4 in vitro and has been connected to colorectal cancer as a risk allele (Tuupanen et al., 2009; Wright et al., 2010). In *Drosophila*, functional HMG sites can bear 20-fold difference in TCF affinity in vitro (Archbold et al., 2014). And there are HMG sites (AGAWAW) that bear no resemblance to the classic consensus, and these sites mediate Wnt/beta-catenin-dependent repression (Blauwkamp et al., 2008). These studies present a broad variety of functional TCF-binding sites and argue that the highest affinity sites determined in vitro might not always be preferred in target finding in vivo.

When methods unbiased for high affinity sites are used, systemic analysis of binding sites in vitro reveals deep binding site degeneracy for many TFs. Using an algorithum based on clustering, affinity, and conservation, CRMs containing imperfect sites for TFs GLIs 1–3, Tcf4, and c-Ets1 are widely predicted and some are validated in transgenic mouse embryos (Hallikas et al., 2006). While this study focuses on binding site with 0-1 substitution from the common consensuses, a complete spectrum of binding site affinity for 104 TFs representing 22 structural

classes can be obtained when the affinity of all possible 8 bp DNA sequences to a certain TF was evaluated by tiled arrays (Badis et al., 2009; Berger and Bulyk, 2009). Almost all TFs being tested exhibit a wide and continuous range of binding site affinity. Interestingly, when high affinity sites are hierarchically clustered, a second consensus ("secondary motif") is observed for nearly half of the TFs. While functional validation is often lacking for this type of study, these results argue that the interaction between proteins and their DNA binding sites could be more flexible than the classic understanding of "one protein recognizes one stringent consensus".

What is the benefit of using lower affinity sites for TF recruitment? One possibility is that variation in TF binding site affinity contributes to the output diversity of transcription. Supporting this, when one of the HMG-Helper site pairs in the nkdUPE2 W-CRM is altered into a high affinity pair, the reporter becomes highly activated in tissues that show no detectable activity of the original reporter (Archbold et al., 2014). Such alteration might not always increase the sensitivity of reporters, but can also change the expression mode driven by the original low affinity sites. When low affinity Ci/Gli sites mediating the response to Hedgehog (Hh) signaling are changed into high affinity sites, multiple reporters exhibit expression patterns that are locked in the default repression by Ci/Gli in the absence of Hh signaling but are no longer activated by Hh in imaginal discs (Ramos et al., 2013). More studies have suggested that high affinity sites direct expression in more restricted region with higher signaling activity (level of signaling molecule) (Liang and Levine, 1993; Parker et al., 2008). One possible mechanism explaining affinity-specific expression

involves other TFs, that degeneracy better allows binding of multiple TFs to the same site. For example, ChIP-seq analysis in mammalian cell culture revealed that induced HNF4alpha expression lowers TCF4 recruitment to several genomic loci containing degenerate HMG sites, some of which are validated for TCF and HNF4alpha binding and regulation in a competitive manner (Vuong et al., 2015).

To summarize, studies on TCFs and other TFs argue that TFs have a wide spectrum of binding site affinity, but the functional importance and universal usage of degenerate sites awaits further investigation. It should be kept in mind that the affinity is often measured in vitro and might not reflect how much the in vivo chromatin locus attracts TF. Swapping experiment is an important approach to verify that it is the binding site rather than the surrounding DNA sequences in the CRM that dictates the output, but how to bring such validation from single case level to systematic analysis requires more breakthroughs in the field.

IV. Signaling-induced transcriptional repression

Despite of the common notion that TFs such as TCF bind to CRMs and mediate signal-induced activation of target gene, genes are also repressed by Wnt signaling. Microarray and RNA-seq analysis has found many targets that exhibit relatively higher basal activity when the signaling is absent, i.e. signal-induced repression, in comparison to signal-induced activation where target expression is higher with the presence of signal (van de Wetering et al., 2002; van de Flier et al., 2007; Kavak et al., 2010; Hoverter et al., 2012; see the Wnt homepage for an earlier list:

http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes_microarray). However, only in very few cases have detailed molecular mechanisms been studied regarding the cis- and trans-acting factors involved (Scully et al., 2000; Olson et al., 2006; Pyrowolakis et al., 2004; Jamora et al., 2003; more cases are cited in this section). For most signal-repressed targets, it remains unknown whether the regulation is direct and which TFs are used in the regulation. "Direct" here means no protein synthesis after signal induction is needed for the target repression to occur. This section will look at three categories of mechanisms that signaling pathways utilize for target repression, with focus on the first two mechanisms using the same TFs for signal-induced activation, as they are more relevant to my thesis study. Several examples of TCF mediated target repression are shown in Figure 1.3.

The same TF and TF binding sites are used for signal-induced activation and repression in different contexts

The classic HMG sites recognized by TCF are widely used for Wnt-activated target transcription, but these sites are also found to mediate several Wnt-induced repression cases in *Drosophila* (Piepenburg et al., 2000; Theisen et al., 2007) and mammalian cell culture (Jamora et al., 2003; Delmas et al., 2007). Since the HMG sites stay the same, the flanking DNA sequences often become the source of signal-induced repression due to recruitment of other TFs. For instance, the transcriptional mediator of the Hedgehog signaling Cubitus interruptus (Ci) activates transcription through binding to sites that partially overlap with HMG sites on a CRM

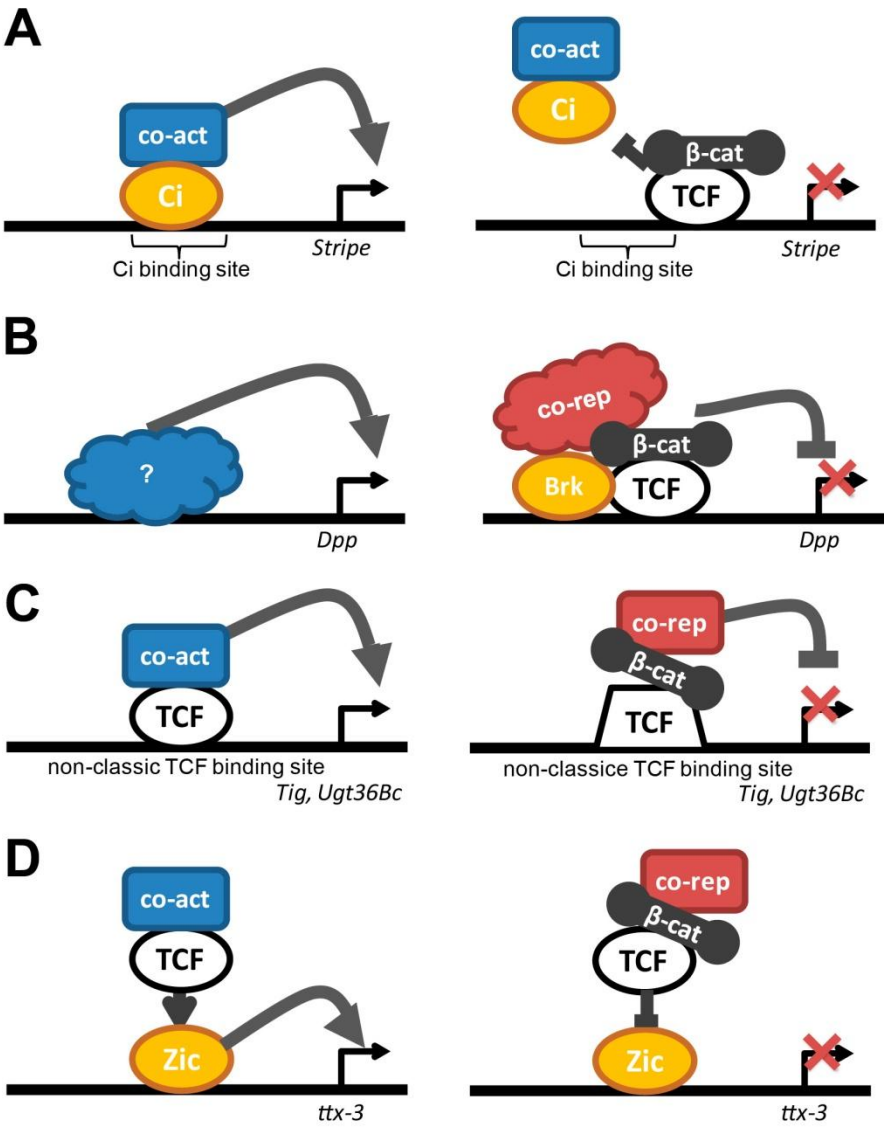

Figure 1.3. Examples of repression of TCF target genes. **(A)** TCF represses *Stripe* in cells with high Wnt signaling through competeing with Ci for DNA binding. **(B)** TCF and β-cat/Arm and Brinker together inhibit *Dpp* transcription. **(C)** TCF and β-cat/Arm repress transcription through non-classic TCF binding sites. **(D)** TCF and β-cat repress Zic-mediated transcription of *ttx-3*.

from *stripe*, a gene important for *Drosophila* embryonic epidermis patterning. In this case, TCF competes with Ci for DNA binding and results in transcriptional repression. This occurs in cells with high Wg activity, although it is unclear whether beta-catenin is directly involved in the regulation (Piepenburg et al., 2000; Figure 1.3A). For Dpp and E-cadherin, transcriptional repressors Brinker and Snail contribute to the repression of each gene, respectively, although HMG sites may or may not be required for target regulation by these co-repressors (Theisen et al, 2007; Jamora et al, 2003; Figure 1.3B). For p16INK4a, it is unknown whether direct binding of other TFs on the W-CRM is the mechanism (Delmas et al., 2007). It is also possible that Wnt signaling controls availability of other cofactors that do not directly bind DNA but are recruited by TCF.

Similar mechanisms have also been found in other pathways (reviewed in Affolter et al., 2008; Nawshad et al., 2007; Kumar and Duester, 2014). The *Drosophila* NF-kappaB family member Dorsal activates and represses different target genes in ventral cells. For a repressed target zerknullt (zen), mutation of DNA sequences flanking Dorsal binding sites, which presumably abolishes binding or other TFs, converts Dorsal-repressed CRM reporters into Dorsal-activated ones (Jiang et al., 1993; Kirov et al., 1993). This highlights the necessity of flanking sequences for determining the transcriptional output of the TF of interest, and suggests that transcriptional activation is the default state for Dorsal regulation in these cells.

Allosteric regulation of TF by different binding sites mediating repression and activation

TFs are occasionally found to recognize binding sites distinct from their traditional sites and these new sites mediate signal-induced target repression instead of activation. This might not be too surprising as degenerate TF binding sites seem to be an underappreciated phenomenon that contributes to transcriptional diversity (see last section). Based on the similarity between binding sites used for activation and repression, several somewhat different mechanisms have been described. In a case of p53-mediated repression, it is the orientation of two half-sites that determines activation versus repression (Johnson et al., 2001). For factors including the POU domain factor Pit-1, the retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimers, and the *Drosophila* Smads Mad/Medea, it is the spacing between half-sites that matters (Scully et al., 2000; Kurokawa et al., 1994; Pyrowolakis et al., 2004). In the latter case, a precise spacing of 5 bp between two Smad binding sites along with certain sequence specificity allows recruitment of Schnurri, a large zinc finger protein that is necessary and sufficient for this repression. The Mad/Medea/Shn complex is found to repress several targets including brinker, gooseberry and bag of marbles, suggesting that this is a common mechanism for TGF-beta signaling induced transcriptional repression at least in *Drosophila* (Pyrowolakis et al., 2004). Despite that no signaling pathway is clearly involved for the p53 regulation, the examples above show that the spacing and orientation of TF binding sites can be important determinant of transcriptional output.

Not only half-site organization but also the individual DNA motifs recognized by TFs may dictate activation versus repression. A single nucleotide change in the binding site can turn Dorsal from a transcriptional repressor into an activator (Mrinal et al., 2011). Much less similarity between repressive and activating binding sites is seen in regulations mediated by glucocorticoid (GC) receptor (GR) (Surjit et al., 2011). This study discovered a type of simple but untraditional palindromic DNA motif that is directly bound and repressed by GR upon GC signal induction. Unlike the other studies introduced in this section, these novel GR binding sites might mediate the GC-repression of 600 or more targets, according to RNA-seq analysis and computational search of the repressive binding sites, suggesting that signal-induced direct target repression through binding sites that are different from those used for activation might be a relatively common mechanism. There are more examples in this category where nuclear receptors being the mediator of target repression (Zhu et al., 2006; Lazar, 2003; Rosenfeld et al., 2006; Saatcioglu et al., 1993, Nygård et al., 2006; Sar et al., 2011), but much less is known in the Wnt field. In fact, the only case comes from a previous study from the Cadigan lab where novel TCF binding sites mediate Wnt-induced target repression instead of activation (Blauwkamp et al., 2008; Figure 1.3C). I further characterized the TCF binding sequences required for this regulation, and found that swapping between classic TCF sites used for Wnt-activation and these new repressive sequences can turn a Wnt-activated CRM reporter into a Wnt-repressed one, and vice versa (Zhang et al., 2014). These reports suggest that TF binding site itself can dictate transcriptional activation or

repression, and this mechanism could be more broadly used by TFs than currently appreciated (Figure 1.2, compare A-A' and D-D').

How can different binding sites dictate transcriptional activation or repression? It is often proposed that the DNA allosterically regulates the TF to recruit different cofactors that in turn affects transcriptional output. However, in most cases above this is not often tested and it also remains unknown which cofactors are involved in the repression. A detailed structural analysis comparing between TF conformations bound to activating and repressive sites was done with the POU domain factor Pit-1 (Scully et al., 2000). The POU domain has a reputation for flexible binding to different DNA motifs (Klemm et al., 1994; Jacobson et al., 1997). The 2 bp spacing difference between activating and repressive sites causes several structural differences, including the position of two DNA binding domains on Pit-1 (either on the same or the perpendicular faces of the DNA) (Scully et al., 2000). Less is known about how the TF conformational change alters cofactor recruitment, although studies have showed correlations that as little as single base pair alteration affects TF conformation and the strength (not type) of transcriptional output or the co-activator selection (Meijsing et al., 2009; Leung et al., 2004). Also note that allostery is not simply passed from one DNA motif to the protein bound to this motif to affect the protein function, but DNA can also be allosterically regulated by proteins bound on one site and this conformational change of DNA might affect protein affinity to adjacent DNA sequences and in turn the transcriptional output (Kim et al., 2013).

Mechanisms involving no direct binding of the common TF of the pathway and chromatin

While people often discuss the “major” TFs that mediate most of the known regulations of a certain pathway, signaling pathways also employ “other” TFs to directly bind DNA and transduce the signal. For example, beta-catenin binds to a homeodomain factor, Prop-1, and inhibits Pit-1 expression in the mouse pituitary gland (Olson et al., 2006). This regulation is independent of LEF1, the major TCF/LEF family member expressed in this tissue. Similarly, beta-catenin binds to the NF-κB transcription factor p50 to repress KAI1 expression in prostate cancer (Kim et al., 2005). Both regulations require Reptin as a co-repressor. A slightly different case is that beta-catenin and TCF complex with Zic to repress ttx-3 via a Zic binding site in *C. elegans* neuronal precursors (Sabrina et al., 2015). In the absence of Wnt signaling, TCF binds with Zic to activate transcription. It remains unclear if non-traditional TCF sites exist near the Zic sites and contribute to this regulation (Figure 1.3C). In addition, the TGF-β signaling pathway transducer Smad3 has been found to sequester bHLH factors MyoD and Myogenin from the chromatin to downregulate target genes (Liu et al., 2004). Although the major transcriptional regulatory complex of the signaling pathway is not directly bound to the DNA in these cases, the regulations are still somewhat direct since no protein synthesis after signal induction is required.

Analyzing transcriptional repression allows identification of more target genes

What is the benefit of studying mechanisms of transcriptional repression in such detail? One hope is that knowing the binding site consensus and the required factors will help to identify more direct targets through computational search. Merely based on binding site consensus and conservation between mouse and human, an aforementioned study identified hundreds of genes containing a putative nGRE (negative glucocorticoid (GC) receptor (GR) elements). 135 genes having a putative nGRE nearby are randomly picked for validation by RT-QPCR and 56/135 are suppressed by dexamethasone treatment in one or multiple mice tissues (Surjit et al., 2011). Putative nGREs are also found in high frequency in GR ChIP-seq peaks in GC-repressed genes (Surjit et al., 2011). This is an impressive example where a non-traditional consensus (secondary motif) of a TF is widely used and it mediates target repression instead of activation. In addition to searching for binding sites, integrating information of TF and cofactor recruitment (ChIP-seq) and differential expression upon signal induction (RNA-seq, microarray, etc.) improves computational search for putative target genes (see Hallikas et al., 2006 as a good example).

Primary analysis of individual CRMs is essential to provide accurate consensus information for computational prediction of direct targets. For example, ChIP-seq analysis of TCF4 and GATA3 co-ocupancy suggest that TCF4 is recruited by GATA3 to targets in MCF-7 breast cancer cells (Frietze et al., 2012). This is based on the assumption that TCF4 is not directly bound to DNA if no classic TCF site is found near the TCF4 ChIP-seq peak. However, it is possible that TCF4 is

recruited by non-traditional TCF binding sites characterized by our lab, and if so, the consensus information may improve the search.

In summary, signaling pathways that are well-established for gene activation can also directly repress transcription without de novo synthesis of regulatory factors. Various mechanisms have been characterized and understanding of them may lead to better identification of target genes and deeper understanding of the signaling networks.

V. *Drosophila* hematopoiesis in the lymph gland

The *Drosophila* hematopoiesis shares many similarities with the vertebrate system in both developmental progression and regulatory factors and pathways (Evans et al., 2003; Williams, 2007; Wood and Jacinto, 2007). In the second half of my thesis study, I have been characterizing the biological role of a Wnt target gene, Tiggrin (Tig) (Chapter III). Tig is predominantly expressed in the fly hematopoietic system and it regulates maturation of immune cells (Chapter III; Zhang et al., 2016, paper in revision). While cell cycle regulation seems to be involved, the detailed mechanism of how Tig functions remains to be explored. In this section, I will give an overview of fly hematopoiesis with focus on the larval hematopoietic organ, the lymph gland (LG), and known roles of the Wg signaling pathway.

The two origins of hemocytes in *Drosophila*

The blood cells, termed hemocytes, together with the fat body form the innate immune immune system in *Drosophila* (Kounatidis and Ligoxygakis, 2012). There are evidences of preliminary adaptive immunity in insects such as *Drosophila*, e.g. the potential to express various isoforms of an immunoglobulin-superfamily member Dscam through alternative RNA splicing (Watson et al., 2005; Dong et al., 2006; Little et al., 2005), but the role of this potential remains to be studied.

Like vertebrates, hematopoiesis occurs in multiple waves in *Drosophila*. The first wave of hematopoiesis originates at about stage 11 from the procephalic (head) mesoderm, giving rise to embryonic hemocytes (Tepass et al., 1994). The vast majority of embryonic hemocytes persists after embryogenesis, further amplifies in larvae (Markus et al., 2009; Makhijani et al., 2011; Leitao and Sucena, 2015; Ghosh et al., 2015), and persists till the adulthood (Holz et al 2003).

The second wave also occurs in embryogenesis, where ~20 cells assemble in the dorsal thoracic mesoderm and coalesce flanking the aorta at stage 14, then rapidly develops into several pairs of lobes aligned on the dorsal vessel during larval development (Holz et al., 2003; Jung et al., 2005). These lobes are called the lymph gland (LG) and cells in the LG are not released into circulation until the end of larval stage (Grigorian et al., 2011). Hemocyte differentiation and maturation occurs mainly in the primary lobes (the most anterior pair) of the LG, while the rest contain predominantly immature prohemocytes during larval stages (Jung et al., 2005), so that most of the current studies in the LG have been focusing on the regulations in

the primary lobes. The LG resembles the vertebrate aorta-gonadal-mesonephros (AGM) mesoderm in both developmental hierarchy and molecular regulations, making it an interesting system to study hematopoiesis (Mandal et al., 2004, Evans et al., 2003).

Embryonic specification of the lymph gland

Clonal analysis has suggested a hemangioblast-like cell in the *Drosophila* cardiogenic mesoderm, which can divide into two daughter cells, one developing into the heart or aorta and the other developing into the LG (Mandal et al., 2004). The GATA factor Serpent (Srp) and *Drosophila* Friend-of-GATA homologue U-shaped (Ush) are expressed in LG progenitors (Fossett et al., 2001; Mandal et al., 2004). This lineage is loss in Srp mutant embryo, and Srp overexpression increases the number of LG progenitor cells in the embryo, arguing that Srp specifies the LG lineage in embryo (Mandal et al., 2004). On the contrary, GATA factor Pannier (Pnr) and homoebox protein Tinman (Tin) specifies the cardioblasts and their expression patterns depend on factors Dpp and Heartless (a fly FGF receptor) (Mandal et al., 2004). These data are reminiscent of the roles of GATA factors in specifying vertebrate cardiogenic mesoderm (Fossett et al., 2001). The overall *Drosophila* cardiogenic mesoderm is also regulated positively by Wg and negatively by Notch. Wg mutant embryos lack all cardiogenic lineages (Mandal et al., 2004). These factors are repetitively used in LG development at later stages.

Three zones of cells in the larval lymph gland

During the 3rd instar larval stage, the LG has developed into several pairs of lobes, with the primary lobes being the most differentiated pair. The primary lobes are organized into three zones of cells. Hematopoietic progenitors with stem cell-like properties are maintained in the central part of the LG termed the medullary zone (MZ). Differentiation starts during the late second instar stage and forms the peripheral area termed the cortical zone (CZ), while typical maturation markers first occur in the early third instar (Jung et al., 2005). In addition, a small group of cells termed the posterior signaling center (PSC) has been proposed to act as a niche that maintains the pro-hemocyte population of the MZ and also communicates with the CZ (Krzemien et al., 2007; Mandal et al., 2007; Mondal et al., 2011). A model showing the organization and development of these three zones is shown in Figure 1.4. Challenges of these established roles of each zone will be discussed below.

The PSC controls LG homeostasis

Several factors are found expressed mainly in the PSC among LG cells, maintaining either the PSC itself or also the MZ. The Hox factor Antennapedia (Antp) specifies PSC in the embryo (Mandal et al., 2007). In mutants of the *Drosophila* EBF factor Collier/Knot (Col), the PSC is initially specified, but is entirely loss by the third larval instar stage (Mandal et al., 2007). Serrate-mediated Notch signaling is required for Col transcription (Mandal et a. 2007). Other factors including Wg and Bag of Marbals (Bam) have been shown to positively regulate the pool of PSC cells, while

the Dpp/BMP pathway is a negative regulator (Sinenko et al., 2009; Pennetier et al., 2012; Tokusumi et al., 2015).

The PSC also expresses ligands for the Hedgehog (Hh) and PDGF/VEGF (also called PVF) pathways (Lebestky et al., 2003; Mondal et al., 2011). Patched, the receptor of Hh, is found specifically expressed in the MZ, while the PVF receptor is expressed in the CZ (Mandal et al., 2007; Small et al., 2014). Loss of each ligand does not kill the LG, but compromises the MZ as it differentiates prematurely, suggesting that the PSC plays important roles in MZ maintenance (Krzemien et al., 2007; Mondal et al., 2011).

Despite of these, the necessity of PSC during LG development has been questioned. Two recent reports found that Col is also expressed at a lower level in the MZ. Loss of this expression but not the higher Col expression in the PSC results in premature differentiation of the MZ (Benmimoun et al., 2015; Oyallon et al., 2016). MZ might be self-maintained beyond a certain developmental time point, but careful time-course experiments are needed to test this possibility.

Maintenance of the MZ and balance between MZ and CZ

The MZ has attracted many interests due to its stem cell-like property, i.e. it contains a pool of cells maintained in premature status, receiving niche signals and giving rise to differentiated progenitors without obvious change of the pool size (Jung et al., 2005). One regulatory pathway for MZ maintenance is the JAK/STAT pathway. The JAK/STAT pathway in vertebrates is triggered by ligands including interferon and

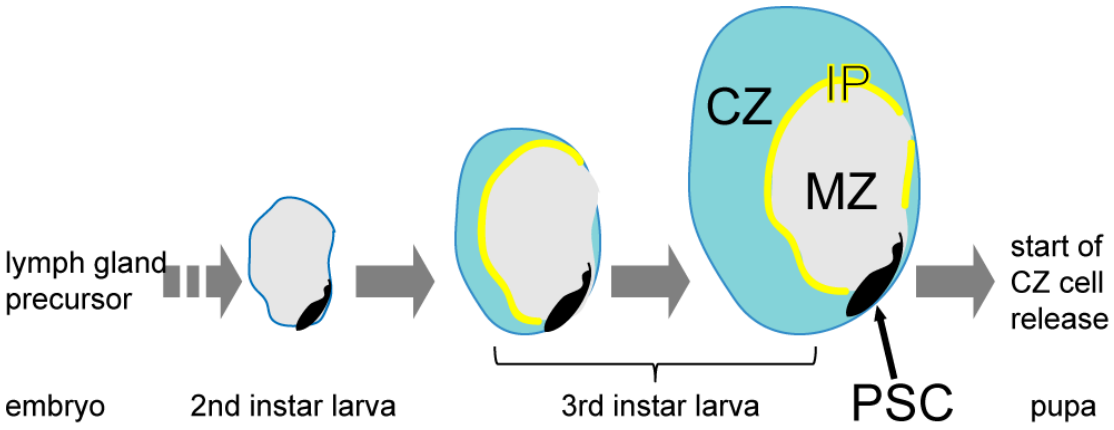

Figure 1.4.The development and organization of the lymph gland. The lymph gland originates from its embryonic precursor and develops into a disc-shaped tissue with three zones, the posterial signaling center (PSC), the medullary zone (MZ), and the cortical zone (CZ). The PSC contains cells that provide a niche for maintaining the MZ. The MZ contains prohemocytes that are immature and differentiate into CZ cells, which are either intermediate progenitors (IP) or matured hemocytes (express maturation marker). The natural population of IP cells is small. The CZ occurs at the end of 2nd instar stage and rapidly expand during the 3rd instar stage. The mature cells in the CZ start to get released into circulation by the end of larval stages. See text for more information. Black area pointed by an arrow: PSC; grey area: MZ; yellow area: IP; blue area: CZ.

interleukin and is widely involved in hematopoiesis and immunity responses (Levy and Darnell, 2002). The *Drosophila* receptor of the pathway, Domeless (Dome), is specifically expressed in the MZ among all LG cells (Jung et al., 2005). This expression pattern is regulated by the pathway itself through binding of STAT92E to a CRM of Dome called Dome-MESO (Rivas et al., 2008; Bourbon et al., 2002). In addition, the MZ marker tep4 is lost in mutant of STAT92E (Krzemien et al., 2007). Another putative receptor of the pathway, Latran/et (Lat), contains a truncated intracellular domain lacking the STAT-binding site and functions as a dominant-negative receptor. Overexpression of Lat in the MZ results in MZ loss and prematuration of the CZ (Makki et al., 2010). These data argue that the JAK/STAT pathway, mainly Dome and STAT92E, are positive regulators of MZ maintenance.

The Wg and TOR pathways also regulate MZ maintenance. Inhibition of the Wg pathway in the MZ by over-expressing dominant-negative receptors Fz^{DN} and $Fz2^{DN}$ shifts MZ toward CZ cell fate with a slight expansion of a population of intermediate progenitors (IP) expressing both MZ and CZ markers (Sinenko et al., 2009). Over-expressing Wg itself in the MZ inhibits CZ formation, and can rescue the premature differentiation of CZ caused by knock-down of Insulin Receptor (InR) (Sinenko et al., 2009; Shim et al., 2012). Components of the *Drosophila* TOR pathway that works downstream of InR, including positive regulators Akt and TORC1 components, and negative regulators Pten, TSC1/2, also promote or inhibit MZ size, respectively (Shim et al., 2012; Dragojlovic-Munther and Martinez-Agosto, 2012; Tokusumi et al., 2012). Interestingly, MZ knockdown of the Pten and TSC2, but not

TSC1, expands both MZ and IP cells; but unlike Wg overexpression, the CZ formation is not blocked in these manipulations, suggesting that the two pathways might not be simply epistatic to each other (Sinenko et al., 2009; Dragojlovic-Munther and Martinez-Agosto, 2012).

The CZ cells also communicate with the MZ and inhibit MZ differentiation. In a network termed the equillibrium pathway, CZ cells receive PSC-secreted Pvr ligands which trigger the release of adenosine deaminase-related growth factor A (Adgf-A) through up-regulation of STAT92E. Adgf-A lowers extracellular adenosine levels in the MZ, keeping pro-hemocytes in an undifferentiated state (Mondal et al., 2011; Mondal et al., 2014). Consistently, loss of STAT92E in CZ clones induces maturation of adjacent cells (Minakhina et al 2011). This role of STAT92E in the CZ is independent of JAK-STAT signaling which functions in the MZ (Mondal et al., 2011).

It should be noted that, although regulators are often discussed from the angle of blocking or promoting the maintenance of MZ, evidences for the MZ cells being a true or typical population of stem cell are not solid. For instance, there has been no evidence for self-renewal of the MZ cells and a preliminary characterization found no typical stem cell markers expressed in the MZ (Krzemien et al., 2010). MZ cells actively proliferate till later third larval instar stages, but the proposed niche of MZ (PSC) also becomes dispensable in these larval stages (Krzemien et al., 2010; Benmimoun et al., 2015). Nevertheless, all CZ cells are developed from MZ cells (Evans et al., 2009).

Regulation of intermediate progenitors at the transition from MZ to CZ

A few reports have described the intermediate progenitors (IPs), a small population (~5% of the primary lobes) of cells that likely represent a transition state from MZ to CZ, possessing higher mitotic activity than mature CZ cells (Krzemien et al., 2010; Dragojlovic-Munther and Martinez-Agosto, 2012). The IP cells have been characterized by either expressing both MZ and CZ markers, lacking both markers, or only expressing general CZ markers but not maturation markers (Krzemien et al., 2010; Minakina et al., 2011; Dragojlovic-Munther and Martinez-Agosto, 2012; Milton et al., 2014). The hierarchy between different descriptions of IP cells remains unclear, i.e. whether and how much do these pools overlap. Markers only found in transitory cells have been suggested but need to be better established with overlay and time-course analysis (Tokusumi et al., 2011; Mondal et al., 2011).

Besides the two cases above that MZ inhibition of the Wg and TOR pathways can expand the IP cells, the only known factor that cell-autonomously regulates the IP population is Pnr. Pnr knockdown or mutant clones in the CZ also inhibits cell maturation (Minakhina et al., 2011). There are two Pnr isoforms with antagonizing functions in other contexts (Fromental-Ramain et al., 2010), and it remains unclear whether both of them participate in the regulation of IP cells. Better characterization of the IP cells such as regulatory factors, their developmental connections with MZ and CZ, and their physiological relevance will greatly improve the current understanding of LG development, but this could be challenging due to their small population and transitory nature.

Overall and environmental regulators of LG growth

In addition to the balancing between MZ and CZ, several regulators of overall LG growth have been identified, including the Toll pathway, Zfrp8/PDCD2, heixuedian (heix) and Rabex-5 (Qiu et al., 1998; Minakhina et al., 2007; Xia et al., 2015; Reimels, 2015). These factors possibly regulate LG growth from a cell-cycle aspect, and are often not restricted to the lymph gland but also regulate the proliferation of circulating hemocytes. For instance, cell cycle regulators Cdc27, mutagensensitive 304 (mus304) and no poles (nopo) are found to suppress the LG overgrowth phenotype caused by loss of Zfrp8 (Tan et al., 2012).

Besides developmental regulation, the LG also responds to several environmental clues and stresses, including olfaction, nutrition, hypoxia, oxidative stress, and infection (Shim et al., 2013a; Shim et al., 2013b; Mukherjee et al., 2011; Sinenko et al., 2011; Krzemien et al., 2010). These conditions all affect MZ maintenance, and it remains an open question that how the LG integrates all these signals and responds in a biologically meaningful way.

Three lineages of mature hemocytes

Both origins of *Drosophila* hematopoiesis can differentiate into three lineages of mature hemocytes, plasmatocytes, crystal cells and lamellocytes. Plasmatocytes are dominant in healthy animals, contributing to about 95% of all mature hemocytes (Crozatier and Meister, 2007; Tepass et al., 1994). They are equivilant of mammalian macrophages, which express phagocytic receptors and are able to clean

both apoptotic debris and foreign materials (Rizki and Rizki, 1980; Wood and Jacinto, 2007). They also support innate immunity (Charroux and Royet, 2009) and participate in tissue regeneration by activating stem cells near the wound (Ayyaz et al., 2015). Crystal cells are large specialized cells containing crystals of enzymes such as prophenoloxidase that facilitate immune responses and wound-healing by causing melanization (Lanot et al., 2001; Rizki and Rizki, 1978). Lamellocytes are rarely found in healthy animals, but their number is significantly increased when larvae are immunologically challenged by infection of a parasitic wasp (Crozatier et al., 2004; Rizki and Rizki, 1992; Sorrentino et al., 2002). Plasmatocytes outside of the LG expressing plasmatocyte-specific maturation markers are able to transdifferentiate into both lamellocytes and crystal cells, suggesting the plasticity and physiological importance of this lineage (Markus et al., 2009; Honti et al., 2010; Leitao and Sucena, 2015).

Several factors have been found regulating the lineages specification in embryonic hemocytes, some of which have also been studied in the LG. In the embryo, the Runx family transcription factor Lozenge (Lz) is required for crystal cells formation (Lebestky et al., 2000). Lz also marks crystal cells in the LG, where its expression in the LG depends on Srp and the Notch ligand Ser (Lebestky et al., 2000; Lebestky et al., 2003). The function of Lz is antagonized by transcription factors Glial cell missing (Gcm) and Friend-of-GATA family member U-shaped (Ush) in the embryo (Evans et al., 2003; Fossett et al., 2003). Ush is expressed in the CZ at earlier 3rd instar stage and inhibits both crystal cell and lamellocyte lineages, possibly

by inhibiting CZ cell proliferation (Sorrentino et al., 2007). Gcm and Gcm2 are both required for plasmatocyte specification in embryonic hemocytes (Bernardoni et al., 1997; Alfonso et al., 2002). Although the same lineage of hemocytes express similar markers despite of their origin (Evans et al., 2014), it is unclear whether the regulations are the same between embryonic hemocytes and the LG.

Within the LG, investigations have been focusing on the balance between crystal cells and lamellocytes. Notch signaling controls the crystal cell-lamellocyte decision, as inhibition of this pathway results in a reduction in crystal cells and a large increase in lamellocytes in healthy larvae (Duvic et al., 2002; Small et al., 2014). Crystal cell number in the LG is also controlled by Hippo signaling, which directly regulates Lz and restricts specification of this cell type in a Notch signaling-dependent manner (Ferguson and Martinez-Agosto, 2014; Milton et al., 2014). This work extends the functions of Hippo signaling beyond cell growth to cell fate determination, and provides a mechanistic view of how Notch signaling regulates LG development and crystal cell specification.

Wg signaling has not been found to specify LG cell fate, but two negative regulators of the pathway, dominant-negative TCF and Shaggy, cause a lamellocyte phenotype in circulating hemocytes (Zettervall et al., 2004). It is unclear whether this effect is specific for the lamellocyte lineage or is just due to induced inflammatory responses.

In summary, the *Drosophila* hematopoietic system, with the LG in particular, serves as a great system for studying hematopoiesis. Many regulatory factors are

shared between the LG and the vertebrate system. Discoveries of LG regulators such as Zfrp8/PDCD2 promoted the understanding of vertebrate hematopoiesis. Great emphasis has been placed on the maintenance of MZ containing undifferentiated stem cell-like prohemocytes, but much need to be learned about the later development in the CZ from lineage study to understand the progression CZ cell maturation to regulations of this process.

Rationale

Wnt/β-catenin signaling is a highly conserved cell-cell communication pathway that plays important and extensive roles in development and disease. Wnt/β-catenin signaling causes various transcriptional outputs and it is important to understand how Wnt/β-catenin signaling regulates transcription in a spatial- and temporal- specific manner. The ability to activate and repress target genes is one source of the specificity, and expression profiling reveal a significant amount of genes that are down-regulated by the Wnt pathway. However, very little is known about Wnt-repressed targets. For example, what is the mechanism of repression, how can Wnt signaling distinguish activated targets from repressed ones, and what are the functions of Wnt-repressed targets? I will try to address these questions in my thesis study.

Previous work from our lab supports a model for TCF and Arm (Armadillo, the fly β-cat) directly repress expression of the *Ugt36Bc* gene in *Drosophila* cells. Repression of the *Ugt36Bc* W-CRM (Wnt responsive cis-regulatory module) requires

interaction between the HMG domain of TCF and novel TCF binding sites, now termed WGAWAWR sites, which are distinct from the classic sites used for Wnt-activated targets. A second DNA binding domain, termed the C-clamp, was found to bind DNA motifs called the Helper sites and this interaction is necessary for the activation of several *Drosophila* and vertebrate Wnt targets. The Helper sites are considered to provide extra clue in DNA sequences and help TCF to be better located to its targets. It was unclear whether the C-clamp is also used for recognizing Wnt-repressed targets.

In Chapter II, I investigated the detailed repression mechanism of Wnt targets. *Tig* was also suggested to be directly repressed by TCF and Arm. I found that *Tig* and *Ugt36Bc* are repressed through similar mechanisms involving recognition of WGAWAWR sites by the HMG domain and repressive-Helper (r-Helper) sites by the C-clamp. These sites are distinct from the classic sites mediating Wnt-activation and are functional in both *Drosophila* cell culture and hematopoietic tissues. Surprisingly, converting both the WGAWAWR and r-Helper sites into those found in Wnt-activated targets (or vice versa) completely reverse the transcriptional output of W-CRM, providing an uncommon example of how transcriptional diversity is achieved through the same transcription factor recognizing multiple types of binding sites.

In Chapter III, I characterized the function of Tig. Tig is essential for survival and proper LG size. Further genetic analysis revealed Tig as a negative regulator of plasmatocyte differentiation. This complements the current understanding of the LG with a cell-autonomous factor regulating the CZ cell fate. Misregulation of the G_2/M

transition of the cell cycle causes phenotypes similar as Tig loss or gain of function, e.g., overexpression of Wee1 which slows the G_2/M transition inhibits plasmatocyte differentiation. Wee1 might effect through Tig as overexpression of Wee1 upregulates Tig transcriptional reporters. Interestingly, overexpression of Tig or Wee1 cause a significant build up of intermediate cells, a small transitory population of cells existing in wild-type LGs, which will allow further analysis or their molecular regulators.

Table 1.1.

Category	Factor	System	Binding Partners and Interaction Domains	Potential Mechanisms	Reference
TCF bound co-repressors which can also disrupt β-catenin/TCF interactions	Coop	*Drosophila*	TCF/Pan: a motif containing HMG domain.	Recruit HDACs. Do not disrupt TCF/DNA interaction.	Song et al. 2010.
	Groucho/ TLE	Vertebrate, *Drosophila* and *C. elegans*	TCFs: central and HMG domains. Gro: Q domain.		Roose et al. 1998; Daniels and Weis, 2005; Sierra et al. 2006; Arce et al. 2009; Cadigan et al. 2012§.
	HIC-5	Vertebrate	TCFs: a motif in the central domain (absent in xLEF1).	Excludes β-catenin on *c-myc* promoter.	Ghogomu et al. 2006; Li et al. 2011.
	MTGs	Vertebrate	A N-terminal motif and the HMG domain on TCF4 contribute to interaction.	MTGR1 interacts with TCF1, TCF4 and LEF1. MTG8/16 could be recruited by Kaiso.	Moore et al. 2008; Barrett et al. 2012.

Table 1.1. List of factors that repress transcription mediated by TCF-β-catenin. Factors are grouped according to general mechanism of action. For the "System" column, Vertebrate denotes factors where positive results were obtained in one or more vertebrate species in vivo, and can also include mammalian cell culture. For the column indicating the interaction domains, the Arm repeats denote the 12 motifs forming central domain of β-catenin (see Valenta et al., 2012). Note that some factors can also promote TCF-β-catenin transcription in some contexts (see Table II). Abbreviations used that are not defined in the text are as follows: HIC-5, Hydrogen peroxide-inducible clone; Hint1, Histidine triad protein; ISWI, imitation switch; p15RS, p15Ink4b-related protein; PIAS, Protein inhibitor of activated STAT; TIS7, 12-O-Tetradecanoylphorbol-13-acetate-induced sequence 7.

Table 1.1. (continued)

Category	Factor	System	Binding Partners and Interaction Domains	Potential Mechanisms	Reference
Bind to β-catenin and disrupt β-catenin/TCF interactions	Chibby	Vertebrate and *Drosophila*	β-catenin: Arm C-terminus.	Besides directly disrupt β-catenin binding, Chibby and 14-3-3 together sequester β-catenin in the cytosol.	Takemaru et al. 2003; Li et al. 2008; Love et al. 2010; Enjolras et al. 2012.
	Duplin/ CHD8	Vertebrate	β-catenin: Arm repeats1-7 shows strongest interaction.	Unclear.	Sakamoto et al. 2000; Thompson et al. 2008; Nishiyama et al. 2012.
	ICAT	Vertebrate	β-catenin: Arm repeats 10-12.	ICAT masks Arm repeats 5-10 and competes for β-catenin binding. TCF4 and LEF1 were tested.	Tago et al. 2000; Daniels and Weis, 2002; Graham et al. 2002; Hasegawa et al. 2007.
	p15RS	Mammalian cell culture	TCF: N-terminus. β-catenin: Arm repeats 6-8.	Also binds to TCF with the same domain (RPR domain).	Wu et al. 2010.
	Sox6, Sox9, Sox17	Vertebrate	β-catenin: Arm repeats 4-10 for interaction with Sox9, 1-6 with Sox17 and 1-4 with Sox6. TCF3 and TCF4: HMG domains.	Sox9 can also promote β-catenin degradation.	Akiyama et al. 2004; Kan et al. 2004; Sinner et al. 2007; Topol et al. 2009; Kormish et al. 2010§.
β-catenin bound co-repressors	Reptin/ Tip49b	Vertebrate and *Drosophila*	β-catenin: unspecified.	Inibitory effect requires its DNA-dependent ATPase activity. Might antagonize Pontin.	Bauer et al. 2000; Rottbauer et al. 2002; Kim et al. 2005; Olson et al. 2006.
	TIS7*	Mammalian cell culture	β-catenin: unspecified.	Unclear.	Vietor et al. 2005.
Modify TCF and inhibit its function	CBP/ P300*	Mammalian cell culture, *Drosophila* and *C. elegans*	TCF/Pan: HMG domain. Acetylation site: TCF/Pan K25; TCF/POP-1 K185, K187 and/or K188.	Acetylation of TCF/Pan reduces its affinity to β-catenin. Acetylation of TCF/POP-1 enhances its nuclear retention.	Waltzer and Bienz, 1998; Gay et al. 2003.; Li et al. 2007.

Table 1.1. (continued)

Category	Factor	System	Binding Partners and Interaction Domains	Potential Mechanisms	Reference
Modify TCF and inhibit its function	PIASy*	Vertebrate	LEF1: a motif containing HMG domain. SUMOylation site: K25 and K267 of LEF1.	SUMOylates LEF1, which is then sequestered into nuclear bodies and inhibited.	Sachdev et al. 2001; Roth et al. 2004.
Recruited to WREs in parallel of TCF	CtBP*	Vertebrate and *Drosophila*	APC: 15 aa repeats	Recruited to WREs in a TCF-independent manner, functioning as a homo-oligomer; can also divert β-catenin/APC complexes away from TCF.	Hamada and Bienz, 2004; Fang et al. 2006; Bhambhani et al. 2011.
	ISWI*/ SNF2H & 2L, SNF5	Mammalian cell culture and *Drosophila*	Unspecified.	Interacts with ACF1 and antagonizes histone acetylation on Wnt targets.	Liu et al. 2008; Eckey et al. 2012; Mora-Blanco et al. 2013.
	Kaiso*	Vertebrate	Unspecified.	Recruit co-repressors to WREs. May also disrupt TCF/DNA interaction. Can activate Wnt targets in *Xenopus.*	Park et al. 2005, 2006; Ruzov et al. 2009; Iioka et al. 2009; Hong et al. 2012.
Misc	Eaf1, Eaf2	Vertebrate	β-catenin: Arm repeats 1-12 and C-terminus. TCF4: unspecified.	Interact with both TCF4 and β-catenin.	Liu et al. 2013.
	NCoR & SMRT	Mammalian cell culture	β-catenin: Arm repeats1-12. TCF4: a motif containing HMG domain.	Known chromatin modifiers that interact with both TCF and β-catenin.	Song and Gelmann, 2008.
	Osterix	Vertebrate	Unspecified.	Disrupt TCF/DNA interaction.	Zhang et al. 2008; Chen et al. 2012.

* factors that can both inhibit and activate Wnt/β-catenin signaling

§ reviews.

Table 1.2.

Category	Factor	System	Binding Partners and Interaction Domains	Potential mechanisms	Reference
Interacts with N-terminal half of β-catenin	BCL9/Lgs and Pygo proteins	Vertebrate and *Drosophila*	β-catenin: Arm repeat 1 interacts with BCL9/Lgs; D172 (fly) and D164 (mouse) are cruicial for the interactions.	BCL9/Lgs recruits Pygo, which in turn recruits many other transcription co-activators. They also help to retain β-catenin in the nucleus.	Kramps et al., 2002; Parker et al. 2002; Thompson et al. 2002; Brembeck et al. 2004; Jessen et al. 2008§; Valenta et al. 2011.
	Pontin/ TIP49	Vertebrate and *Drosophila*	Arm repeats 1-4.	DNA-dependent helicase that can complex with histone acetyltransferases (HATs). Also binds to TBP, another co-activator.	Bauer et al. 2000; Rottbauer et al. 2002; Feng et al. 2003.
Interacts with C-terminal half of β-catenin	CBP & P300*	Vertebrate and *Drosophila*	Arm repeats 10-12 and C-terminus.	HATs.	Hecht et al. 2000;Sun et al. 2000;Takemaru and Moon, 2000; Kioussi et al. 2002; Ma et al. 2005; Sierra et al. 2006; Li et al. 2007.
	Brg-1/ Brm	Vertebrate and *Drosophila*	Arm repeats 7-12.	ATPase-dependent chromatin remodeller.	Barker et al. 2001; Major et al. 2008; Mahmoudi et al. 2010.

Table 1.2. List of factors that activate transcription mediated by TCF-β-catenin. Factors are grouped according to general mechanism of action. Vertebrate systems and Arm repeats of β-catenin are defined as in Table I. Note that some factors listed can also repress TCF-β -catenin transcription (see Table I). Abbreviations used that are not defined in the text are as follows: Brm, Brahma; MED12, Mediator 12; TAF, TBP-associated factor. TBP, TATA-box binding protein; TRRAP, Transcription/transformation domain-associated protein.

Table 1.2. (continued)

Category	Factor	System	Binding Partners and Interaction Domains	Potential mechanisms	Reference
Interacts with C-terminal half of β-catenin	ISWI*/ SNF2H & 2L	Mammalian cell culture and *Drosophila*	Arm repeats 11-12 and C-terminus.	ATPase-dependent chromatin remodeler.	Sierra et al. 2006; Song et al. 2009.
	Hyrax/ Para-fibromin	Mammalian cell culture and *Drosophila*	Arm repeat 12 and C-terminus.	Member of the PAF1 complex, which is involved in transcription initiation and elongation. Target activation is dependent on Pygo. Activity is regulated by SHP2.	Mosimann et al. 2006; Takahashi et al. 2011.
	Med12 & Med13	*Vertebrate and Drosophila*	Arm repeats 11-12 and C-terminus.	Subunit of the mediator complex. Also found to be recruited by Pygo.	Kim et al. 2006; Carrera et al. 2008; Rocha et al. 2010.
	TAF complex	Mammalian cell culture and *Drosophila*	TBP interacts with Arm repeats 11-12 and C-terminus.	TBP is recruited by β-catenin. TAF4 is recruited by Pygo. These are members of the TFIID complex.	Hecht et al. 1999; Wright et al. 2009; Simoneau et al. 2011.
Other histone modifiers	MLL1/ MLL2	Mammalian cell culture	Arm repeats 11-12 and C-terminus.	MLL2 was also shown to be recruited by Pygo2. MLL1/2 are Histone methyltransferases (HMTs). Catalyzes H3K4 mono-, di- and tri-methylation.	Sierra et al. 2006; Chen et al. 2010.
	Dot1L (Dot1)	Vertebrate and *Drosophila*	Might be recruited through TRRAP/Tip60.	Found in several complexes with MLL partners and has HMT activity. Catalyzes H3K79 methylation.	Mohan et al. 2010; Mahmoudi et al. 2010.
	SET8	Vertebrate	Interacts with a TCF4 fragment spanning from N-terminus to the end of HMG domain.	HMT. Catalyzes H4K20 mono-methylation.	Li et al. 2011.
	PRMT2	Vertebrate	Interacts with β-catenin.	HMT. Catalyzes H3R8 methylation.	Blythe et al. 2010.

Table 1.2. (continued)

Category	Factor	System	Binding Partners and Interaction Domains	Potential mechanisms	Reference
Other histone modifiers	Carm1	Mammalian cell culture	Interacts with β-catenin but not LEF1.	HMT.Catalyzes H3R17me2.	Ou et al. 2011.
	TRRAP p400 & TIP60	Mammalian cell culture	Arm repeats 11-12 and C-terminus.	HAT complex. Might also mediate β-catenin ubiquitination through Skp1/SCF.	Sierra et al. 2006; Sustmann et al. 2008.
Facilitating β-catenin/ TCF interaction	Jerky/ Ebd1	Mammalian cell culture and *Drosophila*	Interacts with β-catenin, LEF1 and Pygo2.	The localization of Ebd1 on polytene chromosomes requires a DNA-binding protein called NRF-1/Ewg.	Benchabane et al. 2011; Xin et al. 2011.
	RNF14	Vertebrate	Interaction requires the N-terminal half of TCF	Contributes to β-catenin recruitment on the chromatin.	Wu et al. 2013.
	TBL1/ TBLR1	Mammalian cell culture	TBL1 interacts with TCF4. TBL1 and TBLR1 both interact with β-catenin.	TBL1 and TBLR1 are SUMOylated in response to Wnt signaling, which releases these factors from the NCoR complex, increasing recruitment to WREs.	Li and Wang, 2008; Choi et al. 2011.
Misc	APPL1 & APPL2	Mammalian cell culture	Interact with Reptin.	Interact with the co-repressor Reptin and remove it from the chromatin.	Rashid et al. 2009.
	CtBP*	*Drosophila*	Unspecified.	CtBP monomers activate some Wg targets downstream of Pygo.	Fang et al. 2006; Bhambhani et al. 2011.
	PIASy*	Mammalian cell culture	SUMOylation site: K297 of TCF4.	SUMOylates TCF4 and increases its activity. SUMOylation of PIASy is required for PIASy activity.	Yamamoto et al. 2003; Ihara et al. 2005.
	TIS7*	Mammalian cell culture	Unspecified.	Unclear.	Nakamura et al. 2013.

* factors that can both activate and inhibit Wnt/β-catenin signaling § reviews.

Table 1.3.

Factors	System	Binding partners and interaction domains	Potential mechanism	Reference
Prop1 and PitX2	Vertebrate	LEF1: can interact with PITX2, domain unspecified. β-catenin: Arm repeats 5-9 with Prop1.	LEF1 could also be involved.	Kioussi et al. 2002; Olson et al. 2006; Amen et al. 2007.
FOXO proteins	*M*ammalian cell culture and *C.elegans*	β-catenin: unspecified.	Could reduce interaction between β-catenin and TCF4.	Essers et al. 2005; Almeida et al. 2007; Hoogeboom et al. 2008.
Sox17*	Vertebrate	β-catenin: Arm repeats 1-6.	TCF4 may also be involved.	Sinner et al. 2004; Kormish et al. 2010§.
MyoD	Mammalian cell culture	β-catenin: Arm repeats 1-9.	Unclear.	Kim et al. 2008
Tbx5 and YAP1	Vertebrate	The co-activator YAP1 interacts with β-catenin while Tbx5 binds DNA.	Tbx5, YAP1 and β-catenin interact with each other. YAP1 and β-catenin colocalize on chromatin. These interactions require phosphorylation of YAP1 by YES1.	Rosehbluh et al. 2012.
HIF-1	Mammalian cell culture	β-catenin: Arm repeats 9-12 and C-terminus.	TCFs could also be involved. β-catenin and HIF-1 can form a ternary complex with androgen receptor, activating androgen-dependent targets.	Kaidi et al. 2007; Mazumdar et al. 2010; Mitani et al. 2012.
Androgen receptor	Vertebrate	β-catenin: Arm repeats 2-7.	Can compete with TCF for β-catenin binding.	Song et al. 2003; Cronauer et al. 2005; Wang et al. 2008; Mitani et al. 2012.

Table 1.3. List of other TFs that bind to β-catenin. The Vertebrate system is defined as in Table I. Abbreviations used that are not defined in the text are as follows: PitX2, Paired-like homeodomain transcription factor 2; Prop1, Homeobox protein prophet of PIT-1; LRH-1, Liver receptor homolog 1; PPAR, Peroxisome proliferator-activated receptor gamma.

Table 1.3. (continued)

Factors	**System**	**Binding partners and interaction domains**	**Potential mechanism**	**Reference**
Vitamin D receptor	Vertebrate	β-catenin: C-terminus; acetylation in K671 and K672 regulates its specificity.	Acetylation of K671/672 on β-catenin promotes TCF targets while inhibits VDR target.	Shah et al. 2006; Palmer et al. 2008.
LRH-1	Mammalian cell culture	β-catenin: Arm repeats, key residues are Y306, K345 and W383.	Unclear.	Botrugno et al. 2004; Yumoto et al. 2012.
PPARγ	Mammalian cell culture	β-catenin and TCF4: domain unspecified.	TCF/β-catenin interactions can antagonize PPARg targets through chromatin loops.	Jansson et al. 2005; Hwang et al. 2012.
Other nuclear receptors	Mainly the RAR, RXR and LXR proteins.			Mulholland et al. 2005§; Beildeck et al. 2010§.

* factors that can both activate and inhibit Wnt/β-catenin signaling

§ reviews.

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Chapter II:

Wnt-mediated repression via bipartite DNA recognition by TCF in the *Drosophila* hematopoietic system

Abstract

The Wnt/β-catenin signaling pathway plays many important roles in animal development, tissue homeostasis and human disease. Transcription factors of the TCF family mediate many Wnt transcriptional responses, promoting signal-dependent activation or repression of target gene expression. The mechanism of this specificity is poorly understood. Previously, we demonstrated that for activated targets in *Drosophila*, TCF/Pangolin (the fly TCF) recognizes regulatory DNA through two DNA binding domains, with the High Mobility Group (HMG) domain binding HMG sites and the adjacent C-clamp domain binding Helper sites. Here, we report that TCF/Pangolin utilizes a similar bipartite mechanism to recognize and regulate several Wnt-repressed targets, but through HMG and Helper sites whose sequences are distinct from those found in activated targets. The type of HMG and Helper sites is sufficient to direct activation or repression of Wnt regulated cis-regulatory modules, and protease digestion studies suggest that TCF/Pangolin adopts distinct conformations when bound to either HMG-Helper site pair. This repressive mechanism occurs in the fly lymph gland, the larval hematopoietic organ, where

Wnt/β-catenin signaling controls prohemocytic differentiation. Our study provides a paradigm for direct repression of target gene expression by Wnt/β-catenin signaling and allosteric regulation of a transcription factor by DNA.

Introduction

It is a common theme in gene regulation that the same transcription factor (TF) can directly activate or repress target gene expression, increasing the transcriptional complexity these TFs can achieve [1,2]. There are several mechanisms by which TFs exhibit this dual regulation. These include TFs interfering with the binding of other TFs to DNA or co-activators [3-5] or signal-dependent changes of co-regulators bound to the TF [6-8]. In many cases, specific differences in the nucleotide sequence of the cis-regulatory modules (CRMs) targeted by these TFs influence the transcriptional outcome.

The sequence specificity that determines the activation/repression choice of TFs can occur in the TF binding sites themselves, or the surrounding sequences. Several TFs that appear to be intrinsic transcriptional activators can also repress transcription when bound to CRMs in conjunction with other TFs [9-11]. In the case of the *Drosophila* NF-κB family member Dorsal, mutation of TF sites flanking Dorsal binding sites converts CRM reporters that are repressed by Dorsal into ones that are activated [12,13]. For other CRMs regulated by nuclear receptors [14,15], P53 [16], the POU TF Pit1 [17] and some Smads [18,19], it is the type of the TF binding site itself that determines output. For the latter cases, it has been proposed that the DNA

binding site allosterically regulates the TF, leading to differential recruitment of co-regulators [17,20].

Dual regulation of transcription has also been seen in Wnt/β-cat (hereafter called Wnt) signaling, an important cell-cell communication pathway that plays various roles throughout animal development, stem cell biology and disease [21-23]. Wnt-induced nuclear accumulation of β-catenin (β-cat) is a key feature of this pathway. Once in the nucleus, β-cat is recruited to CRMs hereafter referred to as Wnt-dependent CRMs (W-CRMs), where it facilitates regulation of Wnt transcriptional targets [24,25].

The best-characterized TFs that recruit β-cat to W-CRMs are members of the T-cell factor (TCF) family [26]. Studies with synthetic W-CRMs containing multiple copies of high affinity TCF binding sites and mutagenesis studies of binding sites in many endogenous W-CRMs support the view that TCF/β-cat complexes are powerful transcriptional activators [26-28]. In many cases, TCFs also mediate default repression by binding to W-CRMs in the absence of signaling [23,28]. This regulation is commonly referred to as the TCF “transcriptional switch” [1,28]. While vertebrate TCFs have become more specialized for either default repression or β-cat-dependent activation, invertebrate TCFs such as *Drosophila* TCF/Pangolin (TCF/Pan) mediate both sides of the transcriptional switch [26,28].

All TCFs contain a sequence-specific DNA binding domain called the HMG domain, whose high affinity consensus is SSTTTGWW, (S=C/G, W=A/T) [29-31]. Invertebrate TCFs and some vertebrate TCF isoforms contain a second DNA binding

domain, C-terminal to the HMG domain, known as the C-clamp [26,32]. C-clamps recognize GC-rich motifs called Helper sites, and this interaction is essential for the activation of many W-CRMs [33,34]. These data support a model where C-clamp containing TCFs recognize W-CRMs in a bipartite manner, via HMG domain-HMG site and C-clamp-Helper site interactions [26].

While TCF/β-cat complexes are commonly associated with transcriptional activation, there are a few cases where they appear to directly repress target gene expression [35-38]. The HMG sites in these repressed W-CRMs are very similar to those found in activated targets. In one case, TCF/β-cat may achieve repression by interfering with the binding of another activating TF [35]. For another target, TCF/β-cat may form a complex with the transcriptional repressor Brinker, and HMG and Brinker binding sites are both required for the repression [38].

In contrast to the aforementioned examples, we previously showed that TCF/Pan mediated Wnt-dependent repression of a W-CRM from the *Ugt36Bc* locus through HMG sites with a consensus that is distinct (WGAWAW) from classic ones [39]. In addition to mediating Wnt-induced repression, TCF/Pan is required for basal expression of *Ugt36Bc* in the absence of signaling [39]. This suggests a “reverse transcriptional switch” occurs at *Ugt36Bc* compared to the switch seen in activated targets. Instead of TCF/Pan default repression and Wnt-dependent activation, the reverse switch consists of TCF/Pan basal activation and Wnt-dependent repression.

In this report, we have explored the mechanism of this reverse switch/direct repression mechanism by TCF/Pan and Wnt signaling in more detail. We identified

another repressed W-CRM from the *Tiggrin* (*Tig*) gene, which contains functional WGAWAW sites bound by TCF/Pan. Regulation of the *Ugt36Bc* and *Tig* W-CRMs by TCF/Pan requires the C-clamp, which binds to Helper-like (r-Helper) sites adjacent to the WGAWAW sites. Swapping these sites in the *Tig* W-CRM to classic HMG and Helper sites converts the W-CRM into one that is activated by Wnt signaling. Conversely, an activated W-CRM from the *naked cuticle* (*nkd*) locus was converted to a repressed W-CRM by replacing its classic HMG-Helper pairs with pairs from the *Tig* W-CRM. Partial protease digestion indicates that TCF/Pan adopts a different conformation when bound to classic or repressive sites, supporting allosteric regulation of TCF/Pan by its binding sites. In addition, we have extended this work from cell culture to the fly, showing that WGAWAW and r-Helper sites mediate basal activation and Wnt-induced repression in the larval lymph gland (LG). Wnt signaling is known to play an important role in regulating hematopoiesis in the LG [40]. Thus, our work provides insight into how TCF/Pan can activate and repress Wnt transcriptional targets, and extends the TCF reverse transcriptional switch mechanism to a physiologically relevant context.

Results

Regulation of Wnt-repressed targets requires the C-clamp of TCF/Pan

Ugt36Bc was originally identified as a candidate for repression by Wnt signaling from a microarray screen performed in Kc167 (Kc) cells [39], a *Drosophila* cell line likely of hemocytic origin [41]. Several other repressed targets were also

identified in this screen, including *Tig* [39], which encodes an extracellular matrix protein that serves as a PS2 integrin ligand [42,43]. *Tig* expression was repressed by DisArmed, a mutated version of Armadillo (Arm, the fly β-catenin) which is defective in gene activation but is still competent for repression [39]. While these results are consistent with *Tig* being directly repressed by Wnt signaling, the cis-regulatory information responsible for Wnt regulation of *Tig* expression had not been identified.

The *Tig* locus is compact, with a small (~1 kb) intergenic region and six introns, only the first of which is larger than 500 bp (Figure 2.1A). The intergenic region possibly also contains elements driving the expression of the adjoining gene, *Fic domain-containing protein* (*Fic*), a gene involved in fly vision [44]. *Fic* was expressed in Kc cells, but was not regulated by Wnt signaling (Figure 2.2). A 1.8 kb fragment containing the intergenic region between *Fic* and *Tig*, as well as the first exon and intron and part of the second exon of *Tig* was cloned upstream of a luciferase gene reporter (Figure 2.1C). This reporter (Tig1) was repressed 2-5 fold by Axin RNAi in Kc cells, similar to the fold regulation of endogenous *Tig* mRNA (Figure 2.1B and 1C). Expression of a stabilized form of Arm (Arm*) [45] also repressed the Tig1 reporter to a similar degree (data not shown). These results suggest that Tig1 contains most of the regulatory information required for Wnt regulation of the *Tig* gene.

To better understand which regions were responsible for basal expression and Wnt-dependent repression of *Tig*, smaller fragments of the regulatory sequences in Tig1 were analyzed. In some cases (Tig2 – Tig4), sequences were cloned upstream of the *hsp70* core promoter, which is unregulated by Wnt signaling [33,39,45], while

the Tig5 reporter used the endogenous *Tig* promoter. These reporters (Tig2 - Tig5) all had basal expression higher than the *hsp70* promoter control (Figure 2.1C). Much of the repressive activity appeared to be contained in a 578 bp fragment containing part of the first exon and most of the first intron (Tig3). However Tig1 was used for further functional experiments, to retain the endogenous promoter and additional cis-regulatory information of the *Tig* locus.

TCF/Pan has previously been shown to activate *Ugt36Bc* and *Tig* in the absence of signaling, and to be required for Wnt-mediated repression [39]. To determine whether the C-clamp of TCF/Pan was required for these activities, RNAi rescue experiments were performed. Endogenous TCF/Pan was depleted from Kc cells using dsRNA corresponding to the 3' UTR of *TCF/Pan*. Cells were then transfected with *Ugt36Bc* or *Tig* reporters, as well as expression plasmids for TCF/Pan, either wild-type control or a C-clamp mutant where five amino acids have been altered [33]. Wnt signaling was activated using Arm*. In control TCF/Pan depleted cells (transfected with empty vector), the *Tig* and *Ugt36Bc* reporters were not regulated by Arm* (Figure 2.3A, B). Wild-type TCF/Pan elevated basal expression and enabled significant repression by Arm*. In contrast, the C-clamp mutant neither activated nor repressed the reporters (Figure 2.3A, B). These data suggest that the C-clamp is required for TCF/Pan-dependent basal activity and Wnt-mediated repression of both reporters.

To ensure that the C-clamp mutant TCF/Pan was functional, a synthetic reporter containing multimerized HMG sites and lacking Helper sites (6xTCF) was

also examined (Figure 2.3C). As previously reported [33], the C-clamp mutant was able to rescue 6xTCF activation by Wnt signaling, albeit not completely under the conditions used (Figure 2.3C). Nonetheless, these data support an important role for the C-clamp in TCF/Pan regulation of the *Ugt36Bc* and *Tig*.

Tig and Ugt36Bc W-CRMs both contain distinct HMG and Helper sites

A search through the Tig1 sequences using the open access program Target Explorer [46] failed to find classic HMG sites (SSTTTGWWS) [29,31] or the Helper sites characterized in activated fly W-CRMs (GCCGCCR) [33]. However, the first intron of *Tig* contained several sequences that were similar to sites in the *Ugt36Bc* W-CRM that were footprinted by the HMG domain of TCF/Pan [39]. Therefore, similar footprinting of a 300 bp region of the *Tig* intron containing these putative sites (Figure 2.4A) was performed, comparing the footprint of GST and GST-HMG domain recombinant proteins (see Material and Methods for details). Several regions of this *Tig* regulatory DNA were protected by the HMG domain (Figure 2.5A), two of which are similar to the three WGAWAW sites previously found in the *Ugt36Bc* W-CRM [39]. Together, the five *Tig* and *Ugt36Bc* motifs defined a consensus of RNWGAWAW (Figure 2.4C). In addition, the regions of the *Ugt36Bc* and *Tig* loci containing the WGAWAW sites were footprinted with GST-HMG and GST-HMG-C-clamp, to identify C-clamp bound sequences. Three additional regions were protected only in the presence of the C-clamp (Figure 2.4B, 2.5A and 6). Alignment of these regions revealed a consensus of KCCSSNWW (K = G/T; Figure 2.4C), which was distinct

from the classic Helper sites found in activated W-CRMs. These motifs are hereafter referred to as repressive-Helper (r-Helper) sites and the HMG bound sequences as WGAWAW sites.

The r-Helper sites in the *Ugt36Bc* and *Tig* W-CRMs are adjacent to the WGAWAW sites (Figure 2.4A), similar to the HMG-Helper clustering in activated W-CRMs [33,34]. To test whether these motifs act together to form a high affinity binding site for TCF/Pan, labeled probes containing a WGAWAW-r-Helper pair from *Tig* and *Ugt36Bc* were synthesized (Figure 2.4D) and analyzed for binding to recombinant GST-TCF/Pan fusion proteins using EMSA (Electrophoretic Mobility Shift Assay). Both probes were bound by GST-HMG-C-clamp, and mutation of the WGAWAW site abolished binding (Figure 2.4E). Mutation of the r-Helper site abolished binding in the case of the *Ugt36Bc* probe, and resulted in a small but reproducible reduction in binding of the *Tig* probe (Figure 2.4E). This difference was also seen with the footprinting data, where GST-HMG-C-clamp protection of the *Ugt36Bc* r-Helper site (Figure 2.4B) was more pronounced than the r-Helper sites in the *Tig* W-CRM (Figure 2.6). Consistent with being C-clamp binding sites, the r-Helper motifs were not required for binding by GST-HMG protein (Figure 2.4F). Taken together, these data support a model in which TCF/Pan binds to the *Ugt36Bc* and *Tig* W-CRMs through bipartite binding of HMG domain to WGAWAW sites and C-clamp binding to r-Helper sites.

To determine whether the WGAWAW and r-Helper sites in the *Tig* W-CRM were functional, site-directed mutagenesis of the Tig1 reporter was performed.

Altering either WGAWAW or r-Helper sites resulted in a strong reduction of basal expression and Wnt-dependent repression (Figure 2.7A). These data were similar to those obtained when the WGAWAW sites in the pHsp-178 *Ugt36Bc* reporter were altered [39]. When the r-Helper site in pHsp-178 was mutated, a similar defect was observed as when the adjacent WGAWAW site was destroyed (Figure 2.7B). These data demonstrate that the distinct bipartite TCF/Pan binding sites found in the *Tig* and *Ugt36Bc* W-CRMs are necessary for basal expression of the reporters. In the absence of these motifs, Wnt signaling causes little reduction in expression of these reporters, either due to loss of basal expression and/or loss of active repression by the pathway.

In addition to the two WGAWAW sites in the *Tig* intronic W-CRM, five additional sequences were footprinted by the HMG domain, most of which were enriched with a TG-rich motif (Figure 2.5A). All five motifs were mutated, but the expression of these mutant reporters were not affected in a significant manner (Figure 2.5B). While it is possible that these motifs are functionally redundant, they were not analyzed further in this study.

The type of HMG and Helper sites determines transcriptional output of TCF/Pan through allosteric regulation

Since WGAWAW and r-Helper sites contribute to both basal activation and Wnt-mediated repression of *Tig* and *Ugt36Bc* W-CRMs (Figure 2.7) [39], these bipartite TCF binding sites could be sufficient for this regulation. To test this, a

synthetic reporter containing two repeats of a small stretch (40 bp) from the *Tig* W-CRM (each repeat contains two pairs of WGAWAW and r-Helper sites) was constructed (Figure 2.8A). This reporter, termed “minR” for “minimal repressed W-CRM”, was repressed about two-fold by Axin RNAi or Arm* expression in Kc cells (Figure 2.9A; data not shown). Like the *Tig* and *Ugt36Bc* W-CRMs, the basal expression of the minR reporter is dependent on the WGAWAW and r-Helper sites (Figure 2.10). These results demonstrate that these bipartite TCF sites are necessary and sufficient for the “reverse TCF/Pan transcriptional switch” that regulates targets repressed by Wnt signaling.

The behavior of minR is the qualitative opposite of classic HMG-Helper site pairs, which are highly activated by Wnt signaling [33]. This suggests that the TCF/Pan sites themselves dictate whether a W-CRM is activated or repressed by the Wnt pathway. To test this, the HMG-Helper sites in the *nkd*-IntE W-CRM, which is activated by Wnt signaling in Kc cells and flies [33,47], were replaced by WGAWAW-r-Helper sites (see Figure 2.8B for base pair changes). The basal activity of this “TCF sites swapped” *nkd*-IntE was significantly higher than either the original *nkd*-IntE or minR, suggesting a synergistic effect between the repressive TCF sites and the remaining sequences of *nkd*-IntE (Figure 2.9B). Strikingly, this W-CRM was repressed upon activation of Wnt signaling (Figure 2.9B).

To determine whether the *Tig1* W-CRM could be converted into an activated W-CRM, the functional WGAWAW and r-Helper sites identified in Figure 2.7 were converted into classic HMG and Helper sites (Figure 2.8C). This swapped Tig1

reporter was robustly activated by Wnt signaling (Figure 2.9C). To assess the individual contribution of each type of binding site to the switch in transcriptional output, r-Helper site only (H-only) and WGAWAW site only (W-only) swaps were constructed in the Tig1 reporter (Figure 2.8C). These “partial swap” W-CRMs lost the high basal expression of Tig1, and lacked the high activation seen when both motifs are swapped (Figure 2.9D). Taken together, these data argue that both the HMG domain and C-clamp binding domains are instructive in determining whether a W-CRM is activated or repressed by Wnt signaling.

Our findings that the transcriptional output can be reprogrammed by altering the TCF binding sites suggests that DNA is allosterically regulating TCF/Pan. To test this, recombinant HMG-C-clamp protein was incubated with excess oligonucleotides containing activating or repressed TCF sites followed by partial digestion with two proteases, chymotrypsin or endoproteinase Glu-C. The digested product was then separated on SDS-PAGE gels. The digestion patterns between HMG-C-clamp bound with a classic HMG-Helper site pair (TH) and WGAWAW-r-Helper pair (WH) were distinct, with several proteolytic fragments observed with TH that were not detectable with WH (Figure 2.11A and 11B). Analyzing HMG-C-clamp mobility on a native gel indicates that the majority of the protein was complexed with either the TH [33] or WH probe (compare the shift with a control SS probe which does not bind TCF in Figure 2.11C). These data strongly suggest that the conformations of the HMG and/or C-clamp domains are distinct when bound to activating or repressing TCF sites.

The HMG domain of LEF1 (a vertebrate TCF) is known to induce a sharp bend in DNA when bound to a classic HMG site [48]. Therefore, the possibility exists that differences in DNA bending could contribute to the transcriptional specificity of activated and repressed W-CRMs. To address this, probes where the position of the binding site was altered were tested via EMSA (Figure 2.S6). If protein binding induced a bend in the DNA, mobility will be slowest when the binding site was present in the middle of the probe [49]. Consistent with the LEF1 data, the HMG domain of TCF/Pan exhibited bending when bound to a classic HMG site (Figure 2.12B). In addition, GST-HMG could bend a WGAWAW site probe, though the bend was slightly less than the classic HMG site (Figure 2.12B). The presence of a C-clamp in the protein and a Helper site in the probe did not alter the degree of bending (Figure 2.12C). Likewise the reduction of bending of the WGAWAW site was still observed when paired with an r-Helper site and bound by GST-HMG-C-clamp (Figure 2.12D). The data demonstrated a small difference in bending between the activated and repressed binding sites, which could contribute to the transcriptional specificity.

Natural and synthetic WGAWAW, r-Helper containing W-CRMs function in the *Drosophila* hematopoietic system

To extend the analysis of Tig1 and minR reporters to the whole organism, these W-CRMs were cloned into P-element Pelican vectors [50], carrying the LacZ reporter gene plus insulators to minimize position effects, either using the endogenous *Tig* promoter (Tig1) or a heterologous one from *hsp70* (minR).

Transgenic lines were established and analyzed for LacZ expression in embryos and larva. Both reporters were active in embryonic hemocytes, as indicated by co-localization with MDP-1, a hemocyte marker (Figure 2.13A-H) [51]. We also found staining of both reporters in the larval lymph gland (LG), fat body and circulating hemocytes (Figure 2.14; data not shown). These patterns are similar to that of endogenous Tig in the LG (Figure 2.14A-C), as well as embryonic hemocytes and fat body [42]. These results indicate that both reporters can be used to study regulation by Wnt signaling in vivo.

The Tig1 and minR reporters are both expressed at much higher levels in the cortical zone (CZ) of the LG, an irregularly shaped region containing mature hemocytes enriched in the periphery of the LG (Figure 2.14B, D, H). This pattern is largely non-overlapping with Wingless (Wg, a fly Wnt), which is enriched in the medullary zone (MZ) containing prohemocytes [40] (Figure 2.8E and 8I). The Wg pattern is more apparent in younger late 3rd instar larvae, i.e., ~96-104 after egg laying (~96-104 AEL; Figure 2.14D-K), but the lacZ reporters expressed highest in older late 3rd instar larvae (~104-112 AEL; Figure 2.14A-C). The expression of the reporters did not overlap with Lozenge-Gal4>>UAS-GFP (Lz>>GFP), which marks crystal cells, a hemocyte lineage found in the CZ that often has high Wg expression [40] (Figure 2.15). While the presence of Wg in the MZ doesn't necessarily imply active Wnt signaling, these results support a model where Wnt signaling represses Tig and minR expression in this portion of the LG.

To test whether the Tig1 and minR reporters were repressed by Wnt signaling in the LG, the Gal4 misexpression system [52] was used to modulate the Wnt pathway. Serpent-Gal4 (Srp-Gal4), which is active throughout the LG [53], was combined with UAS lines expressing Arm* or DisArmed in a background containing either reporter. Expression of either Arm* or DisArmed in the LG repressed the minR (Figure 2.16A, D and G) and Tig (Figure 2.16J, M and P) reporters with 100% penetrance. Under the conditions employed, no detectable change in expression of Cut, a CZ marker (Figure 2.17) [53], was observed (Figure 2.16B, E, H, K, N and Q), ruling out a gross change in cell fate in the LG being responsible for the loss of reporter expression. With stronger or longer expression of Arm*, we did observe a strong reduction of the CZ cell fate as previously reported (Figure 2.18) [40]. The results indicate that Wnt signaling can repress the *Tig* and minR reporters in the CZ without detectably altering cell fate. In addition, the finding that DisArmed can mediate this regulation suggests that the transcriptional activation activity of Arm is not required for this regulation.

To test whether the Tig1 and minR reporters were repressed by Wnt signaling in embryonic hemocytes, we expressed Arm* or DisArmed under the control of two embryonic hemocyte drivers, Srp-Gal4 or Croquemort-Gal4 (Crq-Gal4). No detectable repression was observed (data not shown). To examine whether the negative results were due to perdurance of LacZ, we assayed circulating hemocytes from mid 3rd instar larvae (~88-96 AEL). This is prior to release of LG hemocytes, so all circulating hemocytes are of embryonic lineage at this developmental stage [54].

Hemese-Gal4 (He-Gal4) [55], a circulating hemocyte driver, was used to drive the expression of UAS-Arm* or UAS-DisArmed. Expression of either transgene resulted in a significant repression of the minR reporter (Figure 2.19), demonstrating Wnt repression of this reporter in the embryonic hemocyte lineage.

Our working model is that TCF/Pan activates Tig1 and minR expression in the CZ of the LG, while Wnt signaling represses these reporters in the MZ. To test this, we examined reporter expression when dominant-negative versions of Frizzled and Frizzled2 (FzDN and Fz2DN) [56,57] were expressed via the MZ driver Dome-Gal4 [58]. We observed a strong expansion of minR in these LGs, but there was also a concomitant expansion of the CZ, indicated by a reduction of Dome>>GFP (Figure 2.20) and increase in the area of Cut expression (data not shown). This is consistent with a previous report demonstrating that Wnt signaling is required for maintenance of the MZ [40]. Depletion of TCF/Pan in the CZ using RNAi caused the predicted reduction in reporter gene expression, but there was also a reduction in the CZ (Figure 2.21; data not shown). In both cases, the change in reporter expression was coupled with a change in cell fate, preventing a definitive demonstration that endogenous TCF/Pan and Wnt signaling regulates the minR and Tig reporters in the LG (see Discussion for further comment).

To confirm that the Tig1 and minR reporters are directly regulated by TCF/Pan in vivo, the WGAWAW sites and r-Helpers in these elements were mutated. Mutation of either motif abolished expression of both reporters in the LG (Figure 2.22). In embryonic hemocytes, the WGAWAW site mutant of minR had no detectable

expression (Figure 2.23 G-I), while there was some residual hemocytic expression in the r-Helper mutant (Figure 2.23 D-F). There was no obvious reduction in the Tig1 reporter in embryonic hemocytes when the two functional WGAWAW or two r-Helper sites identified in Kc cells were destroyed (data not shown). This caveat aside, the results indicate that the reverse transcriptional switch documented in Kc cells ([39] and this report) is also operational in the *Drosophila* hematopoietic system.

Discussion

Bipartite TCF binding sites mediate a reverse transcriptional switch

This study extends our previous work characterizing WGAWAW sites in the *Ugt36Bc* W-CRM [39], identifying additional sites in another repressed target, *Tig*, and refining the consensus of these sites to RNWGAWAW (Figure 2.4C). These sites are distinct from traditional HMG sites (SSTTTGWWS) identified in earlier studies of TCF binding [29,31]. These studies failed to identify WGAWAW sequences as TCF binding sites, perhaps because their experimental designs were biased for the highest affinity sites. However, Badis and coworkers used a microarray of randomized 8-mers to survey DNA binding domains of TFs found WGAWAW sites among the preferred binding sites for HMG domains derived from the four human TCFs [59]. To illustrate this point, we examined where eight functional classic HMG sites from activated W-CRMs and the five WGAWAW sites from the *Tig* and *Ugt36Bc* W-CRMs rank among the nearly 33,000 8-mers tested by Badis and coworkers (Table 2.1). Two classic sites from a *Notum*/*wingful* W-CRM [33] were the top-ranked

site for all four HMG domains, while the third site from this W-CRM ranked 2-4th, depending on the protein. For classic sites in two *nkd* W-CRMs [33,47], the rankings were lower, on average between 112th and 2833rd. The repressive WGAWAW sites we identified ranked between 98th and 4167th (Table SI). This work highlights the diversity of DNA recognition by HMG domains (which was also observed for half of the 104 TFs tested in this study) [59], and reveals that WGAWAW sites are a preferred class of HMG binding for TCF/Pan and vertebrate TCFs.

In addition to HMG domain-WGAWAW site binding, we found that C-clamp interactions with r-Helper sites are required for TCF/Pan to regulate the *Tig*, *Ugt36Bc* and minR W-CRMs. The C-clamp is required for regulating the *Ugt36Bc* and *Tig* reporters (Figure 2.3), and WGAWAW and r-Helper sites in these W-CRMs are required for expression in Kc cells (Figure 2.7) as well as for the *Tig1* W-CRM in the larval LG (Figure 2.22). Multimerized WGAWAW-r-Helper site pairs are sufficient for high basal expression and repression by Wnt signaling (Figures 2.9A, 2.16 and 2.22). The three characterized r-Helper sites share a loose consensus of KCCSSNWW and the spacing between adjacent WGAWAW and r-Helper sites is less than 7 bp among the sites we have examined (Figure 2.4B and 6). More functional WGAWAW, r-Helper site pairs need to be identified to better understand the sequence, spacing and orientation constraints on what constitutes this class of bipartite TCF binding site.

In contrast to the *Ugt36Bc* and *Tig* W-CRMs, in several other cases traditional HMG sites have been found to mediate Wnt repression in *Drosophila* [35,38] and mammalian cell culture [36,37]. An examination of the sequences surrounding the

functional HMG binding sites in the fly repressed W-CRMs did not reveal obvious candidates for r-Helper or Helper sites (C. Zhang and K. Cadigan, unpublished observations). In these cases, TCF/Pan is proposed to act with other TFs, either competing for binding with an activator [35] or acting in concert with the transcriptional repressor Brinker [38, 60]. We favor the view that the mechanism described in this report is distinct from these other examples of Wnt-mediated repression.

The common models for signal-induced repression require the presence of a default activator bound to DNA near the repressive sites [2,18,36]. In contrast, in the TCF-mediated repression described in this report, both basal activation and repression occur through the same TCF binding sites (Figure 2.24). Mutagenesis of WGAWAW sites and r-Helper sites argue that they are both required for basal activation (Figures 2.7, 2.22 and 2.23), while repression of the minR and Tig reporters by Arm* and DisArmed argue that these sites are also responsible for Wnt-dependent repression (Figures 2.9, 2.16 and 2.19). Consistent with a dual role in regulating these W-CRMs, depletion of TCF/Pan via RNAi resulted in a reduction of basal activation and loss of Wnt-repression (Figure 2.3). Our data supports the model of a “reverse TCF transcriptional switch” that we have published previously [39], and this work extends this mechanism to the Tig W-CRM and highlights the importance of the C-clamp and r-Helper sites in this regulation (Figure 2.24).

While we favor the model outlined in Figure 2.13, it is possible that it is an over-simplification and several things remain to be clarified. For example, mutation of

the WGAWAW or r-Helper sites results in a dramatic loss of basal activation (Figure 2.2.7, 2.22 and 2.23) while depletion of TCF/Pan has a more modest reduction (Figure 2.3) [39]. This raises the possibility that other TFs could also act through the WGAWAW and r-Helper sites to achieve basal expression. For example, it is possible that TCF/Pan and Arm inhibit transcription by displacing other activating TFs from W-CRM chromatin. Another possibility is that Arm interaction with TCF/Pan disrupts its ability to bind to the bipartite site, though this model is not supported by ChIP data at the *Ugt36Bc* locus [39]. Further investigation is needed to determine whether additional regulators of these W-CRMs exist and if so, how do they functionally interact with TCF/Pan.

Allosteric regulation of TCF/Pangolin by DNA

Our report provides a dramatic example of how the DNA site can influence the transcriptional output of the TF binding to the site. Replacing classic HMG and Helper sites in a W-CRM (*nkd*-IntE) with low basal expression and a high degree of Wnt activation completely inverted the regulation: the altered W-CRM had high basal expression and was repressed by Wnt signaling (Figure 2.9B). Just as strikingly, changing 22 bps in the 1.8 kB Tig1 reporter, which converted two WGAWAW and two r-Helper sites into classic motifs, resulted in a W-CRM that behaves like a conventionally activated W-CRM (Figure 2.9C). Both the HMG and C-clamp binding sites needed to be swapped for this switch in regulation to occur (Figure 2.9D). These results clearly demonstrate that the type of bipartite TCF binding site to which

TCF/Pan binds determines whether it acts as an activator or repressor upon Wnt stimulation.

There are other examples of switching the transcriptional output of CRMs through altering the sequence of TF binding sites. Mutating sequences adjacent to Dorsal binding sites converts a repressed CRM into an activated one, suggesting that for Dorsal, transcriptional activation is the default state [12,13]. Altering the binding site of Thyroid receptor or POU1 converted CRMs from repressed to activated elements [14,16,17]. In these cases, the conversion was only made in one direction, leaving open the possibility that the TF binding sites are not completely sufficient for determining the activation/repression decision.

In our previous report on Wnt mediated TCF/Pan repression, the repressed *Ugt36Bc* W-CRM was converted to an activated one by changing three WGAWAW sites into classic HMG sites [39]. However, Wnt activation was only achieved when the *Ugt36Bc* W-CRM was placed adjacent to the *metallothionein* (*MT*) promoter and a small amount of Cu2+ was added [39]. When the *hsp70* promoter was used, the altered *Ugt36Bc* W-CRM was not active, similar to the HMG site only swap in the Tig1 W-CRM (Figure 2.9D). Our new data strongly suggests that the complications in the prior report were due to our lack of knowledge of Helper sites, which we have now demonstrated to be essential for controlling the transcriptional output of W-CRMs.

The conformation of the HMG and/or C-clamp domains of TCF/Pan is different when bound to a classic HMG-Helper pair compared to a WGAWAW-r-Helper pair, as judged by protease digestion patterns (Figure 2.11). In addition, the degree of

bending of the DNA by the HMG domain is reduced when it is bound to a WGAWAW site (Figure 2.12). Presumably, these structural differences are transmitted to Arm protein bound to TCF/Pan, leading to differential recruitment of transcriptional co-regulators, as has been suggested for other TFs [20,61]. Our results add to the growing recognition that TF binding sites are not just for recruiting TFs to regulatory DNA, but also have a profound influence on the TF's functional activity.

Wnt mediated repression in the hematopoietic system

Repressed W-CRM reporters, either natural (Tig1) or synthetic (minR), are active in embryonic and larval hematopoietic systems (Figures 2.13, 2.14 and 2.19), and are regulated by Wnt signaling (Figures 2.16 and 2.19). The data in the LG are especially interesting, given that Wnt signaling has been shown to control several cell fate decisions in this tissue. The Wnt pathway is required for maintenance and proliferation of the posterior signaling center (PSC), which functions as a hematopoietic niche in the LG [40,62]. In addition, Wnt signaling promotes prohemocytic cell fate, blocking their differentiation in the MZ of the LG as well as promoting proliferation of crystal cells [40]. The Tig and minR reporters displayed minimal expression in the MZ and crystal cells (Figure 2.2.15 and 2.20), and their high expression in the CZ can be repressed by ectopic activation of Arm and DisArmed (Figure 2.16). Since DisArmed has little/no ability to activate transcription but retains repressive activity [39], these data suggest the existence of Arm-dependent repression of gene expression in the prohemocytes of the MZ.

Wnt-mediated repression of the *Tig* and minR W-CRMs in the LG is likely direct, based on site-directed mutagenesis of the WGAWAW and r-Helper sites (Figures 2.22 and 2.23). However, we were unable to demonstrate that endogenous TCF/Pan and Wnt signaling regulates these reporters, because the genetic manipulations also altered the ratio of pro-hemocytes (MZ) and differentiated hemocytes (CZ; Figure 2.2.20 and 2.21). Thus, we could not uncouple cell fate change from regulation of the reporters in our loss of function experiments. It may be that the thresholds for maintaining the CZ and MZ cell fates and regulating the reporters are too similar. Another possibility is that Wnt signaling works redundantly with another factor to repress these reporters in the MZ. Having said this, it's interesting to note that the expression of Peroxidasin (Pxn), normally restricted to the CZ of the LG, expands into the MZ when Wnt signaling is inhibited [40]. Pxn has also been shown to be repressed by Wnt signaling and DisArmed in Kc cells and embryonic hemocytes [39], suggesting a similar relationship in the LG.

The minR synthetic reporter is regulated by Wnt signaling in Kc cells, as well as hemocytes derived from embryos and the LG (Figures 2.9, 2.16 and 2.19). This regulation depends on the WGAWAW and r-Helper sites in all three contexts (Figures 2.22, 2.23, 2.10). The Tig1 reporter is similarly regulated in Kc cells (Figure 2.1) and the LG (Figures 2.16, 2.22). In contrast, we found no detectable regulation in embryonic hemocytes (data not shown), even though the reporter is expressed there (Figure 2.13) and *Tig* transcripts were repressed by Wnt signaling in these cells [39].

We suspect that the 1.8 kb Tig1 reporter may lack some cis-regulatory information required for Wnt regulation in embryonic hemocytes.

Whether the repressive TCF sites can respond to Wnt signaling in other tissues remains unclear, since the minR and Tig reporters have no basal activity outside the hematopoietic system and fat body. To explore whether WGAWAW and r-Helper sites function outside of these tissues, we utilized a GFP reporter containing binding sites for Grainyhead (GRH), which provide basal activity in the imaginal discs [63]. Classic or repressive TCF sites were placed downstream of the GRH sites and transgenic flies generated and analyzed (Figure 2.25). While classic HMG-Helper site pairs (4TH) displayed strong expression consistent with activation by Wg signaling (Figure 2.25B, G and L), insertion of the minR sequences had no effect on the GRH-GFP reporter (Figure 2.25D, I and N). These results suggest that WGAWAW and r-Helper sites only respond to Wnt signaling in specific tissues (e.g. the LG). Conversely, 6TH and several other reporters that are activated by Wnt signaling in many tissues [33,47] are not expressed in the LG (Figure 2.26; data not shown). These data argue that the mechanism of Wnt gene regulation in the LG is different from other tissues such as imaginal discs, perhaps because the reverse transcriptional switch mechanism plays a greater role in this tissue. Further studies are needed to identify additional W-CRMs that are active in the LG, and to determine whether the regulatory mechanism uncovered in this report underlies Wnt control of PSC, pro-hemocyte and crystal cell fate in the fly LG.

Materials and Methods

Drosophila cell culture, RNAi, qRT-PCR, transient transfection and reporter assays

Kc cells were cultured and transient transfections were carried out as previously described [45]. For RNAi treatments, cells were seeded at 1×106 cells/ml in growth media supplemented with 10 μg/ml dsRNA for 4 days, diluted to 1×106 cells/ml without additional dsRNA, and grown for 3 more days for luciferase assay using Tropix Chemiluminescent Kits (Applied Biosystems) or 2 more days for mRNA preparation using Trizol Reagent (Invitrogen). dsRNAs targeting the 3'UTR of *TCF/Pan* [33] and the ORFs of *Axin* or a control gene (*β-lactamase*) were used [39]. qRT–PCR was performed as previously described [39]. Gene expression among different samples was normalized to *tubulin56D* levels.

Each treatment in reporter assays was done in triplicate wells, each containing 2.5×105 cells. For standard reporter assays, 50 ng luciferase reporter and 6.25 ng LacZ per well were transfected with Axin RNAi or control RNAi. For TCF/Pan rescue assays, same amount of reporter and LacZ plus 50 ng TCF/Pan-expressing plasmid and 250 ng Arm* per well were transfected with TCF/Pan RNAi. pAc5.1-V5/His-A vector was used to equalize DNA content between samples and as a negative control for expression vectors. Luciferase activity was normalized to β-galactosidase activity from pArm-LacZ to control for differences in transfection efficiency among samples. In the figures, each bar represents the mean of biological triplicates and the data shown are representative of three independent experiments. All RLA units are arbitrary units unless otherwise specified.

Plasmids

All luciferase reporter vectors are derivatives of pGL2 or pGL3 (Promega). pHsp-178, Tig1, Tig5 and all site mutants and swaps based on these W-CRMs were cloned into pGL2-basic. Tig2-4, minR, *nkd*-IntE and all site mutants and swaps based on these W-CRMs were cloned into pGL3-basic containing an *Hsp70Bb* minimal promoter. Vector with a *Hsp70Bb* promoter but containing no W-CRM was used to control for basal promoter activity. A *MluI* site was introduced into Tig1 upstream of the TCF sites for the ease of cloning of the swap constructs. Sequence changes were done using site-directed mutagenesis (QuickChange SDM kit, Stratagene) or recursive PCR [64]. Restriction sites and primer sequences are in Table SII or as previously described [33,39].

For expression plasmids, pAc-TCF (WT/C-mut), pAc-Arm*, pGEX-GST, pGEX-GST-HMG and pGEX-GST-HMG-C-clamp (WT/C-mut) have been described elsewhere [33,39]. pArm-LacZ, a derivative of pAc-LacZ (Invitrogen) using the Arm promoter [65] was used as a transfection control.

EMSA and DNA bending assays

EMSAs were performed as previously described [39]. All GST-tagged proteins used in this study were purified from *E. coli*. 4nM biotinylated probe (IDT, Coralville, IA) and 7-20 μM protein were used in each reaction. The conditions in the DNA bending assays were similar to the EMSA assays except for the following modifications: 4 nM biotinylated probe was incubated with 20 μM (for WH and WS), 200 nM (for TH) or 500 nM (for TS) protein before separating on 5% native PAGE gel.

The probes for the DNA bending assays were generated according to a previously described strategy [49]. In short, the indicated TCF binding sites were cloned into pGL2-basic vector, and seven pairs of primers at varied positions on the vector were used. PCR products were digested at both ends by *EcoRI*, whose sites were introduced by the primers, and biotinylated through Klenow reaction using Biotin-16-dUTP (Roche). Probes containing a SS site (with both HMG site and Helper site mutated from TH) were generated to confirm that TCF/Pan has no detectable affinity to the surrounding sequences on the probes (data not shown). The sequences of TCF binding sites are summarized in Table SII. The WH and WS probes have the binding sites from Tig1 used in the EMSAs shown in Figure 2.4B. The sequences of the TH, TS and SS sites are previously described [33].

Fluorescent footprinting

DNaseI fluorescent footprinting was performed as previously described [39]. 20 μM GST-HMG or GST-HMG-C-clamp was used in 50 ul reactions with 12 nM labeled probes. The probes were generated by PCR using one labeled primer and one unlabeled primer (IDT) (Table SII). For comparison between GST and GST-HMG, or GST and GST-HMG-C-clamp, or GST-HMG and GST-HMG-C-clamp, FAM and HEX labeled probes were used in two parallel reactions with different proteins, and combined after digestion. 303 bp in the middle of the *Tig* intronic W-CRM and the full length *Ugt36Bc* W-CRM (178 bp) were footprinted (see Table SII for sequence information).

Partial proteolytic digestion and reverse EMSA

20 µl reactions containing 3-6 µM GST-HMG-C-clamp and 20x of the indicated DNA oligonucleotide were incubated for 5 min on ice and 15 min at room temperature. The buffer was the same as used for EMSA but without poly-dI*dC. Protease was then added (for partial proteolytic digestion) or not (for reverse EMSA) at a final concentration of 5-50 ng/µl for chymotrypsin (Roche) or 50-150 ng/µl for endoproteinase Glu-C (New England Biolabs). The mixture was incubated at 25℃for 2.5-3 hours. Then the digested product was loaded onto 16% tricine SDS-PAGE gel [66], and the undigested mixture was loaded onto 6% native PAGE-gel. After running, the gels were silver stained as previously described [67].

Drosophila genetics

Tig (Tig1) and minR fly reporters were generated by cloning the corresponding sequences into pPelican and pHPelican vectors, respectively [50]. All 3xGRH-W-CRM fly reporters were generated by cloning the corresponding sequences into pDestination-eGFP vectors via pENTR/D-TOPO using the Gateway technique, then injecting into integration site 86Fb [68,69]. Transgenic flies were generated by BestGene Inc. (Chino Hills, CA), Genetic Services Inc. (Cambridge, MA) and Rainbow Transgenic Flies Inc. (Thousand Oaks, California).

All the Gal4 and UAS lines used in this study have been previously described: Srp-Gal4 [70], Dome-Gal4 [71], Lz-Gal4 [72], Cg-Gal4 [73], HmlΔ-Gal4 [74], UAS-Arm* and UAS-DisArmed [39], UAS-FzDN and UAS-Fz2DN [56,75] and the DHH triple marker line containing Dome>>EBFP, Hml>>dsRed and Hh>>GFP [76]. The UAS-TCF/Pan-RNAi was a recombinant of two TCF/Pan RNAi lines, one from

Vienna Drosophila Resource Center and the other from the *Drosophila* RNAi Screen Center.

The Srp>>Arm* and DisArmed experiments were carried out in the presence of tub-Gal80ts. Crosses were set up at 18ºC, and the larvae were transferred to 25ºC for 2 days (Figure 2.16) or 3 days (Figure 2.18) before assaying.

Immunohistochemistry of embryos and LG

3rd-instar larvae were dissected in ice cold PBS from the ventral midline in a similar manner as body wall muscle preparations [43]. For β-galactosidase stainings, exposed LG were fixed in 1% glutaraldehyde at room temperature for 15-20 min, then washed twice and stained in X-gal staining solution [77] with 1-2% X-gal for 10-60 min. Preparation of embryos, immunostaining and microscopy were as previously described, and methods for immunostaining of wing discs were adapted for LG [33]. At least 20 embryos or 12 LGs were analyzed for each condition, and the examples presented are representative.

Primary antibodies were used at the following dilutions: mouse α-wg at 1:150, mouse α-Cut at 1:100 and rabbit α-Tig [78] at 1:75 for LG staining; mouse α-MDP-1 [51] at 1:100 for embryo staining, and rabbit α-LacZ (MP Biomedicals) at 1:400 for embryo or 1:600 for LG staining. Secondary antibodies were described previously [45].

Immunostaining and quantification of circulating hemocytes

Collection and processing of circulating hemocytes were as described previously [76]. Immunostained circulating hemocytes carrying the minR or Tig1 lacZ

reporters were imaged using the Leica SP5 laser scanning confocal microscope with four channels representing LacZ, He>>GFP, P1 (a plasmatocyte marker) [79] and DAPI. Random hemocytes were circled as regions of interest (ROI) and quantified using the Leica LAS AF software. We observed little or no difference between control (He-Gal4>>+) and experimental groups (He>>Arm* or He>>DisArmed) for the DAPI and P1 and some fluctuation in the GFP channel, which could be due to Arm* or DisArmed affecting cell fate/identity. Therefore, we only used hemocytes whose He>>GFP signal intensity falls into the range of control hemocytes. For quantification, 10-15 hemocytes per larvae and 5 larvae per genotype were used.

Acknowledgements

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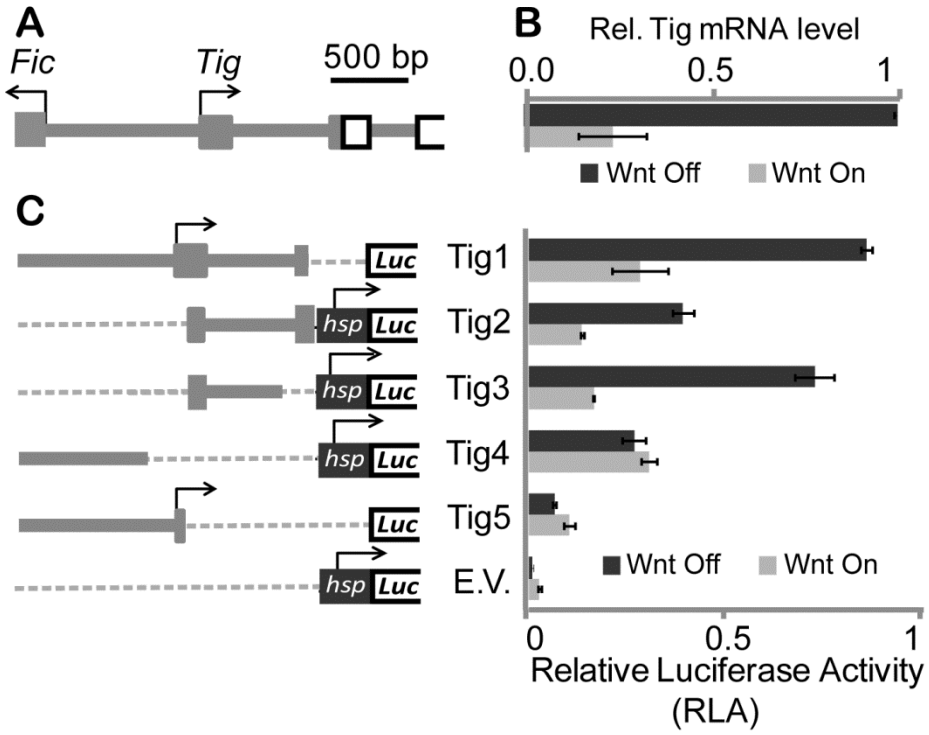


Figure 2.1. Characterization of *Tig* cis-regulatory information in Kc cells. (A) Cartoon depicting the intergenic region between the *Tig* and *Fic* loci. Bent arrows represent the TSSs of each gene, grey boxes the 5' UTRs, and white rectangles the *Tig* ORF. (**B)** *Tig* transcript levels in Kc cells are repressed when Wnt signaling is activated via Axin RNAi as previously described [39]. (**C)** The Tig reporters assayed are depicted on the left. The *hsp70* (*hsp*) promoter is not drawn to scale. Regulation of the luciferase reporters by Wnt signaling (using Axin RNAi) in Kc cells is shown in the graph on the right. See Materials and Methods for details of the transfection conditions.

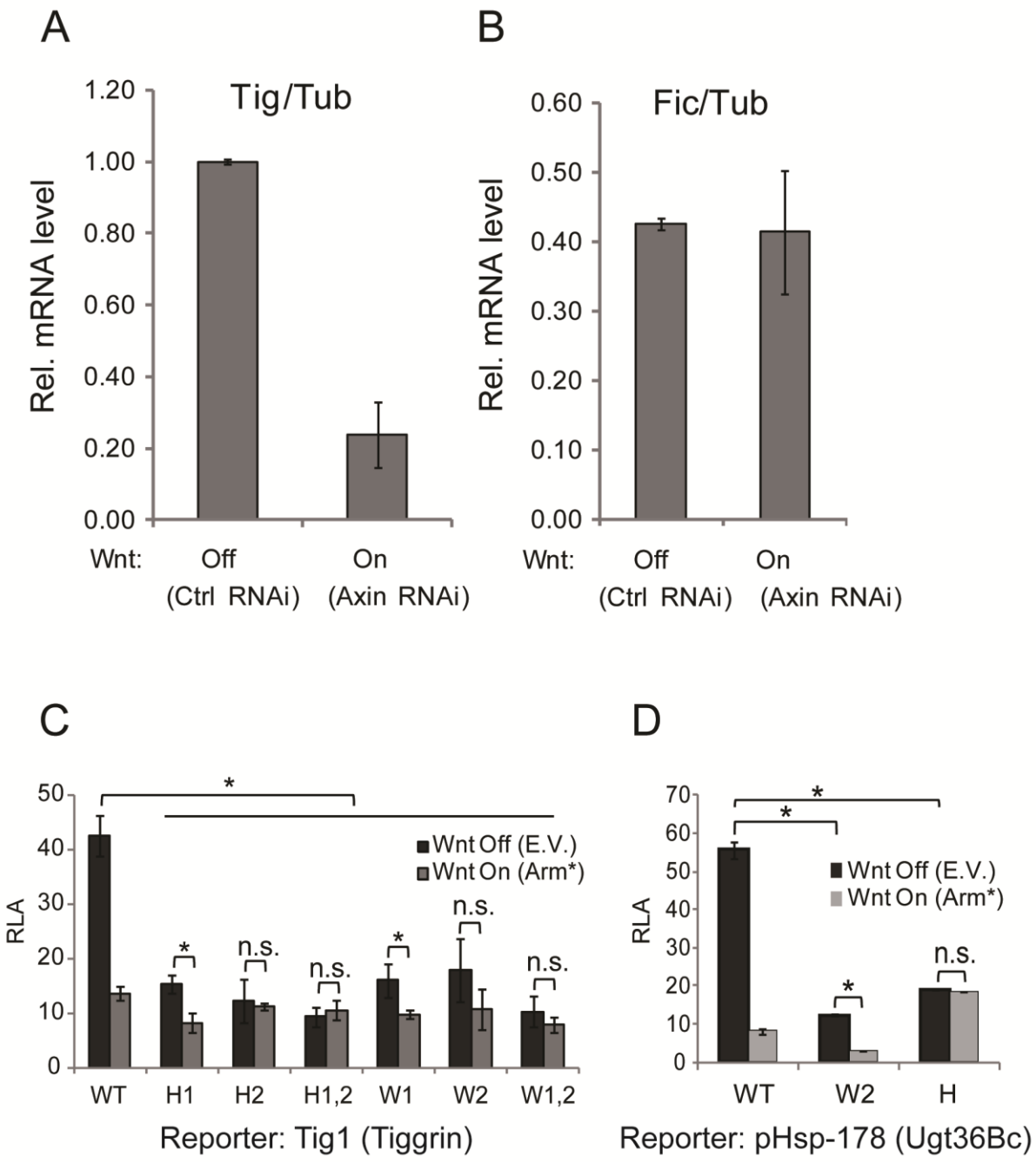


Figure 2.2. Expression of *Fic* is not affected by Wnt signaling. (A, B) Kc cells were treated with control (Wnt Off) or Axin (Wnt On) dsRNA for six days and processed for transcript analysis as described in Materials and Methods. *Tig* expression is repressed by Wnt signaling (A), which *Fic* expression is unaffected (B).

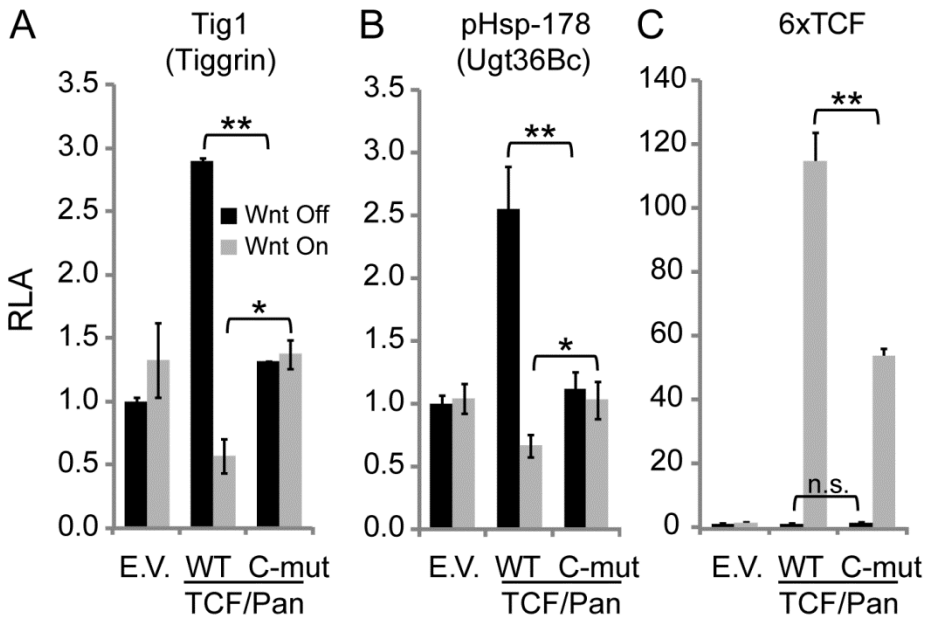


Figure 2.3. The C-clamp domain of TCF/Pan is required for Wnt-mediated repression of *Tig* and *Ugt36Bc* W-CRMs. TCF rescue assays in Kc cells were performed as previously described [33]. Endogenous TCF was depleted with dsRNA corresponding to the *TCF/Pan* 3'UTR for four days before co-transfection of W-CRM reporters with empty expression vector (E.V.) or ones expressing wild-type (WT) TCF/Pan or TCF/Pan containing five amino acid substitutions in the C-clamp (C-mut). Wnt signaling was activated by the over-expression of Arm*. (**A**) The Tig1 reporter is not regulated in TCF/Pan depleted cells. Transfection of WT TCF/Pan rescues basal activation and Wnt-mediated repression, but the C-clamp mutant variant does not. **(B)** The *Ugt* W-CRM reporter pHsp-178 [39] behaved similarly to as Tig1. For both reporters, WT TCF/Pan repressed expression to significantly lower levels than the C-clamp mutant (compare the fourth and sixth bars). **(C)** Activation of a synthetic reporter containing six classic HMG binding sites (6xTCF) was rescued by wild-type TCF, while the C-clamp mutant rescued activation about half as well. In each experiment, luciferase activity in the absence of Wnt signaling without TCF expression was normalized to 1.0 for each reporter. *P<0.05. **P<0.01. n.s., not significant (Student's T-test).

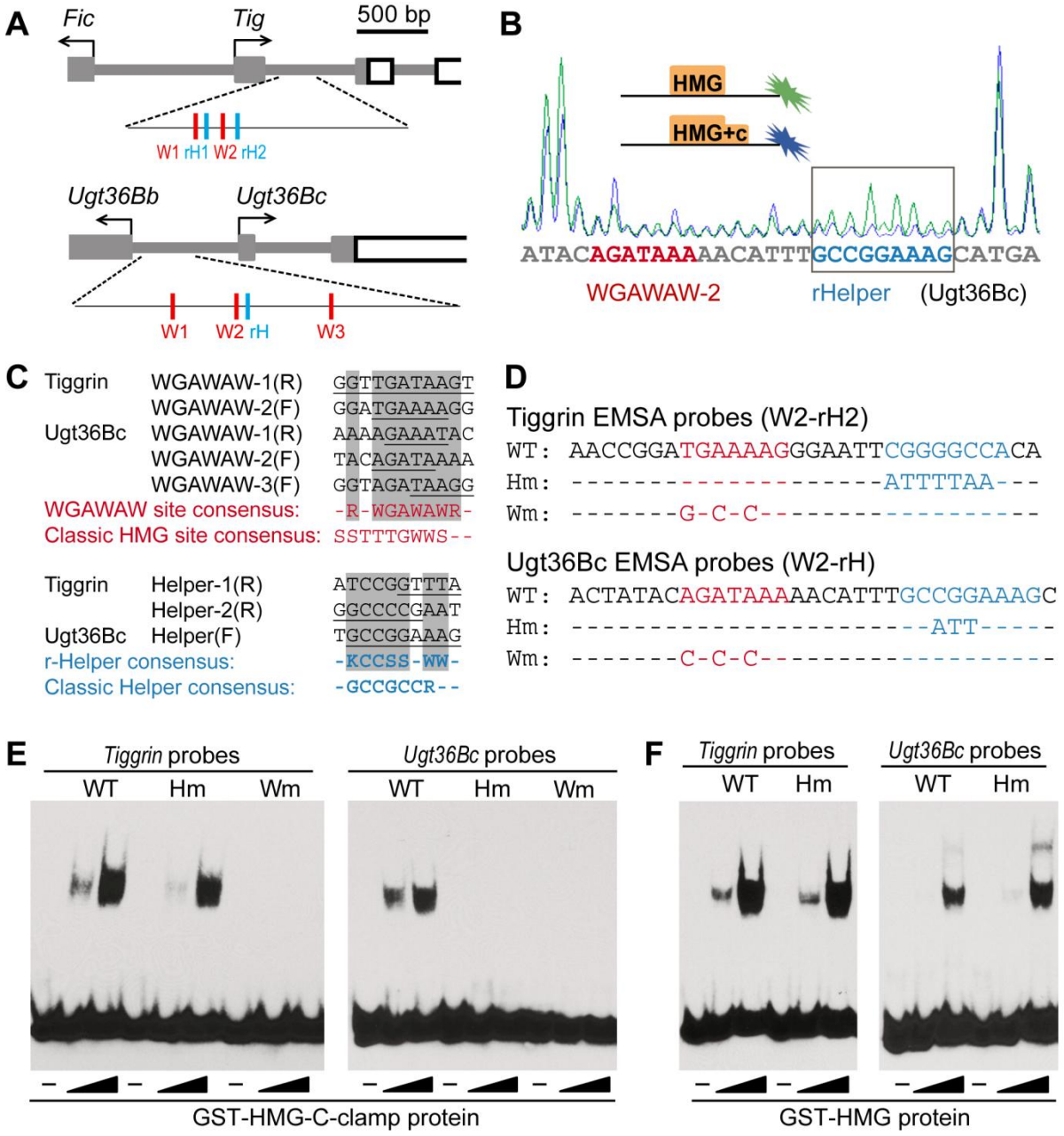


Figure 2.4. TCF recognizes repressed W-CRMs through a bipartite mechanism. **(A)** A cartoon showing the *Tig* and *Ugt36Bc* loci, along with the regions that were footprinted indicating the location of the WGAWAW sites (red) and r-Helper sites (blue). **(B)** Example of a footprinting chromatograph showing the C-clamp-specific protection of the r-Helper in the *Ugt36Bc* W-CRM. The boxed region where the green peaks are higher than the blue indicates sequences protected by GST-HMG-C-clamp and not by GST-HMG. **(C)** Alignment of the WGAWAW and r-Helper sites identified by footprinting from the *Tig* and *Ugt36Bc* W-CRMs. The WGAWAW sites were identified by comparing footprints of GST-HMG and GST, while r-Helper sites were footprinted by GST-HMG-C-clamp and not GST-HMG. In the alignments, the

footprinted sequences are underlined. The consensuses for each motif are shown, along with the classic HMG and Helper site consensuses. **(D)** Sequences of the probes used for EMSA, derived from two endogenous WGAWAW, r-Helper pairs. Mutations in the r-Helper and WGAWAW motifs are indicated. (**E)** EMSA data showing that both WGAWAW sites and r-Helper sites were required for maximal binding with GST-HMG-C-clamp protein. The reduction of binding with the Tig Hm probe was slight but reproducible. (**F)** EMSA showing that r-Helper sites were not required for binding by GST-HMG protein. All footprinting and EMSA experiments were performed at least three times with similar results.

A

gcaggggcccaagaaatgtgttccagaaataagcaatactatatttgggaaaatgca
① ****** ②* * *

tgtgtgacccatttgggggagacgtcttctgtggggccctgaaatcgaaagttacatg
③ ****** ④****** * * *

tcgtcaagtccatgggacttatcaacctaaaccggatgaaaaggaattcggggccac
* * * * * * * * * ********

atacattagtctaattgttgtcaaaa
⑤* ****

Underline: footprinted sites with GST-HMG but not GST.
Red: functional WGAWAWR sites in reporter assay.
Blue: functional r-Helper sites in reporter assay, footprinted with GST-HMG-C-Clamp but not GST-HMG.
Green: additional "TG-rich"motif footprinted by GST-HMG.
*: mutated nucleotides.
①- ⑤: additional footprinted sites (AddFP-1~5).

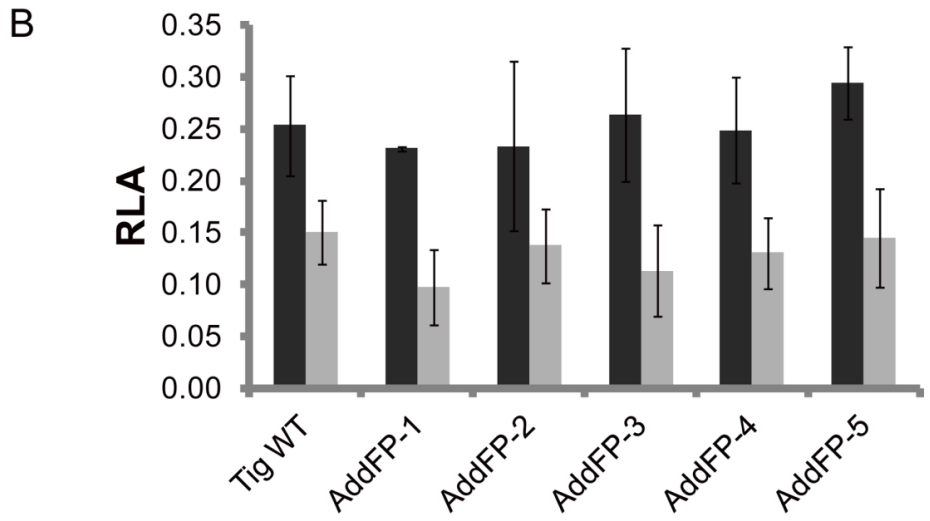


Figure 2.5. Sequences protected by GST-HMG and/or GST-HMG-C-clamp in the *Tig* intron. (A) The 200 bp stretch of the *Tig* probe containing all footprinted regions is shown, with the HMG domain and C-clamp protected regions indicated. Two WGAWAW sites (red) are bound by the HMG domain, as well as several other sites (green). r-Helper sites bound by the C-clamp are shown in blue. The sequences that were mutated for the reporter assays shown in Figure 4 or Figure S2B are indicated with asterisks. (**B)** Tig1 reporters containing mutations in the TG-rich regions footprinted by the HMG domain were similar to the wild-type control.

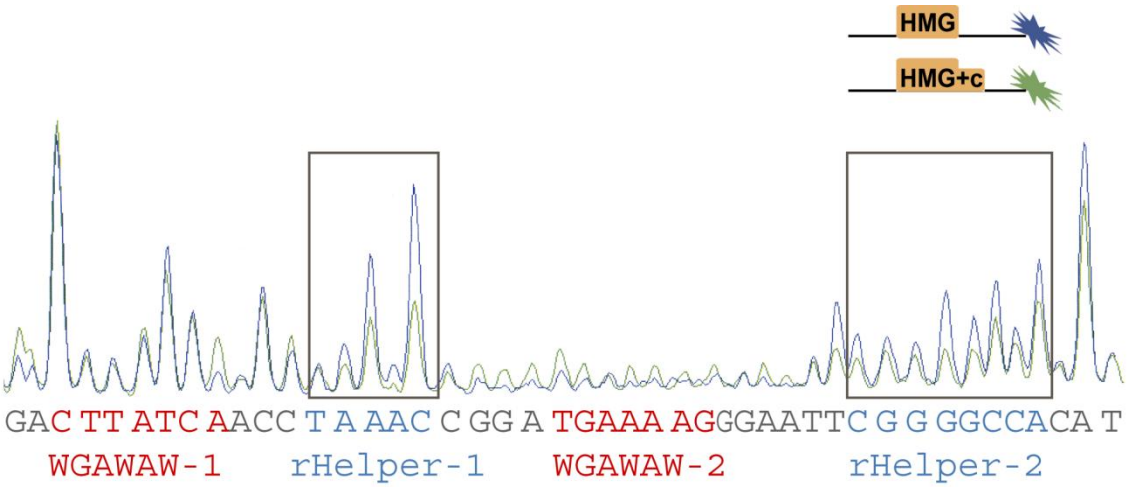


Figure 2.6. Footprinting chromatographs showing the C-clamp-specific protection of the r-Helper sites in the *Tig* W-CRM. Regions where the blue signals are higher than the green signals were protected by GST-HMG-C-clamp and not by GST-HMG. Note that the arbitrary colors are switched compared to those shown in Figure 3B.

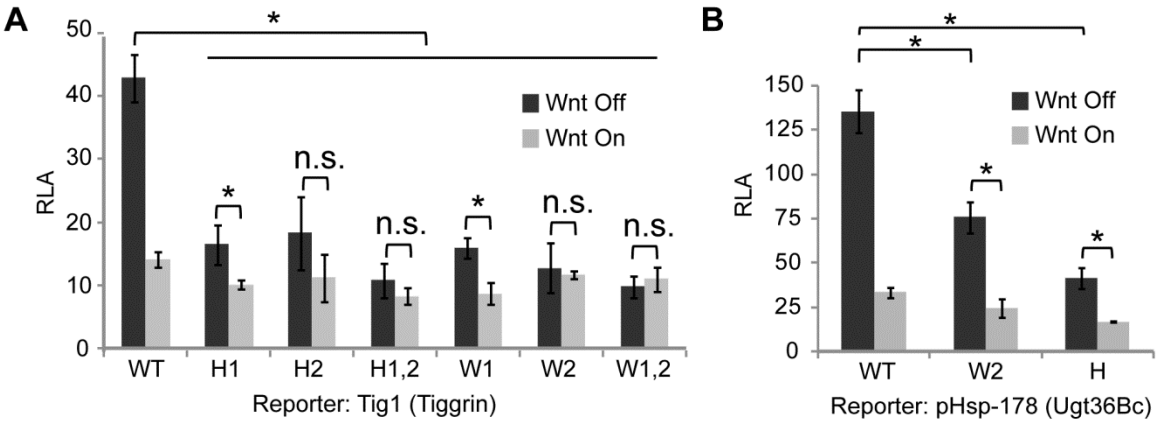


Figure 2.7. r-Helper and WGAWAW sites are required for Wnt-regulation of *Tig* and *Ugt36Bc* W-CRM reporters. (A, B) Mutations in r-Helper sites (H) or WGAWAW sites (W) greatly decrease the basal activity and repression of the *Tig* and *Ugt36Bc* W-CRM reporters in Kc cells by Axin RNAi (A, B) or Arm* expression (data not shown). *p<0.05; n.s., not significant (Student's T-test).

A

minR-WT

CTTATCAACCTAAACCGGATGAAAAGGGAATTCGGGGCCACTTATCAACCTAAACCGGATGAAA
AGGGAATTCGGGGCCA

minR-Hm

CTTATCAACCcAgAtCGGATGAAAAGGGAATTattttaacCTTATCAACCcAgAtCGGATGAAA
AGGGAATTattttaac

minR-Wm

CgTcTaAACCTAAACCGGAgGcAcAGGGAATTCGGGGCCACgTcTaAACCTAAACCGGAgGcAcA
GGGAATTCGGGGCCA

B

Nkd-IntE WT

ATAGTTTGTGTATAGTTAAAATGAAATTGAAAATAATACAAAACGCTTTGTCGGTCATTAGCTT
TGAGTGGACGCCGCCACCGCCGCACTTTGCCGCTTTCGAGTATTTTTTTTGGGGATAGTCGCGT
CATTTGTGCGCTCGGTTTGCTTTGGGTTCGGTTCGGTTCGGTTTGGTTTTGATTATGTCAAAGC
GAAAAAGCCGCCAAACATATGTGCCTCCAACCACATGCCTGCCTGCCCGGTTGCTTTGAGGAA

Nkd-IntE swapped

ATAGTTTGTGTATAGTTAAAATGAAATTGAAAATAATACAAAACttaTcaaCctaaAccggatG
aaaaGggaattcggggccacttatcaacctaaaccggatgaaaaggGaattCGggGccaGCACT
TTGCCGCTTTCGAGTATTTTTTTTGGGGATAGTCGCGTCATTTGTGCGCTCGGTTTGCTTTGGG
TTCGGTTCGGTTCGGTTTGGTTTTGAcggatgaAAAGgGAAttcGggGCCAAACATATGTGCCT
CCAACCACATGCCTGCCTGCCCGGTTGCTTTGAGGAA

C

Tig WT

TGTCGTCAAGTCCATGGGACTTATCAACCTAAACCGGATGAAAAGGGAATTCGGGGCCACATACA

W-only swap

TGTCGTCAAGTCCAcaaGAtcaAaggACCTAAACCaagatcaaaggGAATTCGGGGCCACATACA

H-only swap

TGTCGTCAAGTCCATGGGACTTATCAAgCcgccaCGGATGAAAAGGGAATTgccGcCaACATACA

Both swapped

TGTCGcaaGaTCaAaGGGAaTTgcCgcCaacAACCaagatcAAAGGGAATTgccGcCaACATACA

Red: classic HMG site or WGAWAW site.
Blue: Helper or r-Helper site. For r-Helper sites, blue shows footprinted regions.
Underlined: swapped regions.
Lowercase: changed or added nucleotides.

Figure 2.8. Sequence information of minR and *nkd*-IntE and Tig1 "swapped site" reporters. For all constructs, classic HMG binding and WGAWAW sites shown in red, while Helper and r-Helper sites shown in blue. **(A)** minR W-CRM and variations with the WGAWAW or r-Helper sites mutated (altered nucleoties in lower case). **(B)** The entire 255 bp *nkd*-IntE W-CRM, with sites to be swapped underlined and the sequence of the W-CRM with classic sites converted into WGAWAW and r-Helper sites. **(C)** Portion of the *Tig* first intron containing the two functional WGAWAW and r-Helper sites, plus the sequences where these motifs are swapped into sites typical of activated W-CRMs. The altered nucleotides in the swapped reporters are shown in lowercase.

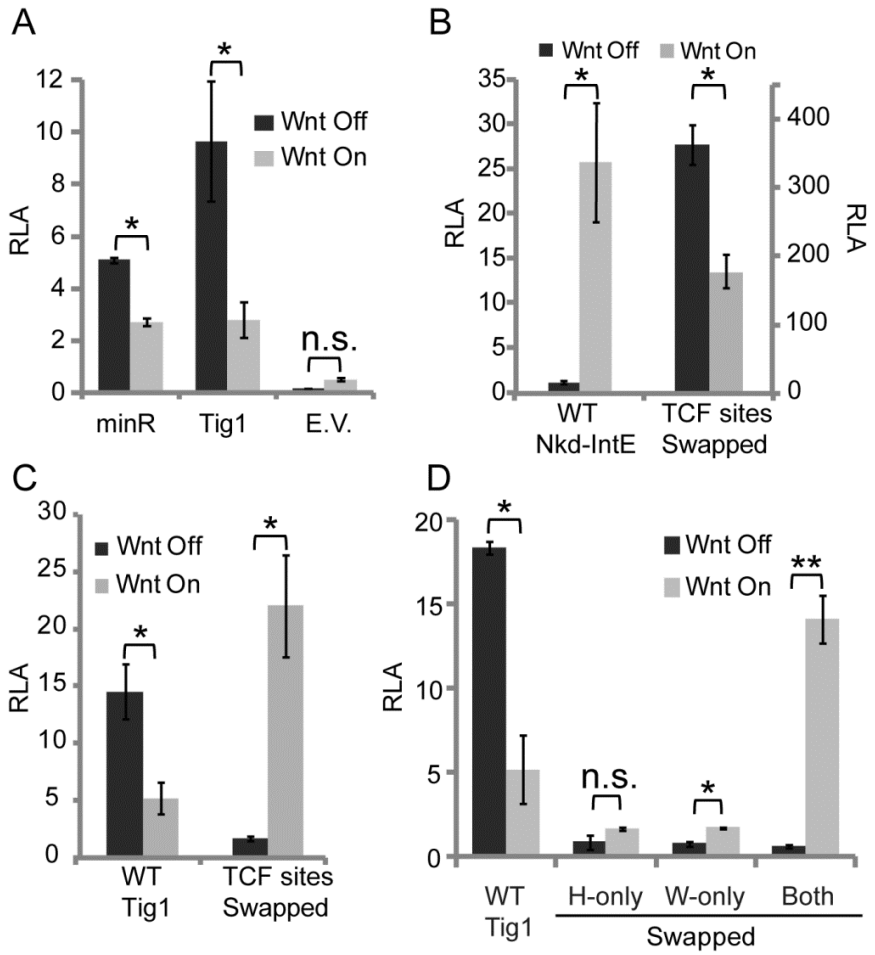


Figure 2.9. Swapping HMG and C-clamp binding sites switches the transcriptional output of W-CRMs. Kc cells were transfected with the indicated reporters with or without Axin RNAi, as described in Figure 1 and the Materials and Methods. Sequences of the reporters used are listed in Figure S3. **(A)** A minR reporter containing two repeats of a 40 bp region of the *Tig* intron (each repeat contains two WGAWAW and two r-Helper sites) cloned upstream of the *hsp70* core promoter is sufficient for driving basal expression and mediating Wnt repression. Tig1 and the *hsp70* core promoter (E.V.) were used as positive and negative controls, respectively. **(B)** The *nkd*-IntE W-CRM reporter, which is activated by Wnt signaling, is converted to a repressed W-CRM when its three functional HMG sites and two Helper sites were replaced by five WGAWAW and r-Helper pairs (see Figure S3 for sequence changes). (**C)** The Tig1 W-CRM reporter is activated by Wnt signaling when two WGAWAW sites and two r-Helper sites were converted into classic HMG-Helper pairs. (**D)** The switch of the Tig1 W-CRM to an activated W-CRM requires swapping both WGAWAW and r-Helper sites. When one motif is swapped without the other, low basal activity and little activation was observed. *p<0.05; **p<0.01; n.s.: not significant (Student's T-test).

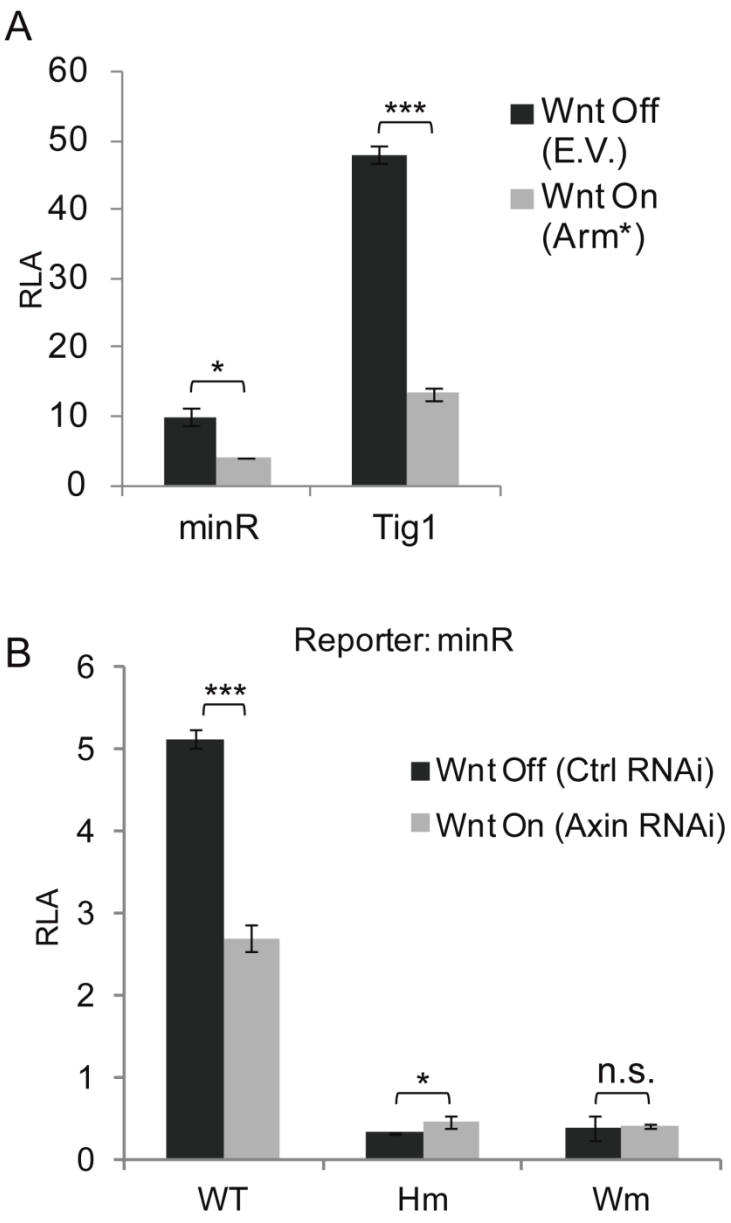


Figure 2.10. The activity of minR is dependent on r-Helper and WGAWAW sites. When either r-Helper or WGAWAW sites were mutated, the basal activity of minR reporter and its response to Wnt signaling (Axin RNAi) were both strongly decreased. *p<0.05; ***p<0.001; n.s.: not significant (Student's T-test).

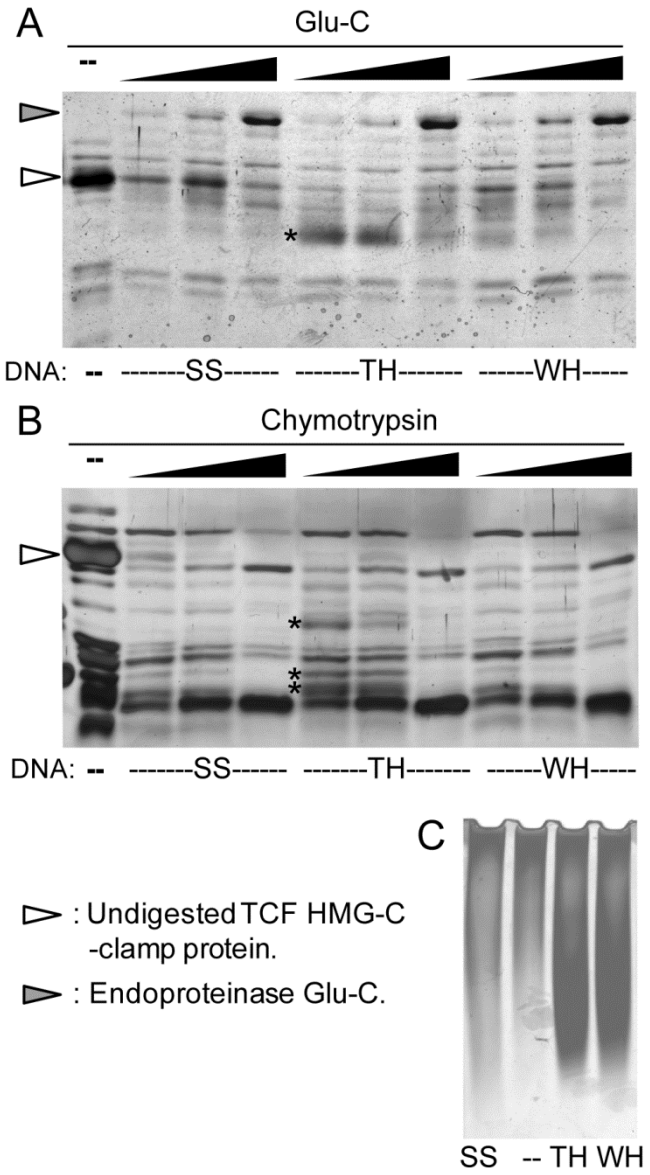


Figure 2.11. The HMG and C-clamp domains adopt different conformations when bound to distinct binding sites. (A, B) Recombinant GST-HMG-C-clamp protein was incubated with 20 fold molar excess of control oligonucleotide (SS), a classic HMG and Helper site pair (TH) and a WGAWAW and r-Helper site pair (WH) (see Table SII for sequences of oligonucleotides). After 20 min to allow binding, the preps were subjected to partial proteolytic digestion with increasing amounts of Glu-C (A) or chymotrypsin (B) and analyzed by SDS-PAGE followed by silver staining. Proteolytic fragments enriched with TH and not WH are indicated with asterisks. **(C)** Silver stained native gel of GST-HMG-C-clamp and different oligonucleotides at the same concentrations used in the proteolytic digestions, demonstrating that a similar amount of protein is bound to TH and WH, while SS has no detectable binding. Each experiment was performed at least three independent times with similar results.

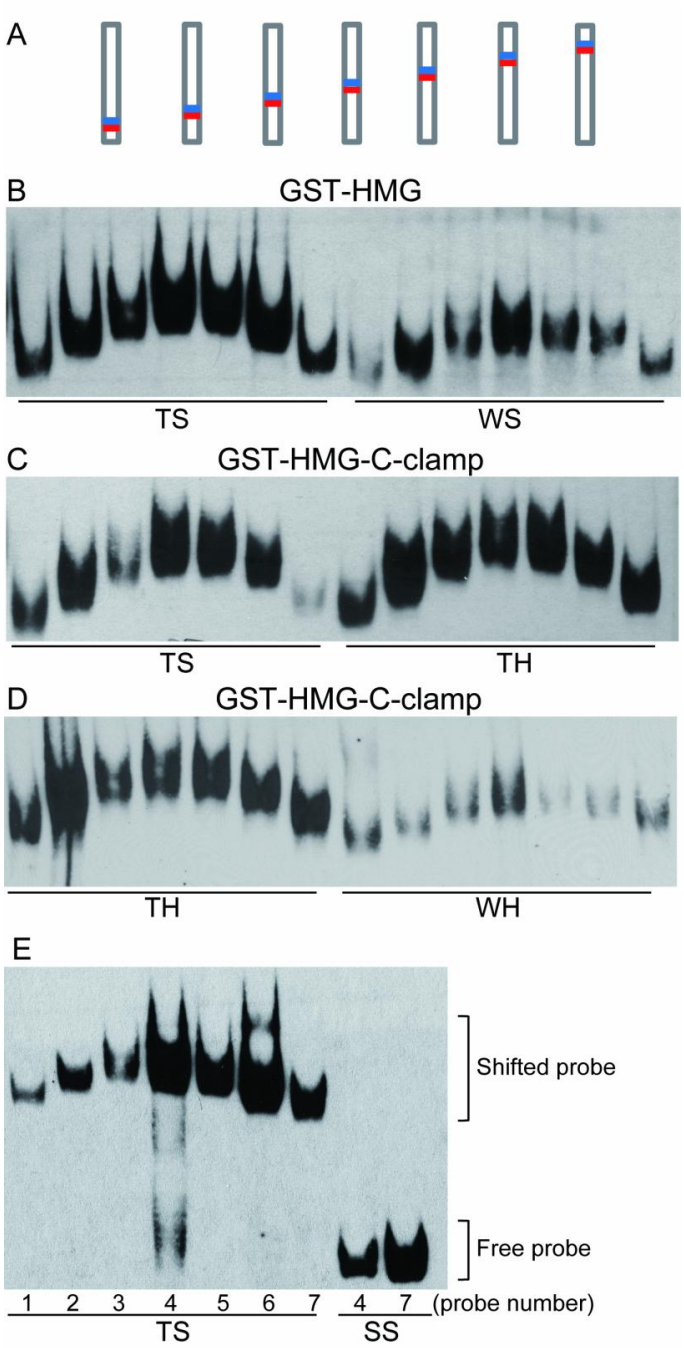


Figure 2.12. DNA bending by the HMG domain of TCF/Pan. (A) Cartoon showing a series of seven probes, each with a bipartite TCF binding site (red/blue boxes) located along the 139 bp oligonucleotide. These TCF sites could consist of a classic HMG or WGAWAW site (TS or WS) or HMG-Helper or WGAWAW-r-Helper pair (TH or WH). If DNA bending occurs upon protein binding, the complex will run slower in an EMSA when the binding site is in the middle of the probe [49]. **(B)** GST-HMG protein bends TS slightly more than WS. **(C)** The presence of a Helper site does not increase the bending observed when GST-HMG-C-clamp binds to a HMG site. **(D)** GST-HMG-C-clamp bends TH slightly more than WH. Each experiment was performed at least three times with similar results.

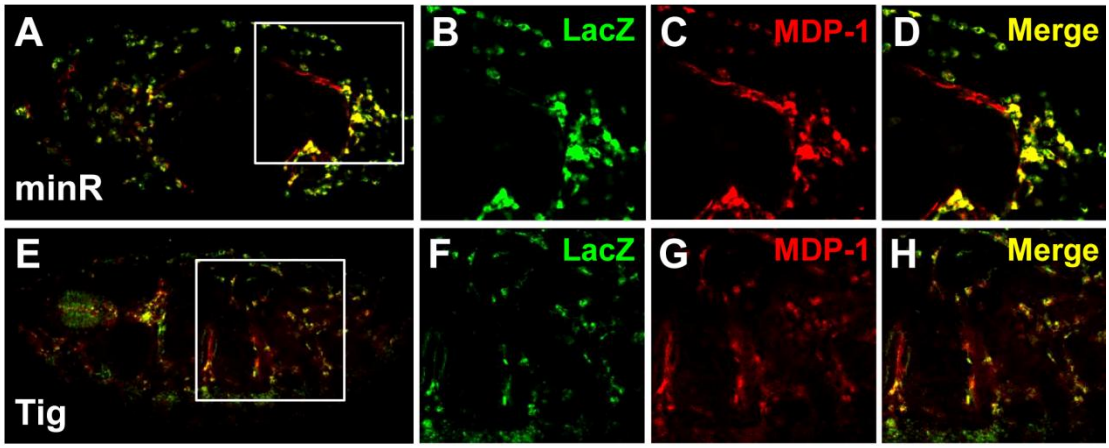


Figure 2.13. Embryonic expression of the Tig and minR reporters. (A-D) Micrographs of a stage 14 embryo containing a minR lacZ reporter immunostained for LacZ (green) and the hemocytic marker MDP-1 (red). Panel A shows the entire embryo while panels B-D are higher magnification insets (white box in A). The majority of lacZ staining is hemocytic. **(E-H)** Stage 16 embryo containing a Tig1 lacZ reporter stained and presented as in panels A-D. There is significant overlap between the reporter expression and hemocytes.

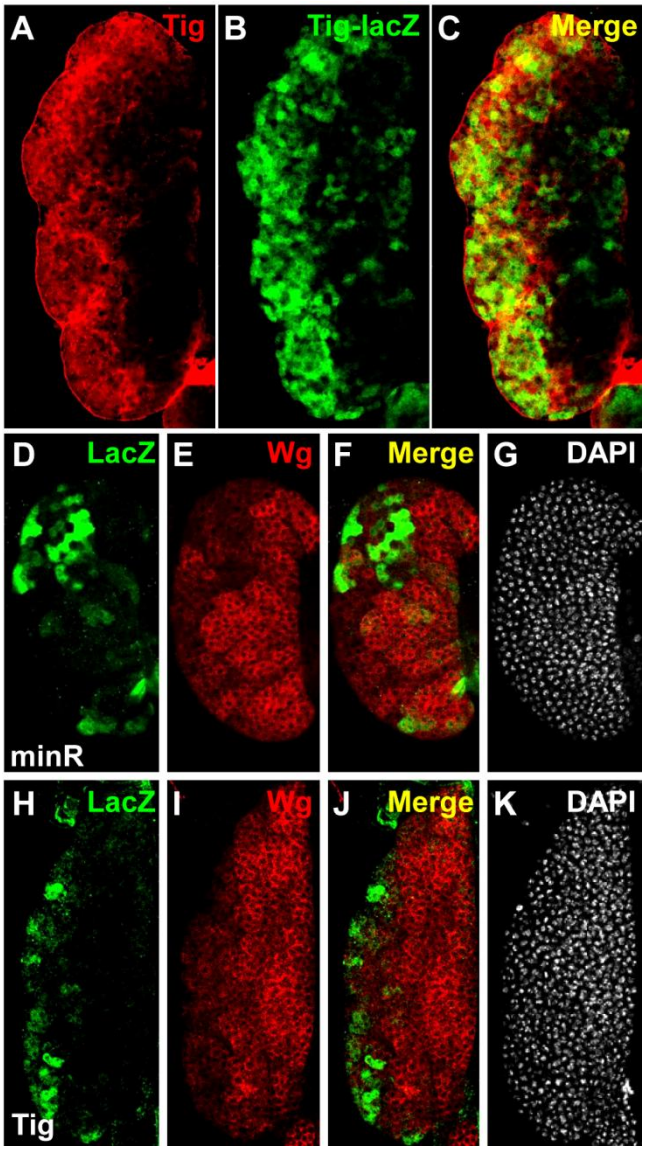


Figure 2.14. Expression of *Tig* and minR reporters in the larval LG. (A-C) Larval LG from older late 3rd instar larvae (~104-112 AEL) containing the Tig lacZ reporter immunostained for Tig protein (green) and LacZ (red). The red and green signals colocalize to the same cells, with most Tig localized extracellularly and LacZ to the cytosol. **(D-K)** Larval LGs from younger late 3rd instar larva (~96-104 AEL) containing the minR (D-G) or Tig1 (H-K) lacZ reporters, immunostained for LacZ (green) and Wg (red). DAPI was used as a counterstain (white). The expression patterns of the reporters and Wg are largely exclusive, suggesting that the reporters are repressed by Wnt signaling.

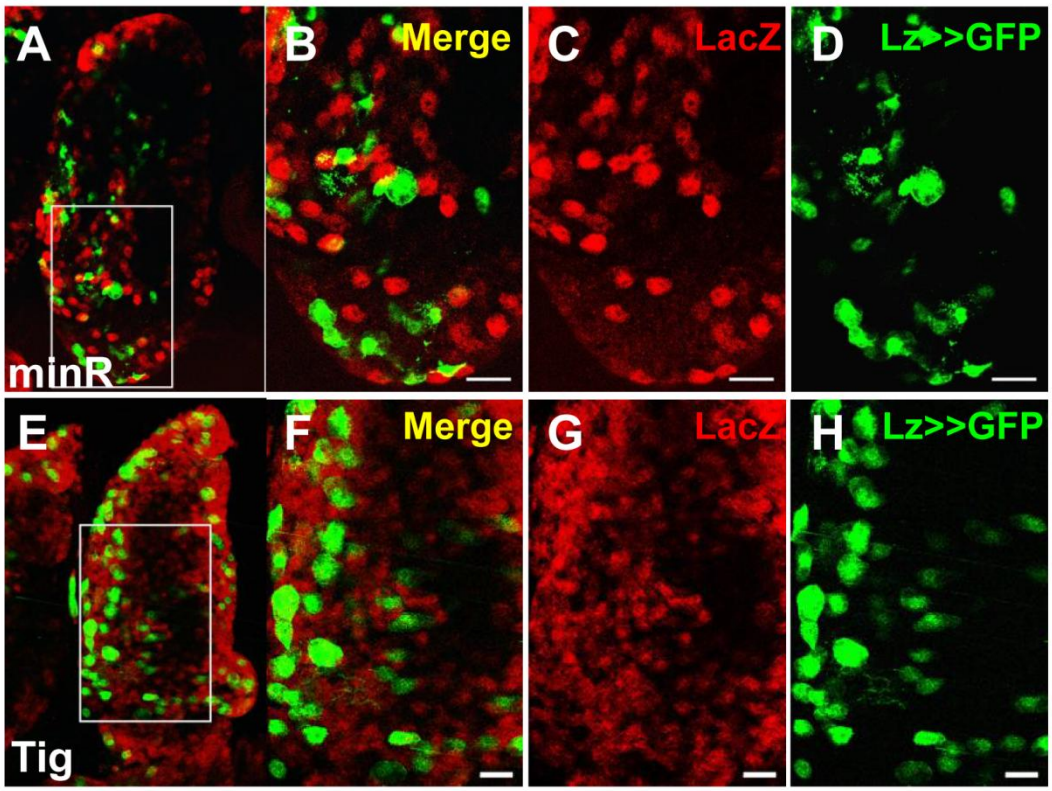


Figure 2.15. The Tig and minR reporters are not active in crystal cells. (A-H) Larval LGs from late 3rd instar larvae containing p[Lz-Gal4] and p[UAS-mCD8::GFP] and the minR (A-D) or Tig1 (E-H) lacZ reporters, with LacZ immunodetection (red). Both fluorescent signals are cytosolic. Panels B-D and F-H are higher magnification of the boxed regions in A and E, respectively. The expression patterns of the reporters are largely exclusive with Lz>>GFP, a marker of crystal cells which often express Wg [40].

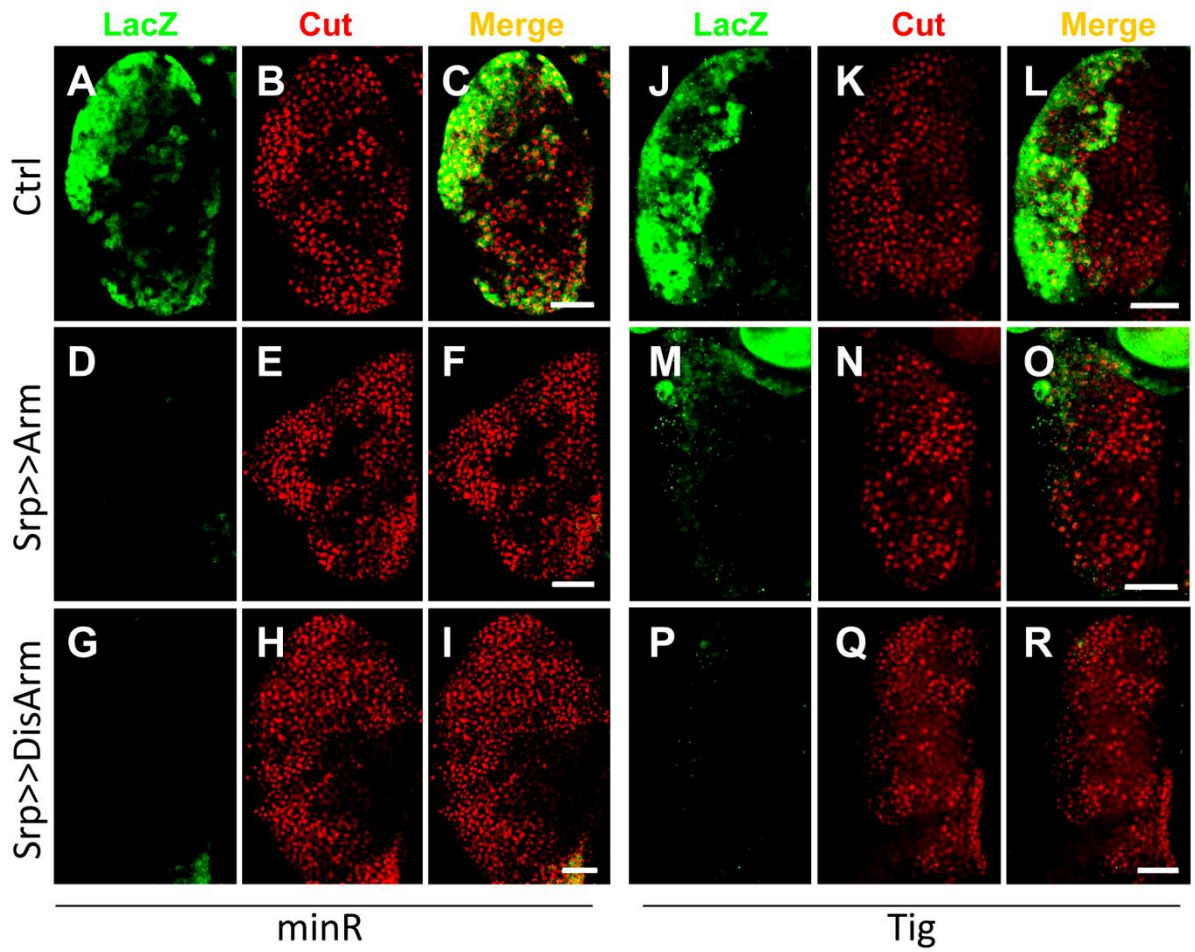


Figure 2.16. The *Tig* and minR reporters are repressed by Wnt signaling in the larval LG. Micrographs of older late 3rd instar larval LGs from strains containing the minR **(A-I)** or Tig **(J-R)** lacZ reporters, combined with P[UAS-Arm*] (D-F, M-O) or P[UAS-DisArmed] (G-I; P-R) transgenes driven by P[Srp-Gal4]. The green signal denotes LacZ and red is Cut, a marker for the CZ [53; Figure S8]. Activation of Wnt signaling by Arm* or DisArmed expression inhibits reporter expression without detectably altering the size of the CZ. Bar = 40 μm.

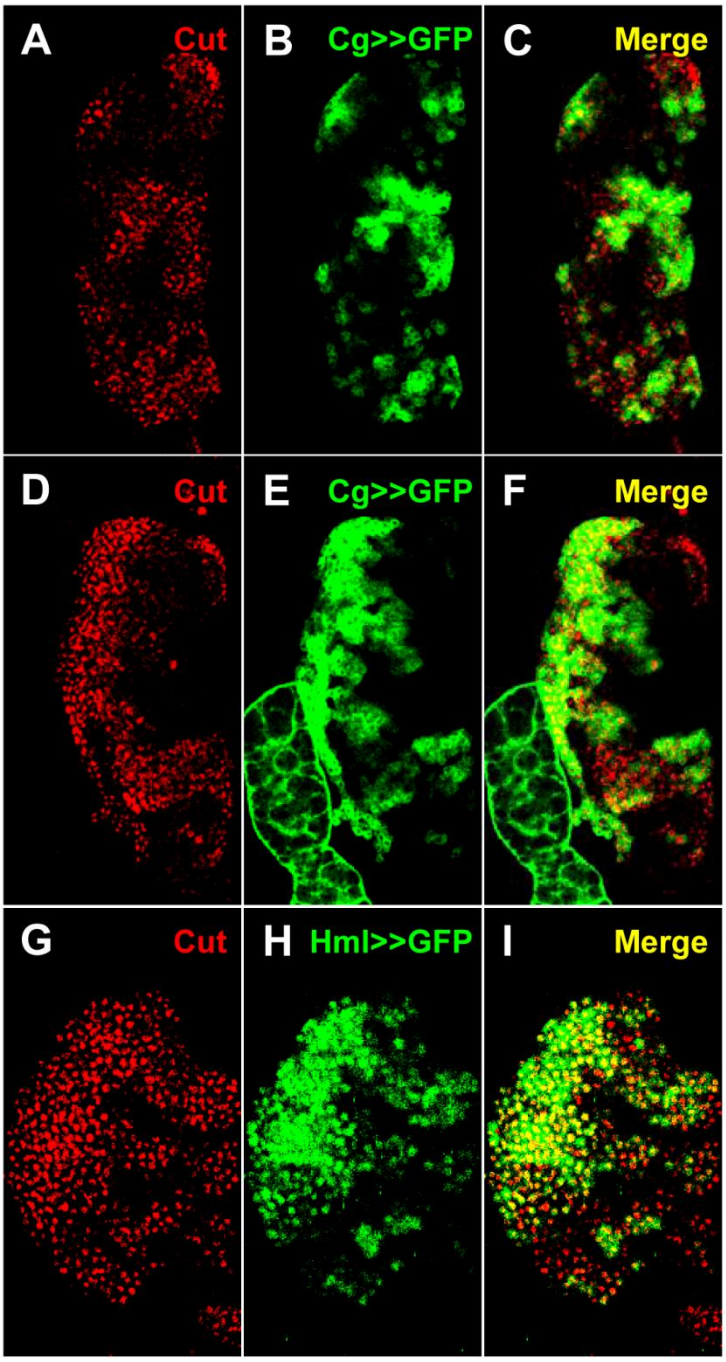


Figure 2.17. Cut immunostaining marks the CZ of the larval LG. Cut (red) colocalizes with Cg>>GFP **(A-F)** and Hml>>GFP (green) **(G-I)**, two established CZ markers [53,76]. Multiple glands are shown to recapitulate the variation in the shape of CZ/MZ. Cut is a nuclear protein, while Hml>>GFP signal is cytosolic and Cg>>mCD8::GFP is localized to the membrane.

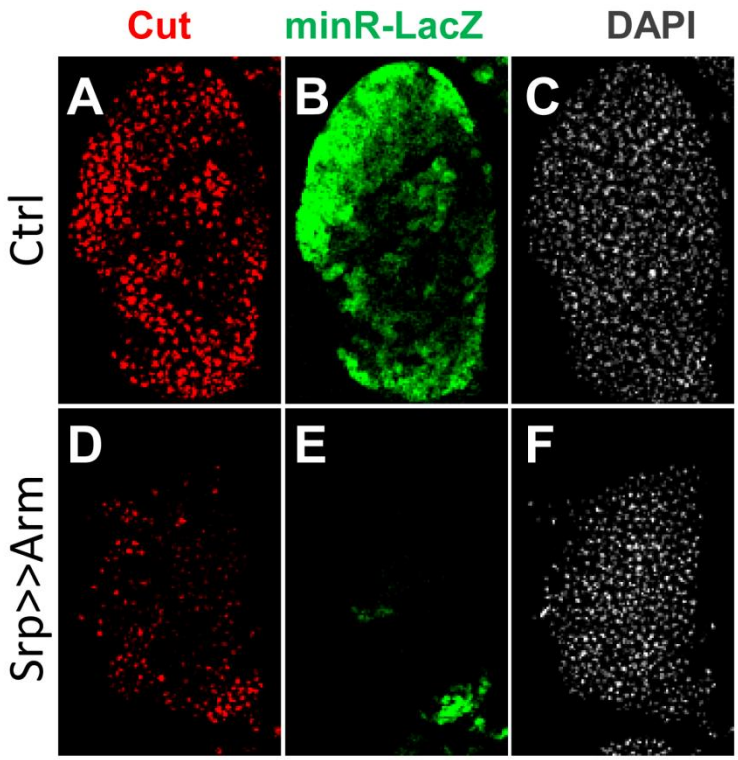


Figure 2.18. Activation of Wnt signaling in the larval LG is able to affect cell fate and reduce the size of the CZ. (A-F) Micrographs of older 3rd instar larval LGs from strains containing the minR reporter and P[Srp-Gal4], without (A-C) or with P[UAS-Arm*] (D-F). Activation of Wnt signaling by Arm* expression greatly reduces the size of CZ, indicated by Cut (red), and expression of the lacZ reporter (green) is greatly reduced.

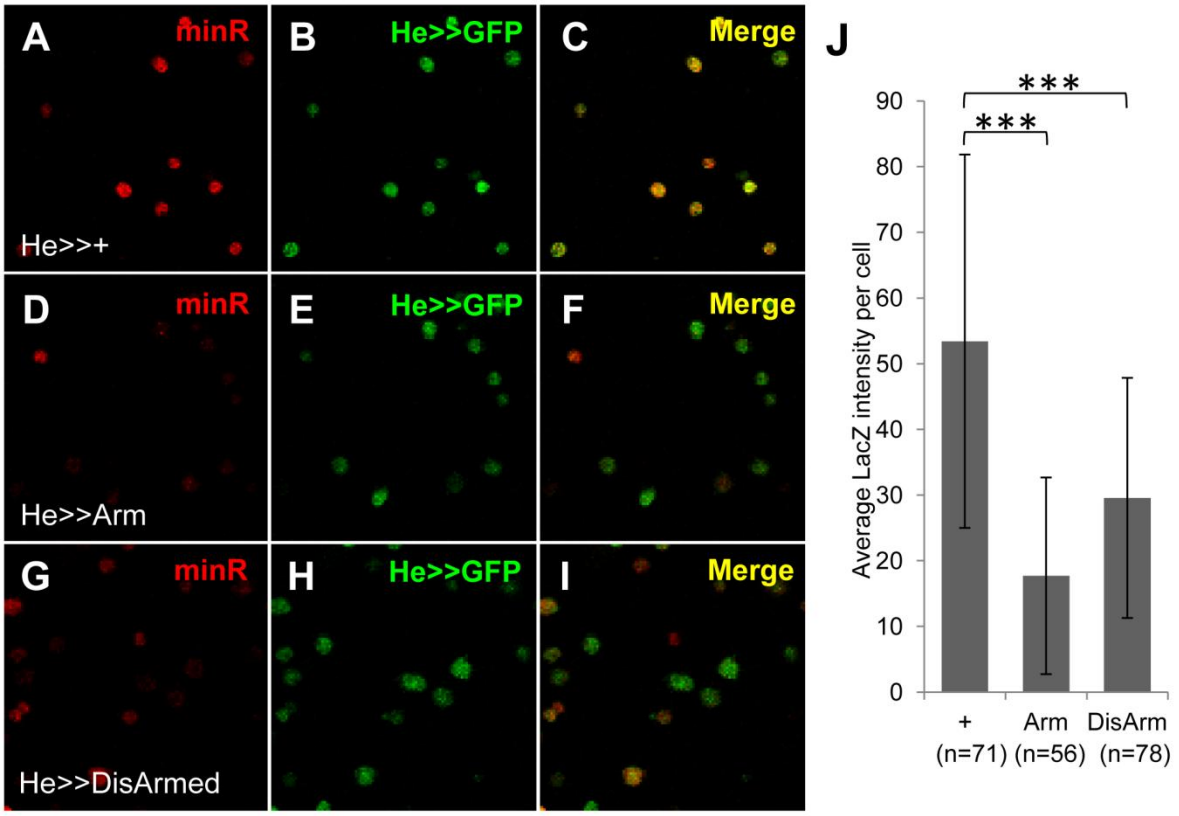


Figure 2.19. The minR reporter is repressed by Wnt signaling in circulating larval hemocytes. (A-I) Micrographs of mid 3rd instar larval (~88-96 AEL) circulating hemocyte smears from strains containing the minR reporter, P[He-Gal4] and P[UAS-GFP] and either + (A-C), P[UAS-Arm*] (D-F) or P[UAS-DisArmed] (G-I) transgenes. Activation of Wnt signaling by Arm* or DisArmed expression inhibits reporter expression in most of the circulating hemocytes. **(J)** Quantification of the data (see Materials and Methods) using 5 larvae for each genotype and 10-15 hemocytes per larvae. ***p<0.001.

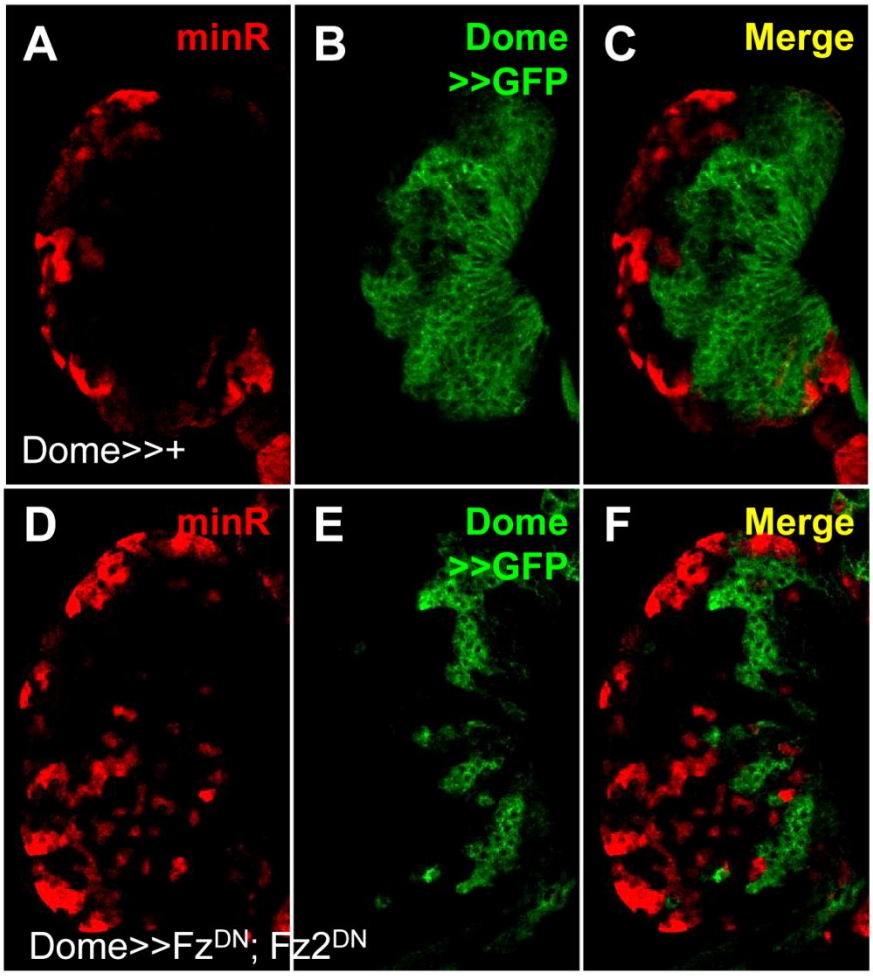


Figure 2.20. Inhibition of Wnt signaling by FzDN and Fz2DN in the MZ derepresses minR signal but also reduces the size of MZ. (A-F) Micrographs of younger late 3rd instar larval LGs (~94-98 hr AEL) from strains containing the minR reporter, P[UAS-mCD8::GFP] and P[Dome-Gal4] without (A-C) or with P[FzDN; Fz2DN] (D-F). Dome>>GFP indicates MZ cells, while GFP positive cells are in the CZ (confirmed by staining of Cut, data not shown). Inhibition of Wnt signaling by FzDN and Fz2DN expression increases the reporter signal (red) in the CZ, but also increases the CZ size and reduces the MZ size.

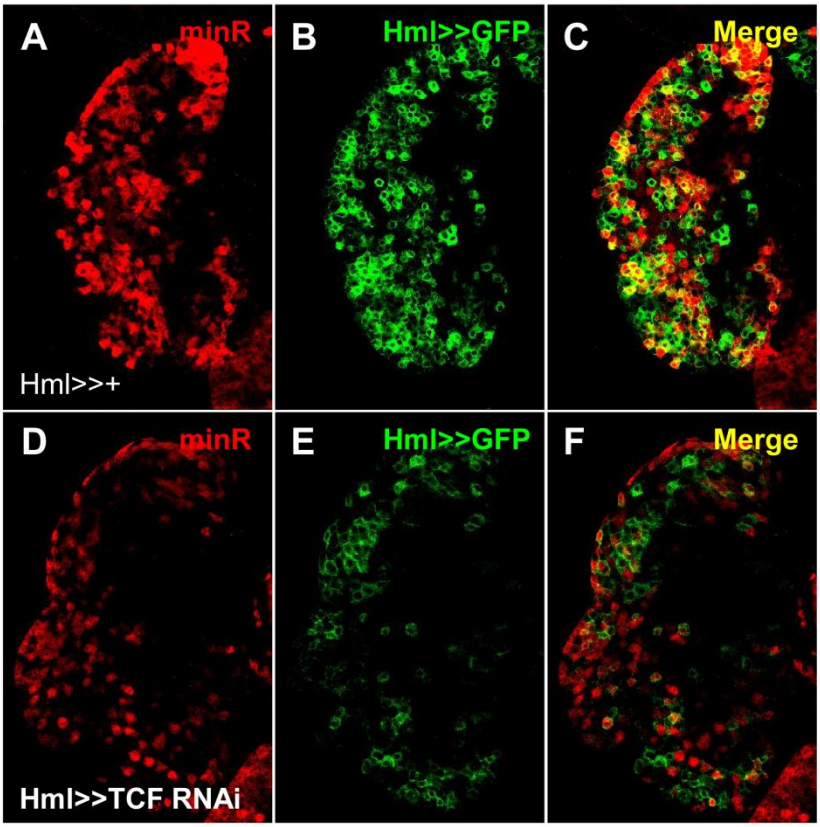


Figure 2.21. TCF/Pan knockdown in the CZ reduces minR expression but also reduces the size of the CZ. (A-F) Micrographs of older late 3rd instar larval LGs from strains containing the minR reporter, P[UAS-mCD8::GFP] and P[Hml-Gal4], without (A-C) or with P[UAS-TCF/Pan-RNAi] (D-F). Depletion of TCF reduces reporter expression (red) in the CZ, but also reduces the GFP signal and the CZ size (confirmed by staining of Cut, data not shown).

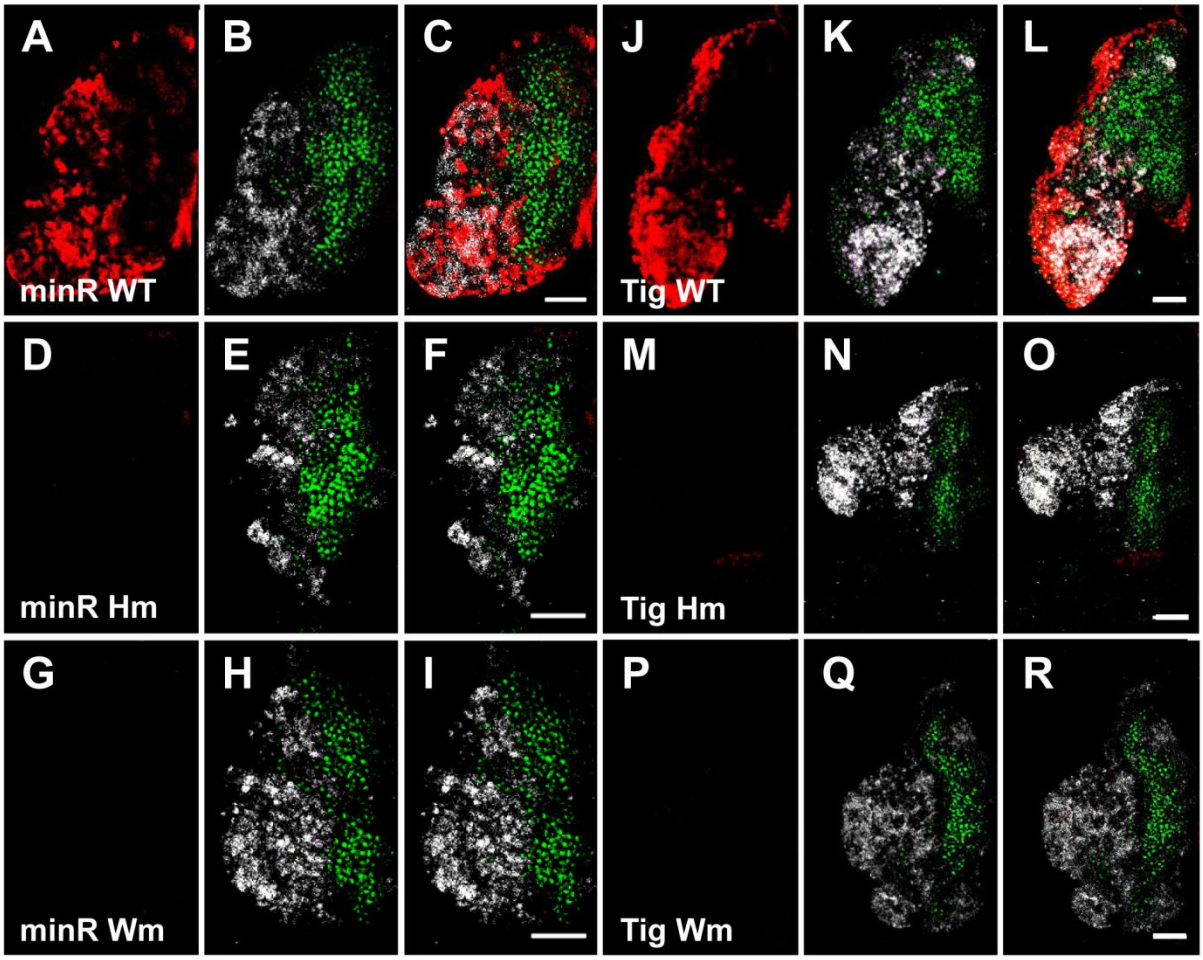


Figure 2.22. The TCF binding sites are required for expression of the *Tig* and minR reporters in the CZ of larval LG. (**A-R)** Older late 3rd instar larval LGs from minR (A-I) and Tig1 (J-R) reporters with mutations in the r-Helper (Hm) or WGAWAW sites (Wm). Mutation of either motif abolishes LG expression for both reporters, indicated by LacZ signal in red. The Dome>>EBFP (green) and Hml>>dsRed (white) mark the MZ and CZ, respectively. When active, the LacZ signal is found in the CZ. Bar = 50 μm.

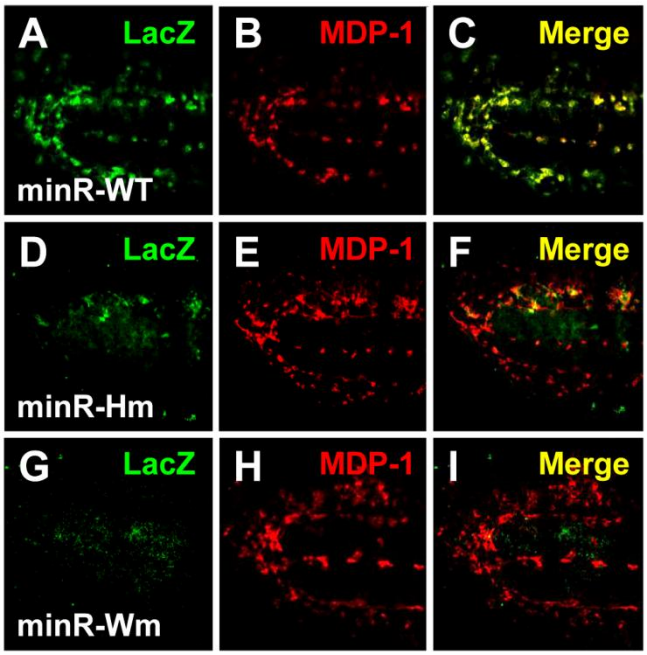


Figure 2.23. The TCF binding sites are required for expression of the Tig and minR reporters in embryonic hemocytes. (A-I) Confocal images of stage 15 embryos containing wild-type minR reporter **(**A-C) and the Hm (D-F) or Wm (G-I) mutants, with immunofluorescence detection of LacZ (green) and MDP-1 (red). Expression of the reporter is greatly reduced when either motif is mutated.

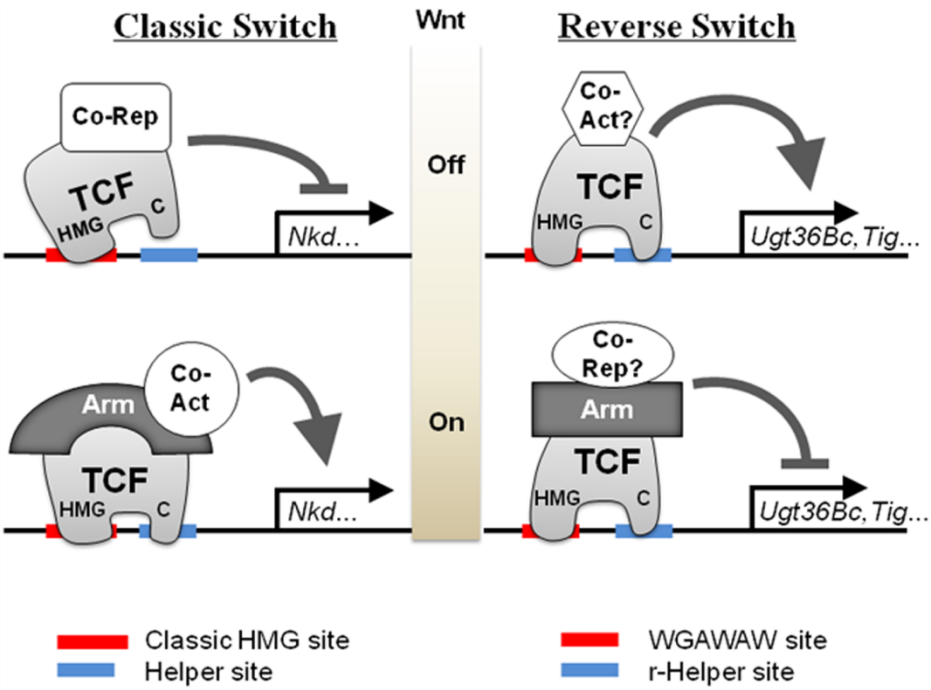
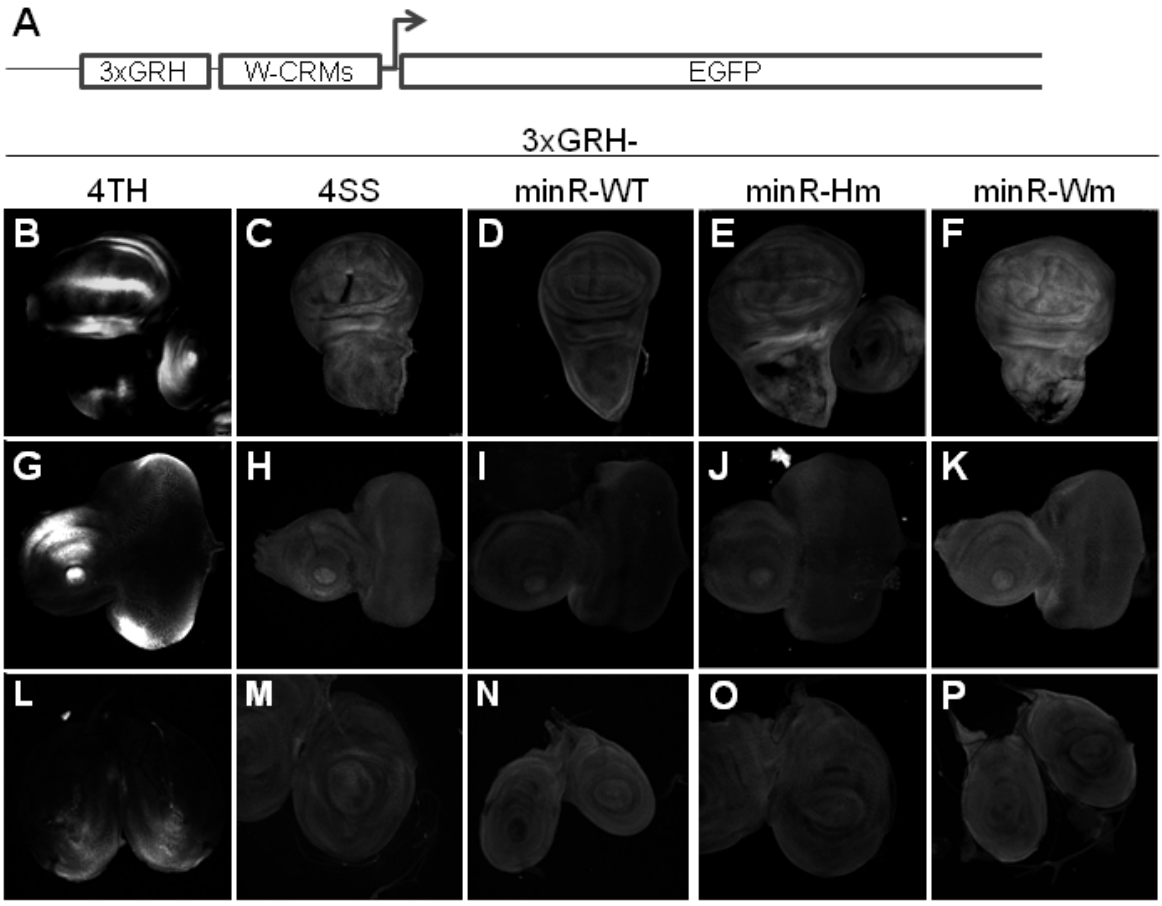


Figure 2.24. Model for allosteric regulation of TCF/Pan and Arm by bipartite TCF binding sites. The cartoon on the left depicts the classic TCF transcriptional switch, where repression in the absence of Wnt signaling occurs through HMG-HMG site interactions, while Wnt-dependent transcription activation requires DNA binding by both the HMG and C-clamp domains [80]. The cartoon on the right depicts the "reverse transcriptional switch", where TCF/Pan activates the W-CRM without signaling and represses when complexed with Arm. HMG-WGAWAW site and C-clamp-r-Helper site interactions are required for both sides of the reverse switch. Unknown co-activators and co-repressors are likely to be involved in this regulation. The allosteric regulation of TCF/Pan is represented by different shapes when bound to either class of bipartite binding site; the allostery is likely passed onto other factors such as Arm.



Q

3xGRH:

AGATCTAAACCGGTTATGCGAGTCTAGACTTGGAAACCGGTTATGCGAGTCTAGACTTGGA
AACCGGTTATGCGAG

W-CRMs:

minR-WH: CGGTTAGGCACCGAGCTCTTATCAACCTAAACCGGATGAAAAGGGAATTCG
GGGCCACTTATCAACCTAAACCGGATGAAAAGGGAATTCGGGGCCACGCGTATAGATCG

minR-Hm: CGGTTAGGCACCGAGCTCTTATCAACCCAGATCGGATGAAAAGGGAATTAT
TTTAAACTTATCAACCCAGATCGGATGAAAAGGGAATTATTTTAAACGCGTATAGATCG

minR-Wm: CGGTTAGGCACCGAGCTCTAAACCACCTAAACCGGAGGCACAGGGAATTCG
GGGCCACTAAACCACCTAAACCGGAGGCACAGGGAATTCGGGGCCACGCGTATAGATCG

4TH: AAGATCAAAGGGGGTAGCCGCCAGTCGGAAGATCAAAGGGGGTAGCCGCCA
CCCGGGAAGATCAAAGGGGGTAGCCGCCAGTCGGAAGATCAAAGGGGGTAGCCGCCA

4SS: CCTCGACCCTTGGGTATAATAACGTCGGACTCGACCCTTGGGTATAATAAC
CCCGGGCCTCGACCCTTGGGTATAATAACGTCGGACTCGACCCTTGGGTATAATAAC

Figure 2.25. WGAWAW, r-Helper site pairs do not affect transcription in several other tissues outside the hematopoietic system. (**A)** A cartoon showing the structure of 3xGRH-W-CRM reporters, containing three Grainyhead (GRH) binding sites which provides basal activity in the tissues being tested and a W-CRM followed by a EGFP reporter gene. (**B-P)** Micrographs of wing (B-F), leg (G-K) and eye-antenna (L-P) discs from 3rd instar larvae carrying indicated 3xGRH-W-CRMs. 3xGRH-4TH contains four classic HMG-Helper site pairs, and displays high expression in regions where Wg is known to be expressed. 3xGRH-SS contains randon sequences and has the low level, ubiquitous pattern previously described [63]. 3xGRH-minR-WT along with the r-Helper (Hm) and WGAWAW (Wm) site mutant versions are all expressed in very similar patterns to 3xGRH-SS, with no hint of basal activation or Wg-dependent repression. (**Q)** Sequence information for the 3xGRH-W-CRM reporters.

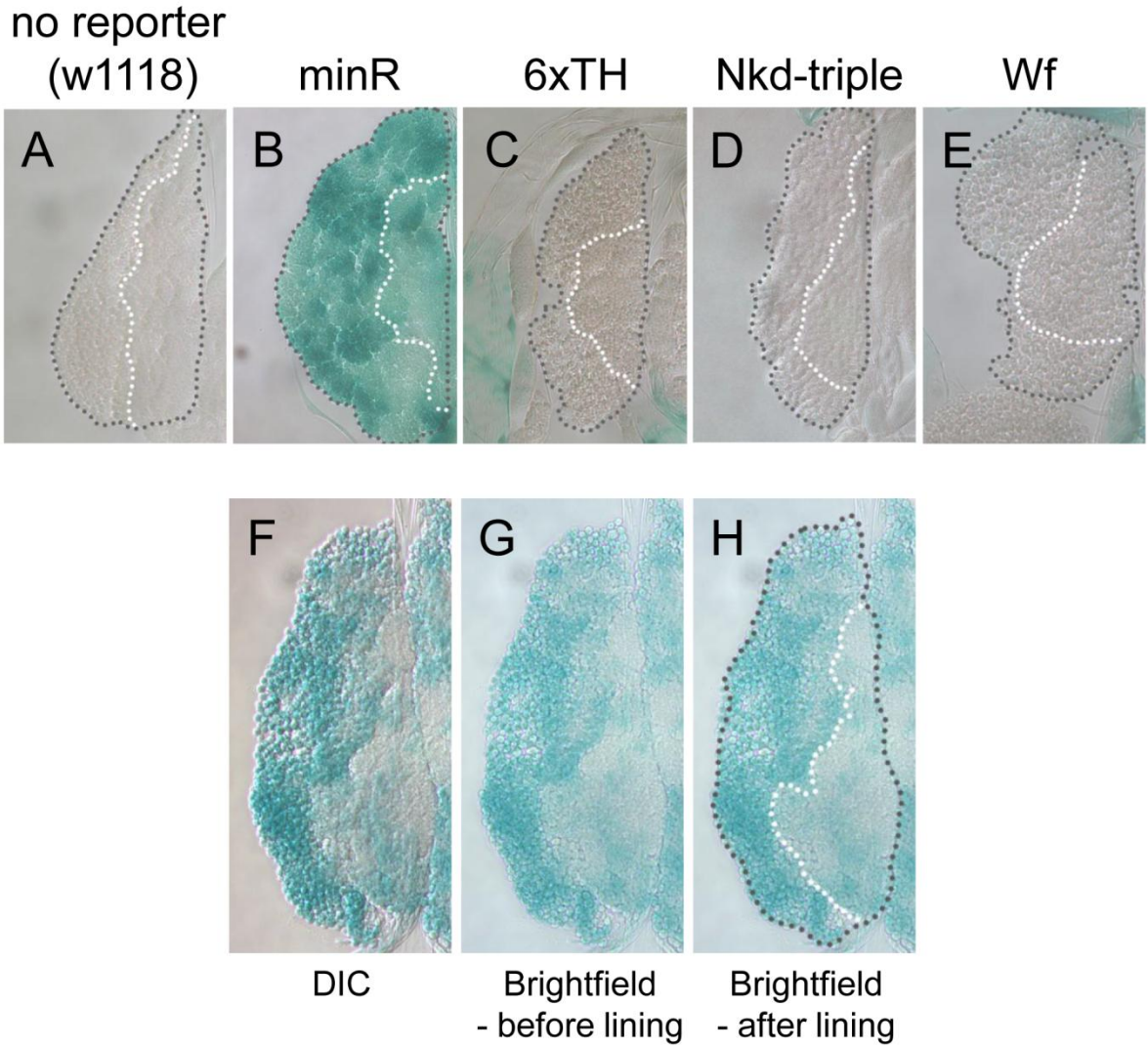


Figure 2.26. Several Wnt-activated W-CRMs have no detectable activity in the LG. (A-E) Comparison between minR and Wnt-activated reporters stained with X-gal. The minR reporter (B) shows strong staining in the CZ, while all the Wnt-activated W-CRMs tested (C-E), as well as the negative control w1118 (A), have no detectable staining. The minR reporter was stained for the same amount of time as the other reporter lines, resulting in over-staining. **(F)** Micrograph of an older 3rd instar larval LG stained with X-gal, taken with DIC optics, highlighting the larger, less densely packed cells of the CZ. **(G)** Brightfield image of the same LG where the LacZ staining is more pronounced. **(H)** Brightfield image of the same LG where the DIC image was used to draw a broken white line separating the CZ and MZ.

Site		Sequence	LEF1		TCF1		TCF3		TCF4	
			#1	#2	#1	#2	#1	#2	#1	#2
Repressive	Tig-1	TGATAAGT	4077	1787	270	55	4411	2794	6671	2955
	Tig-2	GATGAAAA	223	84	1052	122	724	248	280	113
	Ugt-1	AAAGAAAT	332	1574	212	628	81	882	295	889
	Ugt-2	AGATAAAA	137	156	176	52	46	41	111	64
	Ugt-3	TAGATAAG	3671	5210	575	5900	2508	7570	3719	4180
Activating	NkdIntE-1	CGCTTTGT	847	1564	3486	7745	1389	5343	631	1657
	NkdIntE-2	AGCTTTGA	152	148	362	2147	477	709	121	160
	NkdIntE-3	CGCTTTGA	63	109	288	852	138	187	83	129
	NkdUpe-4	CCATTGAT	576	122	1870	2235	1716	619	568	211
	NkdUpe-6	CTTTGTTC	51	70	54	378	56	180	45	59
	Notum-1	CTTTGATG	3	4	4	2	3	2	3	3
	Notum-2	CTTTGATC	1	1	1	1	1	1	1	1
	Notum-3	CTTTGATC	1	1	1	1	1	1	1	1

Table 2.1. Preference of WGAWAW binding by the HMG domains of vertebrate TCFs. Taken from Badis et al., (2009). HMG binding sites from five repressed and eight activated W-CRMs were analyzed [33,39,47]. Numbers represent ranking out of a pool of 32896 8-mers tested by Badis and co-workers [59]. Two data sets from each TCF family member are shown (#1 and #2). The underlined sequence denotes the HMG binding site from each W-CRM within a specifc 8-mer. These sequences are found in more than one 8-mer; the ones with the highest ranking are shown and the highest ranking 8-mer containing each binding sequence is highlighted in yellow. While the sites from the Notum/wingful W-CRM are found in the highest ranked 8-mers, the range for the other sites from activated W-CRMs are similar to those found in repressed W-CRMs.

Purpose	Primer name	Primer sequence	Restriction site added (if any)
Cloning of Tig WREs	Tig1-F	ccgGGTACCCCGTTTCTGATATAAATCGCAACG	KpnI
	Tig1-R	ctaCGGCCGCCTGTGATGCGCTGCAAATG	EagI
	Tig2/3-F	CCGACGCGTACCCACATAGTGTCCTGAATCC	MluI
	Tig2-R	CCGCTCGAGCCTGTGATGCGCTGCAAATG	XhoI
	Tig3-R	ccgCTCGAGGGCGTTGATAAGGGGAGGATG	XhoI
	Tig4/5-F	ccgGGTACCCCGTTTCTGATATAAATCGCAACG	KpnI
	Tig4-R	ccgACGCGTATGAATGAATCTCGCCATGACC	MluI
	Tig5-R	ctaCGGCCGAGTCGAGATGAAACCGCTGC	EagI
Footprinting	Tig-fp1F	AGCGATACGTTCGTTAGTTCG	
	Tig-fp1R	GAAGCTCACTGCCCACTTG	
	Ugt-fp1F	ATATGCGAAATTTCAGTTGATATGA	
	Ugt-fp1R	TAATAAATGGTTTCTTTTCTGCTTA	
qRT-PCR of Fic	Fic-RT-F	CTGACTGCACGGAGAAGACG	
	Fic-RT-R	CCGTCTGGATGAGCATAGGG	
DNA bending and partial proteolytic digestion	WH	AACCGGATGAAAAGGGAATTCGGGGCCACA	
	WS	AACCGGATGAAAAGGGAATTATTTTAAACA	
	TH	AAGGAAGATCAAAGGGGGTAGCCGCCAGTA	
	TS	AAGGAAGATCAAAGGGGGTATAATAACGTA	
	SS	AAGGCCTCGACCCTTGGGTATAATAACGTA	

Table 2.2. Oligonucleotide sequences used in this chapter. Note that the sequences for DNA bending assay were presented in a longer probe (see Materials and Methods for more information).

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Chapter III:

The Matrix Protein Tiggrin Regulates Plasmatocyte Maturation in the *Drosophila* Lymph Gland

Abstract

The *Drosophila* lymph gland is an established model system for studying hematopoiesis. In this tissue, hemocyte precursors (prohemocytes) are amplified and maintained in the medullary zone (MZ), and progress towards maturation in the cortical zone (CZ). Plasmatocytes, the functional counterpart of mammalian macrophages, comprise the vast majority of mature hemocytes. Previous studies have uncovered genetic pathways that regulate prohemocyte maintenance and control the cell fate choice between plasmatocytes and other hemocyte lineages. However, less is known about how the plasmatocyte pool is established and matures. Here we report that Tiggrin, a large extracelluar matrix protein expressed in the CZ, plays an essential role in regulating plasmatocyte maturation. *Tiggrin* mutants have a reduced CZ and exhibit precocious maturation of plasmatocytes. Conversely, overexpression of Tiggrin blocks plasmatocyte maturation, resulting in an expanded CZ filled with a population of intermediate progenitors that express both MZ and CZ genes, but lack mature plasmatocyte markers. These intermediate cells are also found in normal LGs and likely represent a transitionary state in prohemocyte to

plasmatocyte differentiation. In addition, we found that the rate of the G_2/M transition in the cell cycle has a profound effect on plasmatocyte maturation. Overexpression of the Wee1 kinase, which slows G_2/M progression, results in a phenotype similar to that of Tiggrin overexpression. Further analysis reveals that Wee1 inhibits plasmatocyte maturation through activation of *Tiggrin* transcription. Importantly, none of the aforementioned manipulations significantly alter the crystal cell and lamellocyte lineages. Our result provides a deeper understanding of the maturation of plasmatocytes in the LG, and elucidates connections between cell cycle regulators, the extracellular matrix and hematopoiesis.

Introduction

Like vertebrates, hematopoiesis occurs at multiple developmental stages in *Drosophila*. The first wave of hematopoiesis takes place during fly embryogenesis when a population of hemocytes arises from the procephalic (head) mesoderm (Lebestky et al., 2000; Tepass et al., 1994). These hemocytes undergo further amplification during larval stages (Makhijani et al., 2011; Markus et al., 2009). In parallel to this wave, embryonic precursor cells of the lymph gland (LG) assemble in the dorsal thoracic mesoderm and coalesce, and rapidly develop into several pairs of lobes aligned on the dorsal vessel throughout larval stages (Holz et al., 2003; Jung et al., 2005). At the beginning of pupation, the LG dissembles, releasing mature hemocytes into circulation, where they assist with tissue remodeling during metamorphosis (Grigorian et al., 2011; Lanot et al., 2001).

During the 3rd instar larval stage, the disk-shaped primary pair of lobes of LGs typically contain a few thousand cells divided into three domains. Hemocyte precursors with stem cell-like properties are maintained in the central part of the LG termed the medullary zone (MZ), while differentiating hemocytes are primarily in the peripheral area termed the cortical zone (CZ) (Evans et al., 2009; Jung et al., 2005). In addition, a small group of cells termed the posterior signaling center (PSC) has been proposed to act as a niche that maintains the pro-hemocyte population of the MZ (Krzemien et al., 2007; Mandal et al., 2007; Mondal et al., 2011). However, this model has been called into question by a recent study demonstrating that genetic ablation of the PSC had no effect on prohemocyte maintenance (Benmimoun et al., 2015). While much remains to be understood, the fly LG has developed into a powerful model for hematopoiesis and stem cell/progenitor regulation (Crozatier and Meister, 2007; Crozatier and Vincent, 2011; Evans et al., 2003; Martinez-Agosto et al., 2007; Morin-Poulard et al., 2013; Shim et al., 2013).

There are three major lineages of mature hemocytes in *Drosophila*: plasmatocytes, crystal cells, and lamellocytes, all of which can be produced by the LG. Plasmatocytes contribute about 95% of all mature hemocytes in healthy animals (Crozatier and Meister, 2007; Tepass et al., 1994). These cells are the equivalent of mammalian macrophages, which are able to clean both apoptotic debris and foreign materials (Rizki and Rizki, 1980; Wood and Jacinto, 2007). They also play important roles in innate immunity (Charroux and Royet, 2009) and participate in tissue regeneration by activating stem cells near the wound (Ayyaz et al., 2015).

Crystal cells are specialized non-phagocytic cells that facilitate immune responses and wound-healing by causing melanization (Lanot et al., 2001; Rizki and Rizki, 1978). Lamellocytes are rarely found in healthy animals, but their number is significantly increased when larvae are immunologically challenged by infection of a parasitic wasp (Crozatier et al., 2004; Rizki and Rizki, 1992; Sorrentino et al., 2002).

The genetic control of cell fate in the LG has been extensively studied, and several signaling pathways are known to be important for proper LG development. For example, the Wnt gene Wingless (Wg) is expressed in the MZ, where it promotes pro-hemocyte proliferation and maintenance (Sinenko et al., 2009). Differentiating hemocytes also communicate with the MZ, through JAK/STAT signaling-dependent release of the adenosine deaminase-related growth factor A (Adgf-A), which lowers extracellular adenosine levels in the MZ, keeping pro-hemocytes in an undifferentiated state (Mondal et al., 2011; Mondal et al., 2014). Within the CZ, many studies have investigated the factors that control the balance of the three hemocytic lineages. For example, Notch signaling controls the crystal cell-lamellocyte decision, as inhibition of this pathway results in a reduction in crystal cells and a large increase in lamellocytes in healthy larvae (Duvic et al., 2002; Small et al., 2014). Crystal cell number in the LG is also controlled by Hippo signaling, which restricts specification of this cell type (Ferguson and Martinez-Agosto, 2014; Milton et al., 2014). By comparison with crystal cells and lamellocytes, the maturation process of the largest hemocyte population in the LG, the plasmatocytes, remains relatively obscure.

The working model of the larval LG states that plasmatocytes are derived from pro-hemocytes. Consistent with this, a population of cells that express both MZ and CZ markers has been observed in the LG (Dragojlovic-Munther and Martinez-Agosto, 2012; Sinenko et al., 2009) as well as cells that possess CZ and lack mature plasmatocyte markers (Minakhina et al., 2011) or lack both MZ and mature plasmatocyte markers (Krzemien et al., 2010). These intermediate progenitors (IPs) are typically found near the MZ and have a higher mitotic capacity than differentiated plasmatocytes (Krzemien et al., 2010). There are some reports of factors controlling this IP pool, e.g., the transcription factor Pannier (Minakhina et al., 2011), but it has been difficult to pin down their roles, due to the transitory nature of this population. The ability to “lock” cells in this intermediate stage would be an important tool to better understand their role in LG cell homeostasis.

We previously reported that Wg signaling represses the expression of Tiggrin (Tig) in hemocytes and in the MZ of the larval LG (Blauwkamp et al., 2008; Zhang et al., 2014). Tiggrin encodes a large extracellular matrix (ECM) protein that binds to integrins and is important for muscle attachment and cell-cell adhesion (Bunch et al., 1998; Fogerty et al., 1994; Graner et al., 1998; Zhang et al., 2010) and has also been implicated in axonal pathfinding (Oliva et al., 2015; Stevens and Jacobs, 2002). The repression of Tig by Wg signaling is noteworthy, as it occurs through a direct mechanism involving novel binding sites for the transcription factor TCF/Pangolin (TCF/Pan), which mediates Wg gene regulation in flies. The data support a model where TCF/Pan and Armadillo (the fly β-catenin), which normally promote

transcriptional activation, are allosterically regulated by the novel TCF/Pan binding sites to promote repression (Zhang et al., 2014).

Here we report on the biological role of Tig in the larval LG, using a combination of loss and gain of function approaches. We found that *Tig* mutants have a reduced LG, with a severe reduction in the size of the CZ, which appears to be due to premature differentiation of plasmatocytes. Interestingly, this phenotype could be rescued by mild expression of a *Tig* cDNA in the CZ. Higher levels of Tig expression resulted in a block in plasmatocyte differentiation, and a large buildup of IP cells that express both MZ and CZ markers. These manipulations of Tig levels did not affect the number of crystal cells and had only a minor effect on lamellocyte number, indicating that Tig is predominantly a plasmatocyte regulator. Expression of a Tig mutant that lacks an integrin binding domain had the same effect as wild-type Tig, suggesting that Tig's function in this context is not due to integrin signaling. In addition, we found that overexpression of the Weel1 kinase, which slows the G_2/M transition, specifically blocks plasmatocyte differentiation and causes a large buildup of IP cells. Wee1 activates *Tig* transcription and epitasis experiments suggest that Wee1 acts through Tig to disrupt hematopoiesis. These results highlight the connection between the cell cycle and the matrix protein Tig in the regulation of plasmatocyte differentiation.

Results

The ECM protein Tig is required for maintaining the LG hemocyte population

Tig is an essential gene, with loss-of-function mutants dying as pupae, with a few adult escapers (Bunch et al., 1998). This pupal lethality is likely due to defects in muscle attachment, morphology and function (Bunch et al., 1998). Tig is thought to be secreted at muscle attachment sites by circulating hemocytes (Bunch et al., 1998; Fogerty et al., 1994). In addition to its expression in circulating hemocytes, we have previously reported that Tig protein and two reporters containing *Tig* cis-regulatory sequences are expressed in the CZ of the LG (Zhang et al., 2014). This raised the possibility that Tig is playing a role in larval hematopoiesis. To test this hypothesis, we examined LGs in a *Tig* transheterozygous mutant background (Tig^{X}/Tig^{A1}). The Tig^{X} allele is a small deletion removing the entire *Tig* locus and parts of two adjacent genes, while the Tig^{A1} allele is an EMS-induced point mutation that fails to complement the muscle phenotype of Tig^{X} (Bunch et al., 1998). *Tig* mutants displayed a dramatic reduction in LG size in late 3rd larval instars (Figure 3.1A,B). Quantification shows that the size of both the CZ and MZ are reduced in *Tig* mutants compared to wild-type (Figure 3.1C), but the PSC cell number is unaffected (Figure 3.1D). These results reveal a previously unexpected role for Tig in the larval hematopoietic system.

To confirm the specificity of the *Tig* LG phenotype, we carried out a rescue analysis by expression of a P[UAS-Tig] transgene with *Hml*-Gal4 (Hml>Tig), which is specifically active in the CZ of the LG and circulating hemocytes (Goto et al., 2003). In an otherwise wild-type background, *Hml>Tig* animals displayed no detectable difference in LG size (Figure 3.1E, first two bars). However, this combination

efficiently rescued the reduced LG phenotype of Tig^{X}/Tig^{A1} (Figure 3.1E, last two bars). These data locate the site of Tig action in the LG to the CZ, and suggest that the MZ size reduction in *Tig* mutants is a secondary effect (see Discussion for further comments). In addition, *Hml>Tig* also resulted in a partial rescue of the pupal lethality of Tig^{X}/Tig^{A1} animals (Table I). These data support that the mutant phenotypes observed in Tig^{X}/Tig^{A1} mutants are due to loss of *Tig* gene activity.

One possible explanation for the reduced CZ size in *Tig* mutants is a lower level of cell proliferation. However, no obvious reduction in the S-phase index was observed in Tig^{X}/Tig^{A1} mutants (data not shown). On the other hand, Hml driven expression of Tig, under conditions (29˚C) where higher expression than used in the rescue experiments (Figure 3.1E) resulted in an increase in CZ size (Figure 3.2A,B). The average cell size of the CZ (Hml>GFP^{+}) cells was not changed by Tig overexpression (Figure 3.2C), suggesting that the bigger CZ size (Figure 3.2D) is due to increased cell number. Indeed, we observed that Tig induces S-phase entry in CZ cells, as a higher percentage of CZ (Cut^{+}) cells are also labeled by the nucleotide analog EdU (Figure 3.2E-K). Cut is a nuclear CZ marker that largely overlaps with Hml>GFP (Zhang et al., 2014), and is used in this analysis because its nuclear localization simplifies quantification of the EdU/Cut overlay. In addition to the increased cell proliferation in the CZ, more EdU labeled cells are also found in the MZ (compare Figure 3.2G & J), which we think is an indirect effect of Tig's action in the CZ (see Discussion).

Tig inhibits plasmatocyte maturation

Plasmatocytes are the major type of hemocyte in the CZ (Crozatier and Meister, 2007). To examine whether Tig regulates plasmatocyte cell fate, we stained LGs with the P1 antibody, which recognizes Nimrod C1 (NimC1), a phagocytosis receptor expressed in mature plasmatocytes (Kurucz et al., 2007). At the late 3rd instar larval stage, there were numerous P1^{+}, Hml^{+} cells in wild-type LGs (Figure 3.3A,B). Strikingly, high levels of Hml driven Tig expression resulted in a dramatic reduction in P1^{+} cells (Figure 3.3F), while the overall number of Hml^{+} cells was increased (Figure 3.3G). Immunostaining revealed that the level of Tig overexpressed in the Hml>Tig LGs was much higher than wild-type (Figure 3.3C, H), but the residual P1^{+} cells had no detectable Tig signal (Figure 3.3J). These results indicate that Tig overexpression represses plasmatocyte differentiation.

Tig has been suggested as a ligand for integrins, based on similarities between the mutant phenotypes of *Tig* mutant and *myospheroid* (*mys*), which encodes a βPS2 integrin (Brabant et al., 1996; Bunch et al., 1998; Stevens and Jacobs, 2002). In addition, Tig-coated surfaces provide excellent substrates for αPSβPS2 integrin-mediated cell spreading (Bunch et al., 1998). Tig contains a RGD tripeptide motif that is commonly found in integrin ligands (Fogerty et al., 1994). Substitution of these residues (to LGA) greatly reduces integrin-mediated cell spreading and causes a dramatic reduction in the ability of transgenic Tig to rescue the muscle attachment defects and lethality of *Tig* mutants (Bunch et al., 1998). However, Tig^{LGA} expression in the CZ resulted in the same phenotype, i.e., inhibition

of P1+ cells and expansion of Hml^{+} cells, as wild-type Tig (Figure 3.3F-O). This suggests that Tig inhibits plasmatocyte differentiation in an integrin-independent manner.

To confirm that the ability of Tig to inhibit P1 expression was due to a loss of mature plasmatocytes, we examined a second marker, Eater-dsRed. This reporter is driven by an enhancer from the Eater loci, which encodes a phagocytosis receptor expressed specifically in plasmatoctyes (Kocks et al., 2005; Tokusumi et al., 2009). Both $Hml>Tig^{WT}$ and $Hml>Tig^{LGA}$ LGs displayed a strong repression of Eater-dsRed expression (Figure 3.3P-R'). In addition, immunostaining with Lozenge (Lz) and L1, which mark crystal cells and lamellocytes, respectively (Jung et al., 2005; Kurucz et al., 2007), revealed no significant change in the frequency of these cell types in LGs overexpressing Tig (Figure 3.3S-U', Figure 3.7). These data support a model where Tig overexpression specifically inhibits plasmatocyte maturation.

To determine the physiological role of Tig in CZ cell fate determination, plasmatocyte development was examined in Tig^{X}/Tig^{A1} mutant LGs. In mid-3rd instar larvae, we found that loss of *Tig* caused precocious maturation of plasmatocytes, with nearly all the Hml^{+} cells expressing high levels of P1 (Figure 3.4A-H). This phenotype was rescued by moderate levels of Hml mediated Tig expression (Figure 3.4I-L). The level of expression from the P[UAS-Tig] transgene used in these rescue experiments did not inhibit P1 expression in an otherwise wild-type background (Figure 3.5). There was no detectable change in crystal cell number in *Tig* mutants (Figure 3.6) and only a slight increase in lamellocytes (Figure

3.7), indicating that the increased number of plasmatocytes was not due to loss of these cell lineages. These loss-of-function experiments provide further support for the model that Tig specifically represses maturation of the plasmatocyte lineage in the LG.

Tig prolongs a pre-plasmatocyte, IP cell fate in the CZ

Overexpression of Tig in the CZ causes the accumulation of Hml^{+} cells that lack the plasmatocyte markers P1 and Eater-dsRed (Figure 3.3). These cells are reminiscent of the IPs that have been previously noted in wild-type LGs (Dragojlovic-Munther and Martinez-Agosto, 2012; Krzemien et al., 2010; Makhijani et al., 2011). Indeed, our examination of Hml>GFP LGs revealed a significant population of cells that were Hml^{+} (indicating they were in the CZ) but were $P1^{-}$ (Figure 3.8A-C'). Hml^{+} cells with very low levels of P1 staining are also evident (arrow in Figure 3.8B). The Hml>GFP signal does not overlap with Lz staining, indicating that the Hml^{+} cells are not crystal cells (Figure 3.8D-E'). Another line of evidence for the presence of IPs is the existence of cells at the MZ/CZ border that are positive for both MZ and CZ markers (Sinenko et al., 2009)Dragojlovic-Munther and Martinez-Agosto, 2012). Cells with these characteristics (i.e., $Dome^{+}$, Hml^{+}) are difficult to locate in control LGs (Figure 3.8G), but Hml^{+} cells with intermediate levels of Dome signal are readily apparent in the CZ of Hml>Tig LGs (Figure 3.8H, I). The data indicate that Tig expression causes a buildup of IPs that cannot proceed with plasmatocyte differentiation.

Tig protein is found throughout the CZ (Zhang et al., 2014), which is seemingly in conflict with a model where Tig promotes an IP fate while inhibiting plasmatocyte maturation. One possibility is that immunostaining of Tig protein detects multiple pools, including Tig that is incorporated in the ECM and no longer able to influence plasmatocyte maturation. When the Tig expression pattern is detected using transcriptional reporters (Zhang et al., 2014), the cells actively expressing the reporters have little overlap with $P1^+$ cells (Figure 3.9). This was observed with Tig-lacZ, which contains a 1.8 kb stretch of genomic DNA including sequences upstream of the *Tig* transcriptional start site and the first intron (Figure 3.9A-C) and the non-overlap is even more pronounced with minR-lacZ, which contains two repeats of a 40 bp minimal Wg responsive element from the *Tig* intron (Zhang et al., 2014) (Figure 3.9D-F). These results suggest that IPs and immature plasmatocytes are the cells most actively expressing Tig in the CZ.

The G_2/M transition regulates plasmatocyte differentiation and Tig expression

Given that Tig affects both plasmatocyte maturation and cell cycle progression in the LG, we wondered if these two processes are related, e.g. whether manipulation of cell cycle regulators affects LG cell fate. During a screen of known cell cycle regulators, we discovered that expression of the Wee1 kinase has a profound effect on plasmatocyte differentiation (Figure 3.10). Wee1 is a key regulator of the G_2/M checkpoint and acts by inhibiting Cdk1, the kinase subunit of maturation-promoting factor (MPF), which promotes the onset of M-phase (Campbell et al., 1995; Price et

al., 2002; Russell and Nurse, 1987). Expression of Wee1 via Hml-Gal4 completely blocks plasmatocyte maturation, as judged by loss of Eater-dsRed expression in late 3rd larval instar LGs, with an accumulation of Hml-GFP^{+} cells (Figure 3.10A-D). Expression of Wee1 does not significantly change the amount of crystal cells and lamellocytes (Figure 3.10 E-H, Figure 3.7). As observed with Tig overexpression, Wee1 causes an accumulation of IPs in the CZ.

To confirm that Wee1 expression caused a slowdown of the G_2/M transition, we utilized the RGB cell cycle tracker (Handke et al., 2014). Wee1 expression caused a marked increase in cells that were positive for EBFP, Tomato and EGFP (arrowheads in Figure 3.13), indicative of delay or arrest in G_2 (Handke et al., 2014). Interestingly, the slowing of the G_2/M transition did not reduce the amount of CZ cells, likely due to increased proliferation of MZ cells (Figure 3.13; see discussion for further comment).

To confirm that the Wee1 effect on plasmatocyte maturation is due to a slowing of the G_2/M transition, we examined LGs where this transition is accelerated. This was achieved by expression of String (Stg), a phosphatase that antagonizes Wee1 function to activate Cdk1 (Edgar and O'Farrell, 1990; Russell and Nurse, 1986). The Hml>Stg background resulted in smaller LGs with an increase in mature plasmatocytes and very few IPs compared to controls (Figure 3.11), similar to *Tig* mutants (Figure 3.4). Taken together, the Wee1 and Stg data suggests that the rate of the G_2/M transition controls plasmatocyte differentiation.

The similarity between the *Tig* and G_2/M perturbation phenotypes raises the

possibility that they act in a linear pathway. To test this, we examined the expression of Tig protein and the Tig-LacZ and minR-LacZ Tig transcriptional reporters under the condition of Wee1 overexpression. All three readouts of Tig are strongly upregulated by Wee1 (Figure 3.12). These data demonstrate that Wee1 activates Tig expression at the level of transcription and suggest that the G_2/M transition regulator Wee1 represses plasmatocyte differentiation possibly through inducing Tig expression.

Discussion

In this report, we demonstrate that the extracellular matrix protein Tig is an important negative regulator of plasmatocyte maturation in the *Drosophila* LG (Figure 3.14A). Loss of function *Tig* mutants display a smaller LG with a reduction in both the MZ and CZ (Figure 3.1). Overexpression of Tig in the CZ inhibits the maturation of mature plasmatocytes (Figure 3.3) and *Tig* mutant LGs have precocious maturation of these macrophage-like cells (Figure 3.4). These manipulations in *Tig* gene activity have little or no effect on non-plasmatocyte lineages, i.e., crystal cell and lamellocytes (Figure 3.3S-U', Figure 3.7 & Figure 3.6). *Tig* mutant phenotypes are rescued by expression of transgenic *Tig* in the CZ (Figure 3.1C, 4I-L), consistent with the endogenous Tig expression pattern (Zhang et al., 2014). Matrix proteins are involved in a variety of structural and signaling processes during development (Rozario and DeSimone, 2010) and are important for stem cell maintenance (Brizzi et al., 2012; Okolicsanyi et al., 2014). Our work demonstrates that in addition to its

function in muscle attachment (Bunch et al., 1998), Tig plays an important role in regulating LG size and plasmatocyte differentiation.

In WT LGs, prohemocytes in the MZ (e.g., marked by Dome-EBFP) undergo a transition as they enter the CZ, becoming cells referred to as IPs which contain residual Dome-EBFP and acquire CZ markers such as Hml>GFP (Dragojlovic-Munther and Martinez-Agosto, 2012; Sinenko et al., 2009). Cells closer to the periphery of the CZ tend to express increasing levels of P1, a plasmatocyte marker (Krzemien et al., 2010; Makhijani et al., 2011) (Figure 3.8A-C'). Thus the prohemocytes in the MZ are $Dome^{+}$, Hml^{-}, $P1^{-}$, IP cells are $Dome^{+}$, Hml^{+}, $P1^{-}$ and maturing plasmatocytes are $Dome^{-}$, Hml^{+}, $P1^{+}$ (Figure 3.14B). Overexpression of Tig "freezes" many cells in the IP fate, leading to an accumulation of cells expressing high levels of Hml reporters and moderate levels of Dome-EBFP (Figure 3.8F-1; Figure 3.14B).

Overexpression of Tig in the CZ results in a dramatic increase in proliferation in the MZ (Figure 3.2). This non-autonomous effect suggests the presence of a feedback signal from IP cells to pro-hemocytes, stimulating the cell cycle. In Hml>Tig (or Hml>Wee1; see Figure 3.13), this signal is elevated. In *Tig* mutant LGs, the precocious differentiation of plasmatocytes could result in a reduction in this mitotic signal, resulting in the smaller MZ observed (Figure 3.1B, C). While speculative, this pathway is reminiscent of the Adgf-A "equilibrium pathway" that has been described where CZ cells signal through an adenosine/JAK/STAT axis to maintain MZ cells in an undifferentiated state (Mondal et al., 2011; Mondal et al.,

2014).

We propose that Tig slows down plasmatocyte differentiation in the CZ, which presumably allows the IP pool to expand and thus generate sufficient progenitors to maintain the appropriate number of plasmatocytes. This model would predict that Tig expression is highest in IPs, but immunostaining revealed that Tig protein is found throughout the CZ (Zhang et al., 2014). We propose the existence of a “regulatory” pool of Tig, that is predominately active in IP cells (Figure 3.14C). Consistent with this, we found that Tig transcriptional reporters were largely active in CZ cells lacking high levels of the P1plasmatocyte marker (Figure 3.9). One possibility is that the regulatory pool is comprised of newly synthesized Tig, which can influence plasmatocyte maturation before it becomes incorporated into the ECM.

How does Tig inhibit plasmatocyte maturation?

Although Tig encodes a large (2186 aa) protein with 16 repeated domains (74-77 aa/repeat), it has no significant sequence similarity outside of Dipterans (www.ncbi.nlm.nih.gov/BLAST/). Tig does contain an Arg-Gly-Asp (RGD) motif at position 1989-1991, which is required for integrin binding in a cell spreading assay (Bunch et al., 1998). Mutation of this tripeptide motif (RGD to LGA) greatly reduced rescue of the muscle attachment phenotype of *Tig* mutants (Bunch et al., 1998). However, the LGA Tig transgene had no detectable defect in blocking plasmatocyte differentiation, compared to similarity expressed WT Tig (Figure 3.3). While we cannot exclude the possibility that the LGA mutation retains the ability to bind to some integrin heterodimers, the data suggests that Tig regulates plasmatocyte

differentiation independently of integrin signaling.

Are there other factors that regulate plasmatocyte development in *Drosophila* which could work in concert with Tig? While screening for suppressors of a LG overgrowth phenotype, three genes, *visgun* (*vsg*), *SHC-adaptor protein* (*shc*) and *Adgf-A*, were identified where loss of function results in precocious plasmatocyte differentiation (Tan et al., 2012). *vsg* encodes an ortholog of mammalian endolyn, a endolysosomal sialomucin (Zhou et al., 2006) and *shc* an SH2/PTB adaptor protein required for a subset of receptor tyrosine kinase receptors (Luschnig et al., 2000). Further examination is necessary to determine whether these proteins act in a CZ autonomous fashion like *Tig*, or whether they work by signaling to the MZ similarly to *Adgf-A* (Mondal et al., 2011).

The GATA transcription factor *pannier* (*pnr*) promotes plasmatocyte maturation in the larval LG. Loss of *pnr* results in a cell autonomous reduction in plasmatocytes (Minakhina et al., 2011). *pnr* produces two isoforms and overexpression of the longer one also inhibits plasmatocyte maturation. Unlike Tig, a Pnr-lacZ reporter is expressed in both the MZ and CZ (Minakhina et al., 2011). Despite these complexities, it might be interesting to examine whether *pnr* is epistatic to *Tig* in the CZ.

In embryos, the related transcription factors *Glial cells missing* (*Gcm*) and *Gcm2* are required for producing the full number plasmatocytes (Bernardoni et al., 1997) (Alfonso and Jones, 2002). Embryonic hematopoiesis shares some genetic similarities with the larval LG, e.g. express similar markers (Evans et al., 2014), but

the role of *Gcm*/*Gcm2* in the latter has not been reported. Likewise further studies are required to determine whether *Tig*, which is expressed in embryonic hemocytes (Blauwkamp et al., 2008; Fogerty et al., 1994; Zhang et al., 2014), regulates plasmatocyte development in this context.

Cell cycle regulation and cell fate determination – a case in fly hematopoiesis

Precise coordination between cell cycle progression and cell fate determination is necessary for proper development and tissue homeostasis, e.g., during neural cell lineages (Farkas and Huttner, 2008; Fichelson et al., 2005) and hematopoiesis (Nakamura-Ishizu et al., 2014). In many cases, cells exit the cell cycle upon terminal differentiation (Buttitta and Edgar, 2007) and perturbations that prolong cell cycle progression result in premature differentiation (e.g., (Manansala et al., 2013; Tapias et al., 2014). In other examples, manipulating the cell cycle, while altering cell number, does not affect cell fate specification (de Nooij and Hariharan, 1995; Edgar and O'Farrell, 1990).

Here, we report a particularly dramatic example where the specification of a particular cell fate, i.e., plasmatocytes, is tightly linked to the cell cycle. Expression of Wee1, which prolongs the G_2/M transition, results in a dramatic block in plasmatocyte differentiation and a concomitant accumulation of IPs (Figure 3.10). Conversely, acceleration of G_2/M by expression of Stg causes premature plasmatocyte differentiation (Figure 3.11). In addition, a previous study found that mutation of *Cyc27*, a component of the anaphase-promoting complex (APC/C),

displays a moderate reduction in P1 expression (Tan et al., 2012), which could be due to a prolonged G_2 phase.

While misregulation of the G_2/M transition is known to disrupt morphogenetic movements, e.g., gastrulation, in *Drosophila* and vertebrate systems (Bouldin and Kimelman, 2014), effects on differentiation are rare. Forced expression of Cdc25a (a vertebrate homolog of Stg) blocks muscle differentiation in zebrafish embryos (Bouldin et al., 2014). This is the opposite of what we observe, i.e., Stg overexpression promotes premature formation of plasmatocytes (Figure 3.11). In the case of pluripotent stem cells, cells in the G_1 phase are more likely to undergo differentiation (Bouldin and Kimelman, 2014; Bouldin et al., 2014; Calder et al., 2013; Coronado et al., 2013; Sela et al., 2012; Singh et al., 2013), possibly due to induced expression of key developmental regulators at this stage of the cell cycle (Pauklin and Vallier, 2013; Singh et al., 2015). It's interesting to note that in *Drosophila* wing imaginal discs, retardation of the G_2/M transition shortens the length of G_1 (Reis and Edgar, 2004), raising the possibility that the Wee1 block of plasmatocyte differentiation is due to reduction of the length of G_1.

While it is possible that Wee1 and Stg regulate plasmatocyte differentiation through their ability to regulate the cell cycle, other mechanisms are also possible. There is some evidence that Cyclin dependent kinase 1 (Cdk1), the target of Wee and Stg (Figure 3.14A) affects gene expression through phosphorylation of transcription factors (Lim and Kaldis, 2013; Hu et al., 2009; Hu et al., 2011). Our data suggest another possibility that the regulation is mediated by Tig, as Wee1

activates the levels of both Tig protein and its transcriptional reporters (Figure 3.12). Expression of minR-lacZ (Figure 3.12), a synthetic reporter that containing two TCF/Pan binding sites (from the Tig regulatory region) upstream of a minimal promoter (Zhang et al., 2014) suggests that Wee1 influences Tig transcription via an mechanism that involves TCF/Pan or a factor that associates with this Wnt regulated transcription factor. Further studies of this regulation will deepen our understanding of hematopoiesis and shed more light on the connection between the cell cycle and cell fate determination.

Materials and Methods

Drosophila genetics

pUAST-Tig^{WT} and pUAST-Tig^{LGA} plasmids were kindly provided by Thomas Bunch (Bunch et al., 1998). Transgenic flies were generated by Rainbow Transgenic Flies Inc. (Thousand Oaks, California) in a w^{1118} background. The expression strength of multiple transgenic lines were compared by crossing to *Hemolectin*-Gal4 (*Hml*-Gal4), immunostaining for Tig and comparing signal intensity in each line using imageJ. A pair of P[UAS-Tig^{WT}] and P[UAS-Tig^{LGA}] flies with similar and relatively strong expression levels were used for all experiments. When used for the rescue of *Tig* mutants, cultures containing P[UAS-Tig] were maintained at 25˚C; in all other experiments, cultures were grown at 29˚C to achieve a significantly higher level of expression.

The other fly stocks used in this study have all been previously described: *Tig*^{X}

and *Tig*^{A1} (Bunch et al., 1998), Hml-Gal4 (Goto et al., 2003), Domeless-Gal4 (Dome-Gal4) (Bourbon et al., 2002), UAS-Wee1 (Price et al., 2002), UAS-String (UAS-Stg) (Neufeld and Edgar, 1998), UAS-RGB (Handke et al., 2014), Tig-LacZ and minR-LacZ (Zhang et al., 2014), Eater-dsRed (Tokusumi et al., 2009), and DHH, a line containing Dome-EBFP2, Hml-DsRed and hedgehog-GFP reporters (Evans et al., 2014).

All crosses were set up at 25˚C. Embryos were collected within a 12 hr window, transferred at 24-36 hours after egg laying (AEL) to 29˚C if necessary, and dissected at desired time: 90-102 hours AEL for mid 3rd instar larvae, 96-108 hours AEL for mid/late 3rd instars and 102-114 hours AEL for late 3rd instars. For experiments with UAS-Wee1, collections were longer (24 hrs), because of reduced fertility of these stocks. In those cases, the Hml>GFP expression pattern, which is dynamic through the mid to late 3rd instar larval stages, was used to identify age appropriate animals.

Dissection and Immunohistochemistry

For LG dissection, two previously described protocols were used for either immunostaining (Lebestky et al., 2000) or simple imaging of fluorescent markers (Small et al., 2012). Immunostaining was done as previously described (Zhang et al., 2014); 5% normal donkey serum was used in blocking and antibody incubation, and 0.5% Triton-X100 was used in all steps following fixation. Primary antibodies were used at the following dilutions: mouse α-P1 at 1:75, mouse α-L1 at 1:10 (Kurucz et al., 2007), mouse α-Lz at 1:30 (Developmental Studies Hybridoma Bank,

DSHB), rabbit α-Tig at 1:50 (Deng et al., 2010), mouse α-Cut at 1:100 (DSHB), and rabbit α-LacZ (MP Biomedicals) at 1:1000. For secondary antibodies, donkey anti-mouse/rabbit IgG, Cy5/Cy3 (Jackson ImmunoResearch Laboratories Inc.) and A488 (Life Technologies) were used at 1:300 and 1:1000, respectively.

EdU labeling

Larvae were dissected before being labeled in 10 μM EdU (diluted in PBS) for 70 minutes, then washed 2 x 5 minutes in PBS and fixed in 4% formaldehyde. If combined with immunostaining, blocking, primary and secondary antibodies were added, after which EdU was visualized by the Click-iT EdU Alexa Fluor 555 Imaging Kit (Life Technologies). Samples were then washed 2 x 5 minutes in PBST (PBS + 0.5% Triton-X100), stained with DAPI for 30 minutes, washed 4 x 5 minutes in PBST, and mounted in Vectashield.

Imaging and Data Quantification

All micrographs were taken with a Leica SP5 laser scanning confocal microscope and quantified using Adobe Photoshop and ImageJ. All images, except for Figure 3.3A-E, are thin optical slices, with slices of P1 immunostains taken approximately one-third from the top of the LG (where the Hml>GFP^{+}, low/no P1 population of cells is the most obvious) and Lz immunostains at approximately one-half of the whole LG thickness (where the most Lz^{+} cells are found). At least eight and usually more than twelve LGs per genotype were examined and representative images are shown. LG size was determined by DAPI, CZ size by Hml-dsRed or Hml>GFP (full stack projection), and MZ size was calculated by the

difference between LG and CZ size. For quantification of the size of CZ cells, thin slices were used and cells with clear Hml>GFP and DAPI signals were selected; 7-8 LGs per genotype and 4 cells per LG were quantified. For PSC cell number, full stack projections of Hh>EGFP were used to determine (a) total GFP intensity and (b) average single cell GFP intensity (which was statistically the same between WT and $Tig^{A1/X}$), and PSC cell number was calculated as the ratio between total GFP and single cell intensity.

Acknowledgements

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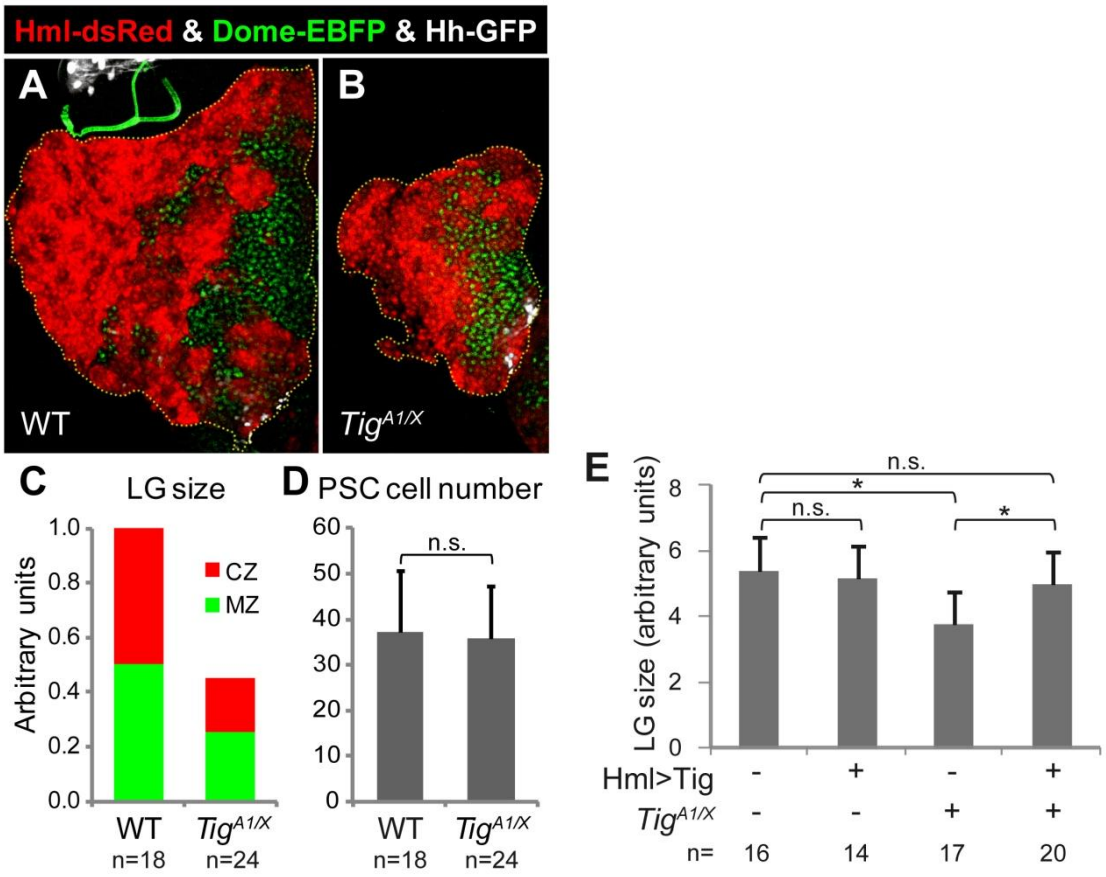


Figure 3.1. Tig is important for LG development. (A-B) Confocal images of LGs from late 3rd instar larvae from WT or *Tig* transheterozygote mutants. The CZ, MZ and PSC are marked by Hml-dsRed (red), Dome-EBFP (green) and Hh-GFP (white), respectively. *Tig* mutants have smaller LGs with less CZ and MZ but the PSC appears unchanged. **(C)** ImageJ quantification shows that the sizes of CZ, MZ and the total LG are significantly different between WT and *Tig* mutants ($p < 0.01$ for all comparisons). **(D)** No detectable change of PSC cell number is observed in *Tig* mutants. **(E)** Size quantification of LGs from late 3rd instar larvae containing P[Hml-Gal4] with or without P[UAS-Tig] and *Tig* mutant alleles. Expressing Tig specifically in the CZ, where Tig is naturally expressed, has no effect on LG size by itself (compare the 1st and 2nd columns) but it does rescue the LG size reduction in *Tig* mutants (compare the 3rd and 4th columns). See Materials and Methods for more details on the quantification in panels C-E. All experiments were performed at 25˚C. *: $p < 0.05$.

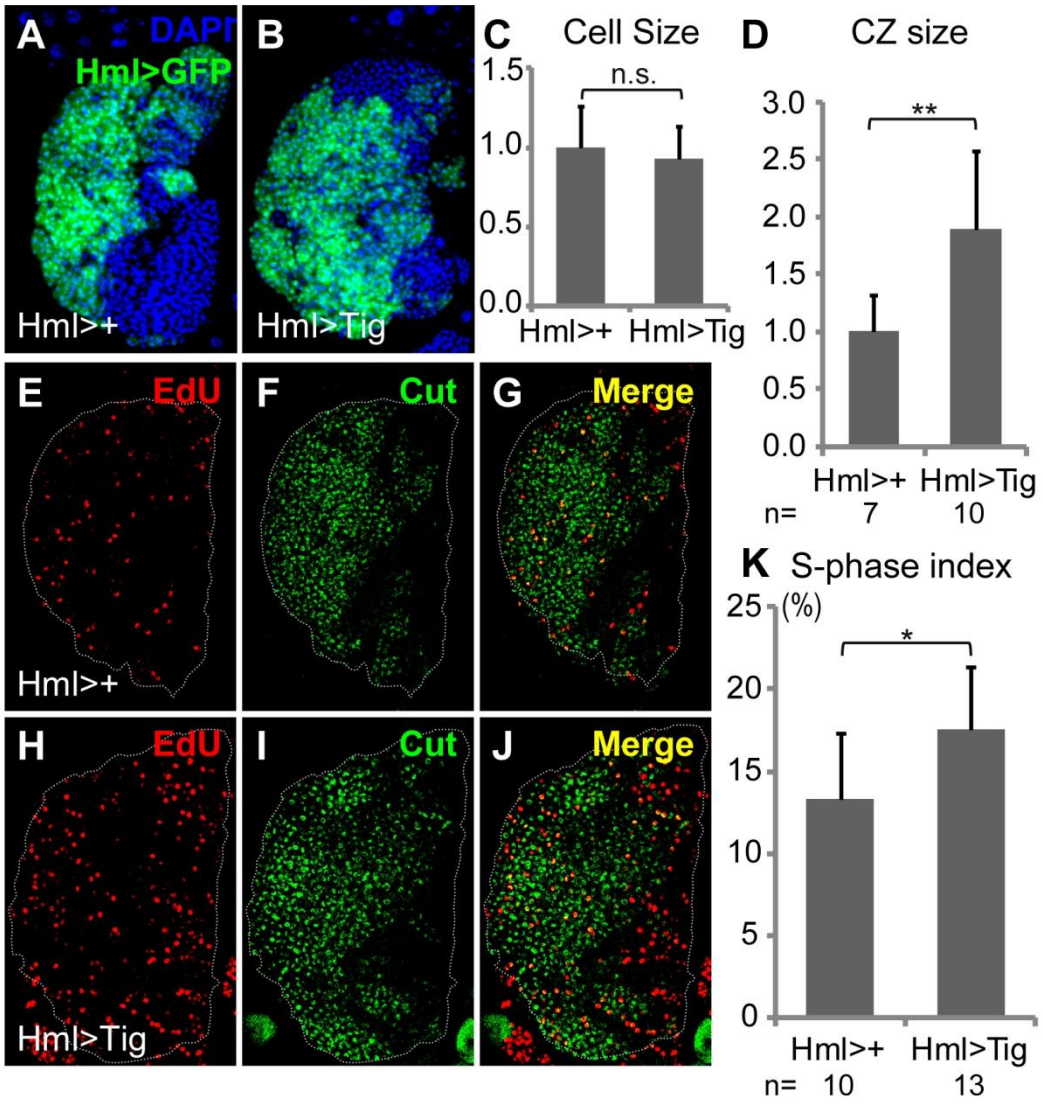


Figure 3.2. Overexpression of Tig expands the CZ through increased proliferation. All confocal images are of LGs from mid/late 3rd instar larvae containing P[Hml-Gal4], P[UAS-GFP] without or with P[UAS-Tig]. **(A-B)** Hml>Tig causes an expansion of the CZ (i.e., GFP^+ cells). **(C)** ImageJ analysis revealed no detectable difference in cell size in Hml>Tig cells (28-32 GFP^+ cells were measured per genotype). **(D)** Quantification of CZ size demonstrates a significant increase in Hml>Tig LGs. **(E-J)** LGs stained for the CZ marker Cut (green) and EdU incorporation (red). Hml>Tig LGs have increased S-phase (EdU^+) cells in both the CZ and the MZ. **(K)** Quantification of the S-phase index in the CZ (i.e., EdU^+& Cut^+/Cut^+ cells) shows a significant increase in Hml>Tig LGs. See Materials and Methods for more details on the quantification in panels C, D and K. Experiments were performed at 29˚C. *: $p < 0.05$. **: $p < 0.01$.

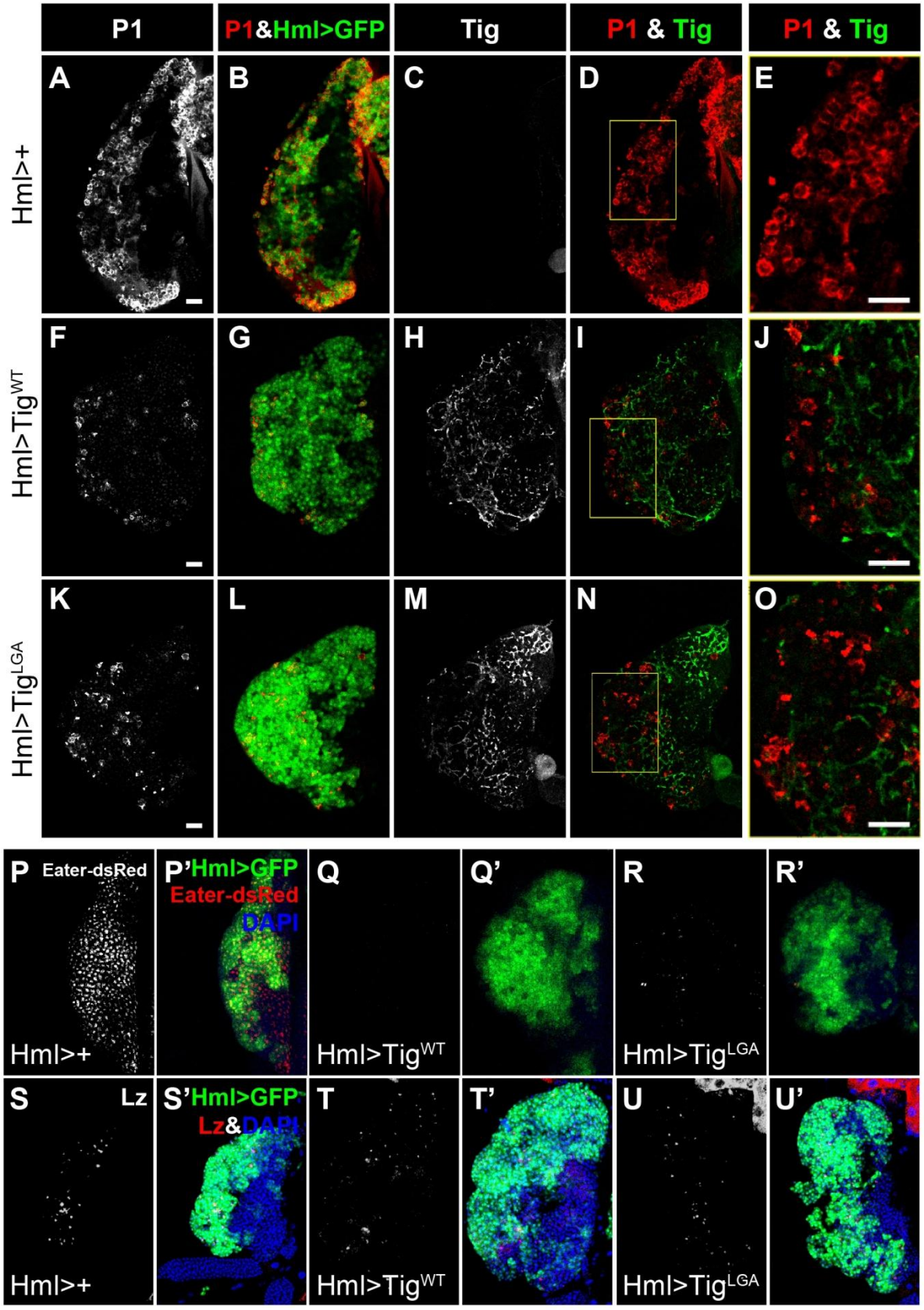

P1
P1&Hml>GFP
Tig
P1 & Tig
P1 & Tig
Hml>+
Hml>Tig^WT
Hml>Tig^LGA
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P Eater-dsRed
Hml>+
P'Hml>GFP
Eater-dsRed
DAPI
Q
Hml>Tig^WT
Q'
R
Hml>Tig^LGA
R'
S Lz
Hml>+
S'Hml>GFP
Lz&DAPI
T
Hml>Tig^WT
T'
U
Hml>Tig^LGA
U'

Figure 3.3. Tig overexpression represses plasmatocyte differentiation independent of a known integrin binding domain. All confocal images are of LGs from mid/late 3rd instar larvae containing P[Hml-Gal4], P[UAS-GFP], without or with transgenes expression wild-type Tig (P[UAS-Tig^{WT}]) or a Tig transgene with the integrin binding domain mutated (P[UAS-Tig^{LGA}]). **(A-O)** LGs stained for Tig and the plasmatocyte marker P1. When over-expressed at similar levels **(C, H, M)**, both Tig^{WT} and Tig^{LGA} strongly repress P1 expression **(A, F, K)**. **(J, O)** Magnification of panels I and N showing that the residual P1 signal is often found at areas of low Tig expression. Bars = 25 μm. **(P-R')** LGs containing an Eater-dsRed transgene expressing Tig proteins. Eater-dsRed is strongly repressed by either Tig^{WT} or Tig^{LGA}. **(S-U')** LGs stained for the crystal cell marker Lz. Tig^{WT} and Tig^{LGA} do not cause a detectable change in the number of crystal cells. All experiments were performed at 29˚C.

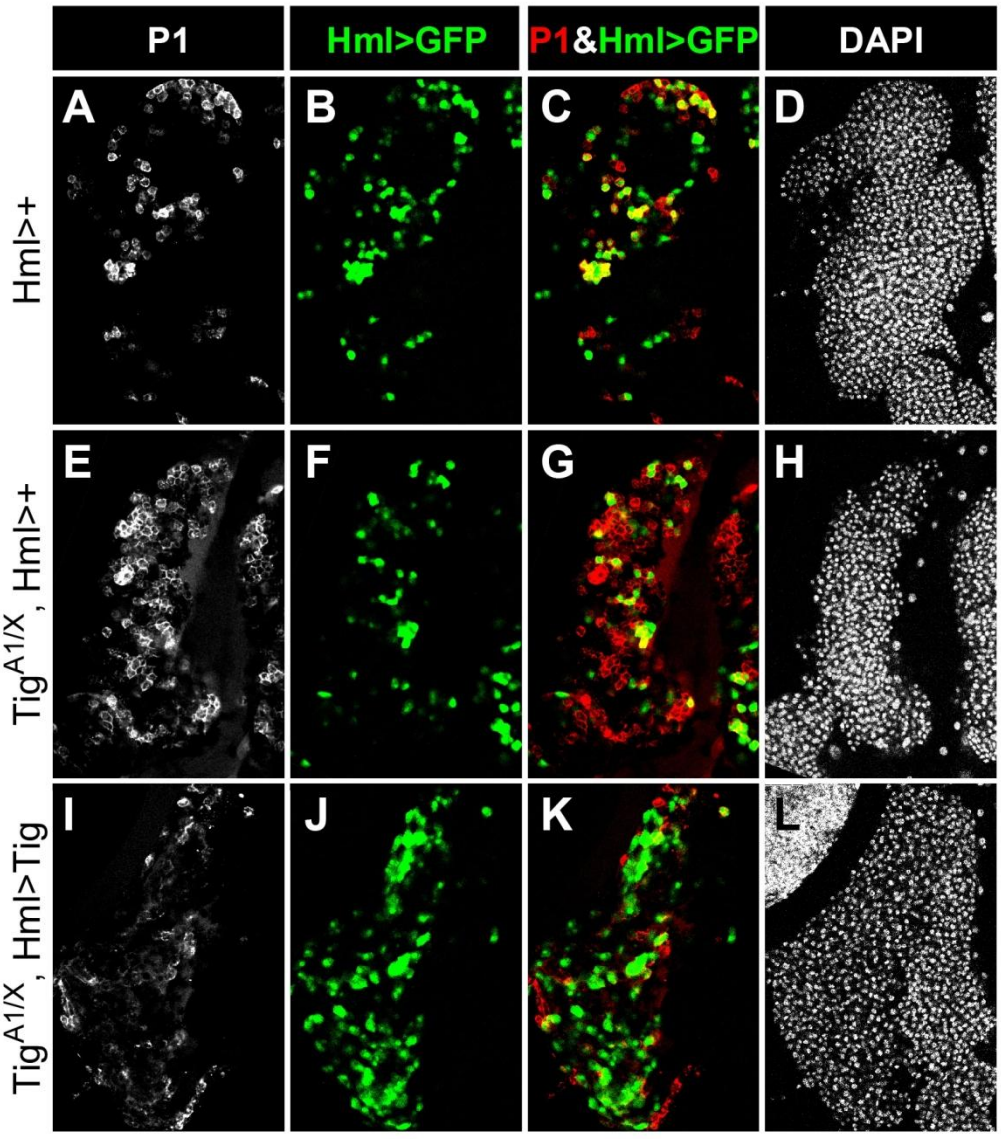


Figure 3.4. *Tig* mutants have precocious plasmatocyte differentiation. Confocal images of LGs from mid 3rd instar larvae. All LGs were stained for P1 and contain P[Hml-Gal4] and P[UAS-GFP], with or without P[UAS-Tig] and *Tig* mutant alleles. **(A-D)** At this stage, many Hml>GFP$^+$ cells lack P1 in WT LGs. **(E-H)** In *Tig* mutant LGs, most Hml>GFP$^+$ cells are also P1$^+$. However, there are also many P1$^+$ cells that lack Hml>GFP. **(I-L)** Expressing Tig in the CZ rescues the precocious plasmatocyte differentiation of *Tig* mutants, i.e., the Hml>GFP$^+$/P1$^-$ population is restored. Experiments were performed at 25˚C to restrict Hml>Tig expression to a moderate level that doesn't inhibit plasmatocyte differentiation in control LGs. Due to the earlier stage and lower temperature, the Hml>GFP signal is weaker than those in the other figures.

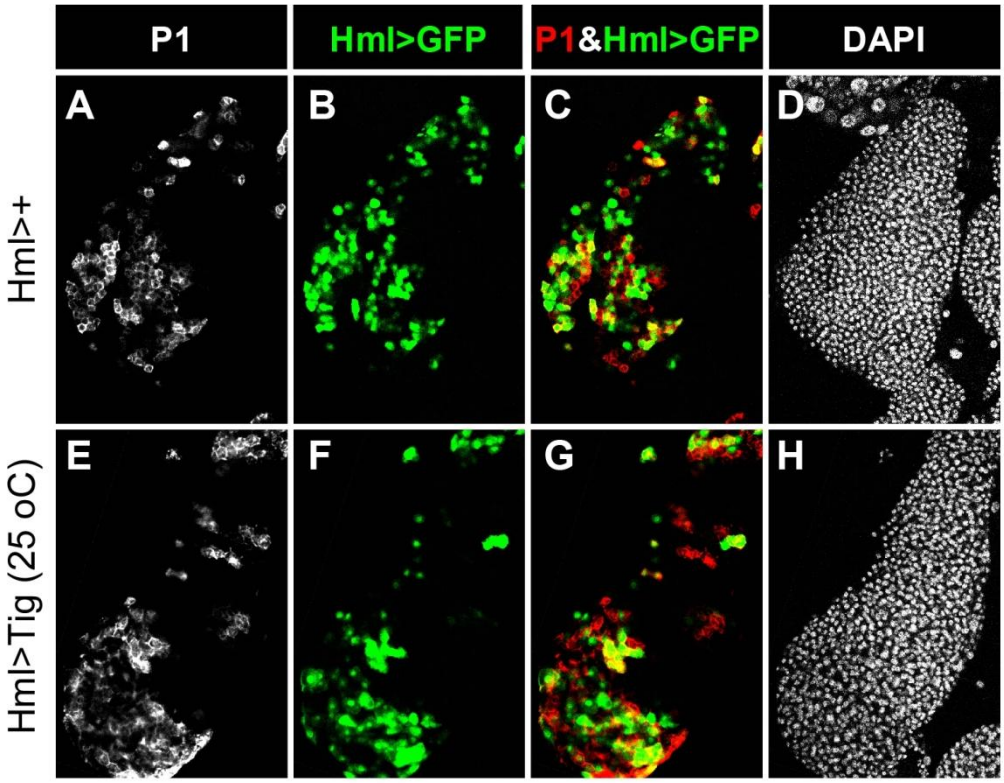


Figure 3.5. Moderate expression of Tig does not affect the plasmatocyte differentiation. Confocal images of mid-3rd instar larval LGs containing P[Hml-Gal4] and P[UAS-GFP] with or without P[UAS-Tig^{WT}] and immunostained for P1. The amount of plasmatocytes **(A, E)** and Hml>GFP+ cells **(B, F)** are not detectably affected by Tig overexpression under these conditions. Experiments were performed at 25˚C to achieve lower expression than used in Figures 3.2, 3.3 & 3.13.

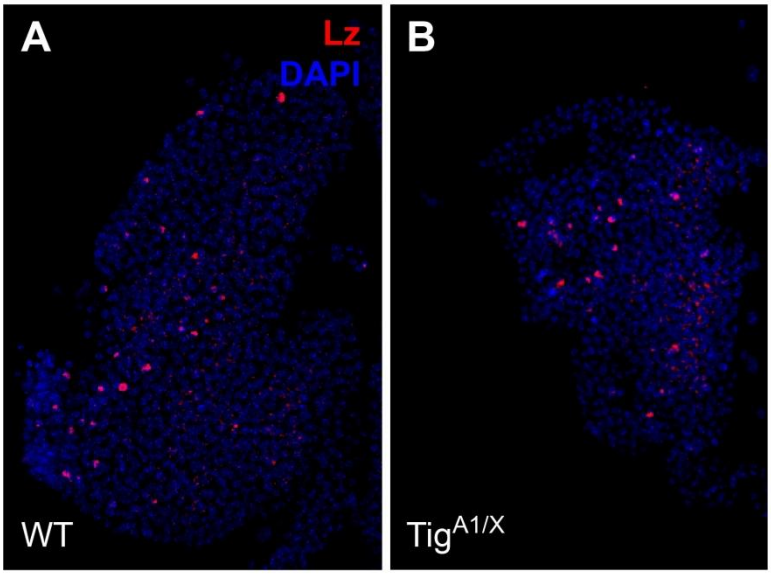
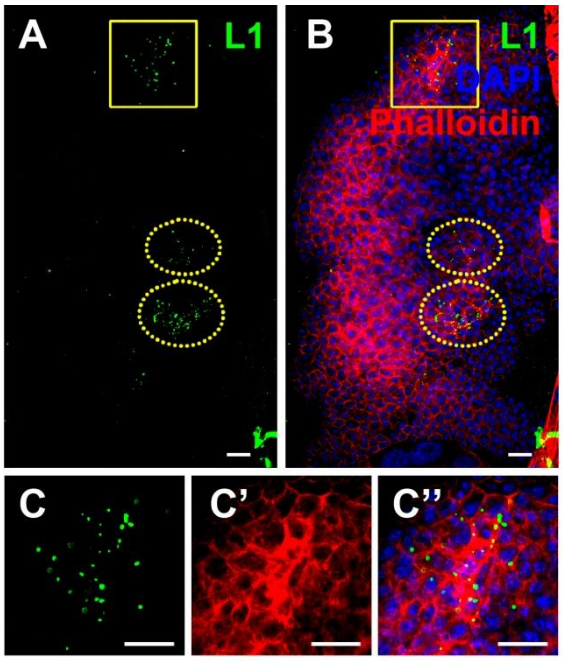


Figure 3.6. *Tig* loss-of-function does not affect crystal cell fate in the LG. Confocal images of LGs from wild-type or *Tig*$^{A1/X}$ mid/late 3rd instar larvae immunostained for Lz. The amount of Lz^{+} cells was similar in both genotypes. Experiments were performed at 25˚C.



D

Geno -Type	WT	Hml> Tig^{WT}	Hml> Tig^{LGA}	Hml> Wee1
Ave. L1+ clusters per LG	0.11 (n=18)	0.17 (n=18)	0.07 (n=14)	0.18 (n=15)

E

Genotype	WT	Tig$^{A1/X}$
Ave. L1+ clusters per LG	0.25 (n=12)	0.5 (n=8)

Figure 3.7. Tig and Wee1 expression do not affect lamellocyte development in the LG. (A-C'') Confocal images of a rare LG from wild-type mid/late 3rd instar larvae that contained a few lamellocytes. The vast majority of wild-type LGs contain very few or no lamellocytes **(D-E)**. LGs were stained with the lamellocyte-specific antibody L1 and counterstained with phalloidin. Lamellocytes are identified by punctate L1 signal and verified by a regional increase in phalloidin signal (boxed area magnified in **(C-C'')**. Each cluster of L1/increased phalloidin was counted as a single lamellocyte. **(D-E)** Summary of the amount of lamellocyte clusters in Hml>Tig^{WT}, Hml>Tig^{LGA}, Hml>Wee1 and *Tig$^{A1/X}$* LGs, compared to wild-type. All LGs contain very few (≤ 0.5) clusters on average. The number of lamellocytes are not significantly induced under these experimental conditions. All experiments were performed at 29˚C.

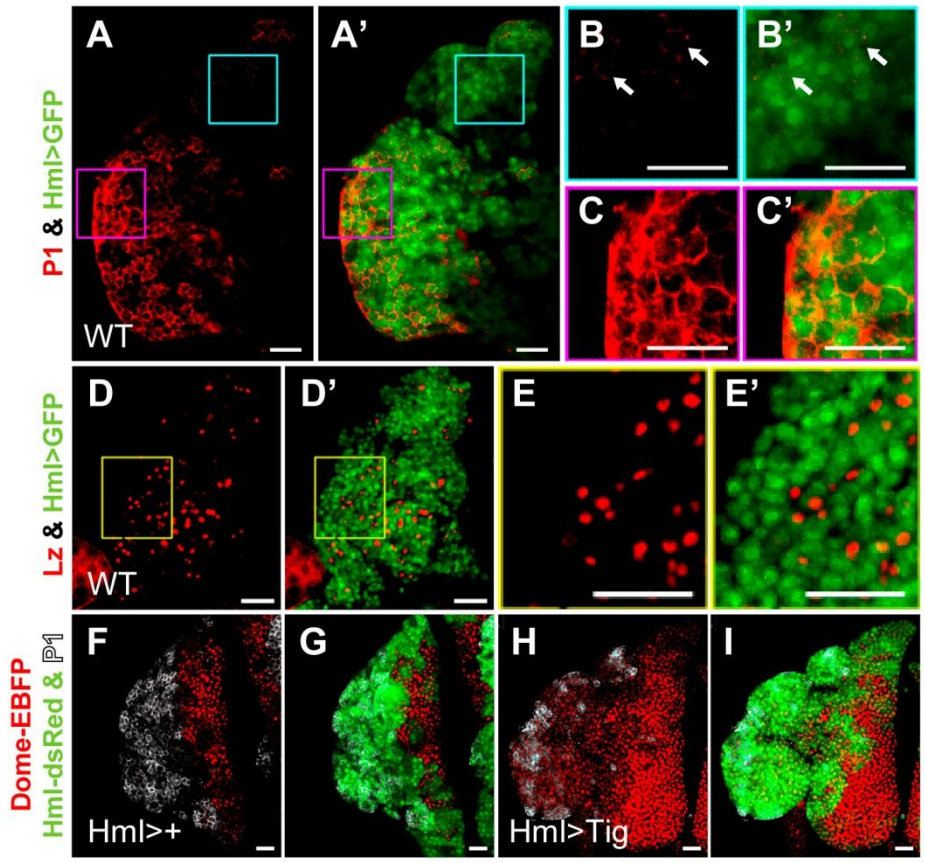


Figure 3.8. The LG contains a pool of Hml>GFP$^+$/P1 negative IP cells. Confocal images of LGs from mid/late 3rd instar larvae. **(A, A')** Stack projections of the surface layer of a LG expressing Hml>GFP (green) and immunostained for P1 (red). **(B-C')** Magnified views illustrating areas with mostly IP cells, i.e, GFP$^+$ with no detectable P1 (**B, B'**; arrows indicate cells with low P1 levels) or mostly mature plasmatocytes, i.e., GFP$^+$ P1$^+$ **(C-C')**. **(D-E')** Stack projection of the surface layer of a wild-type LG expressing Hml>GFP (green) and immunostained for Lz (red). Crystal cells (Lz$^+$) typically have little or no GFP, suggesting that the IP cells in A-C' are not crystal cells. **(F-I)** LGs expressing P[Hml-Gal4] with or without P[UAS-Tig], containing the CZ marker Hml-dsRed (green) and the MZ marker Dome-EBFP (red), and stained for P1 (white). Hml>Tig expands the population of Hml-dsRed$^+$ P1$^-$ cells, which also contain intermediate levels of Dome-EBFP, consistent with these IP cells being in transition from a MZ to CZ identity. Bars = 25 µm. All experiments were performed at 29˚C.

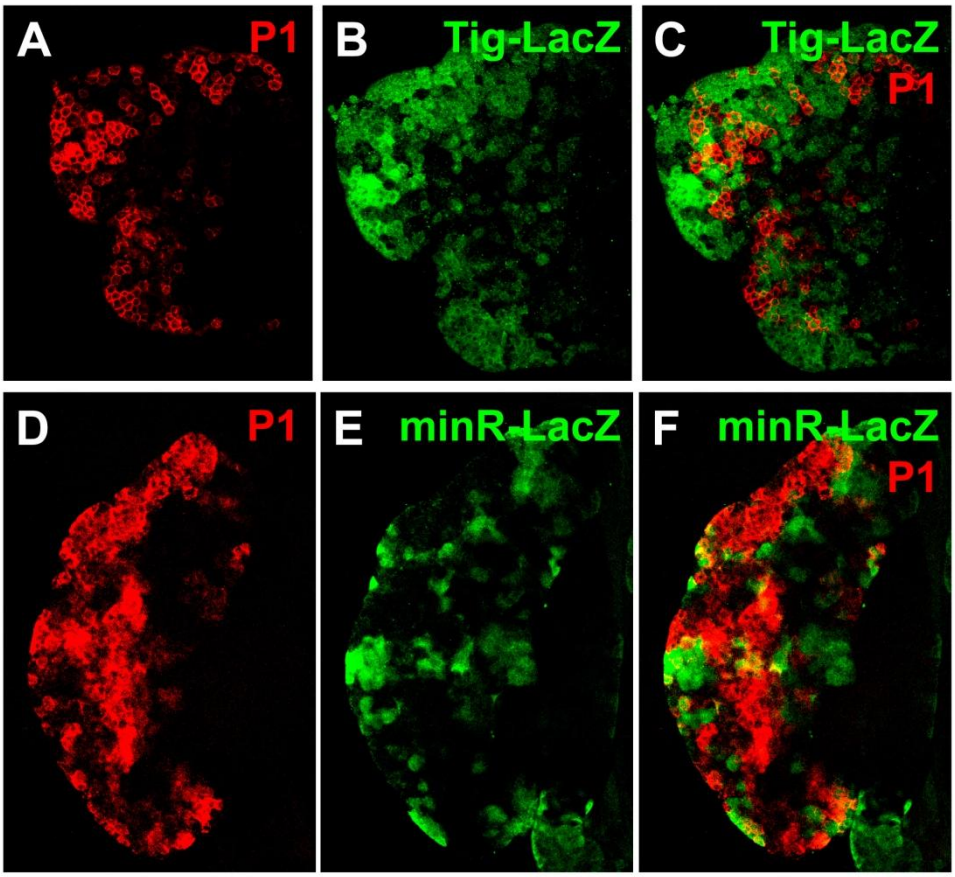


Figure 3.9. Mature plasmatocytes are often found in cells expressing lower levels of Tig transcriptional reporters. Confocal images of mid/late 3rd instar wild-type larvae expressing Tig LacZ or minR-LacZ and immunostained for the plasmatocyte marker P1. Many $P1^{+}$ cells do not express Tig-lacZ **(A-C).** The degree of non-overlap is even more obvious between P1 and minR-lacZ **(D-F).** Experiments were performed at 25˚C.

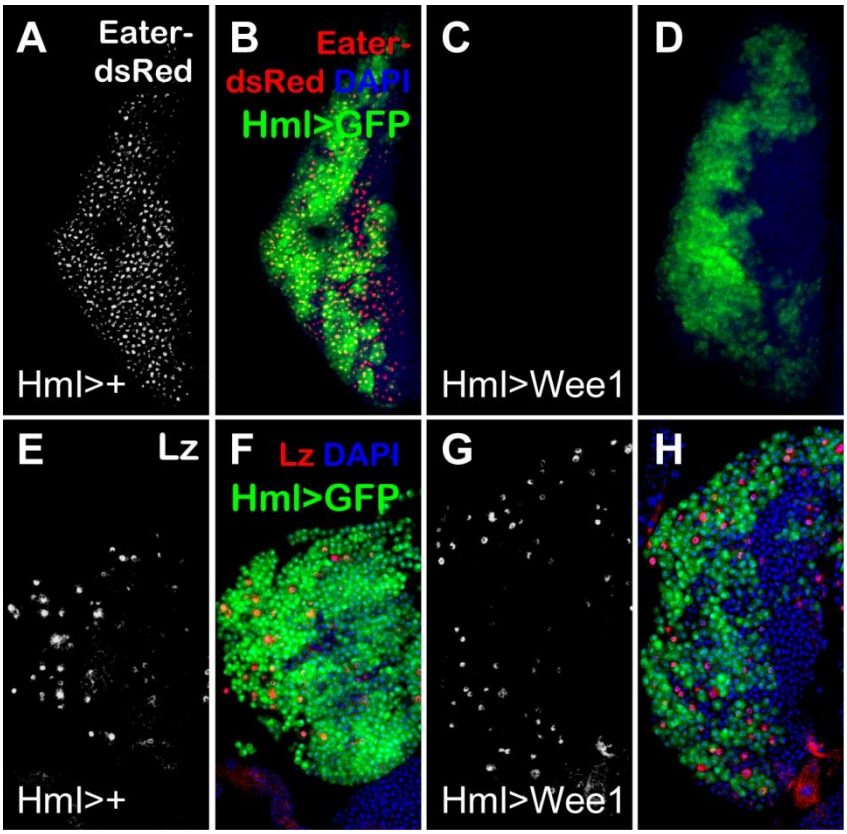


Figure 3.10. Inhibition of the G_2/M transition blocks plasmatocyte differentiation. Confocal images of LGs from mid/late 3rd instar larvae containing P[Hml-Gal4], P[UAS-GFP] with or without P[UAS-Wee1] labeled with different cell fate markers. In Hml>Wee1 LGs, the plasmatocyte marker Eater-dsRed **(A-D)** are strongly repressed, while the crystal cell marker Lz **(E-H)** displays no consistent difference. Experiments were performed at 29˚C.

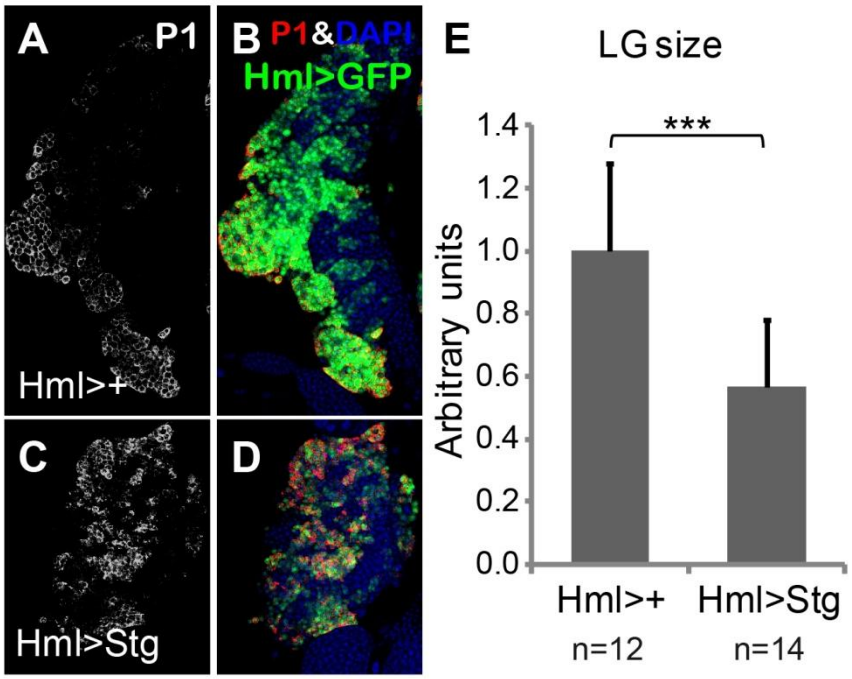


Figure 3.11. Acceleration of the G_2/M transition promotes plasmatocyte differentiation. **(A-D)** Mid/late 3rd instar LGs containing P[Hml-Gal4], P[UAS-GFP] with or without P[UAS-Stg] and immunostained for P1. In Hml>Stg LGs, the vast majority of Hml>GFP$^+$ cells are also P1$^+$, and the IP population (GFP$^+$, low/no P1) is greatly reduced. **(E)** ImageJ quantification confirms that the LG size is greatly reduced in Hml>Stg LGs. Experiments were performed at 29˚C. ***: p<0.001.

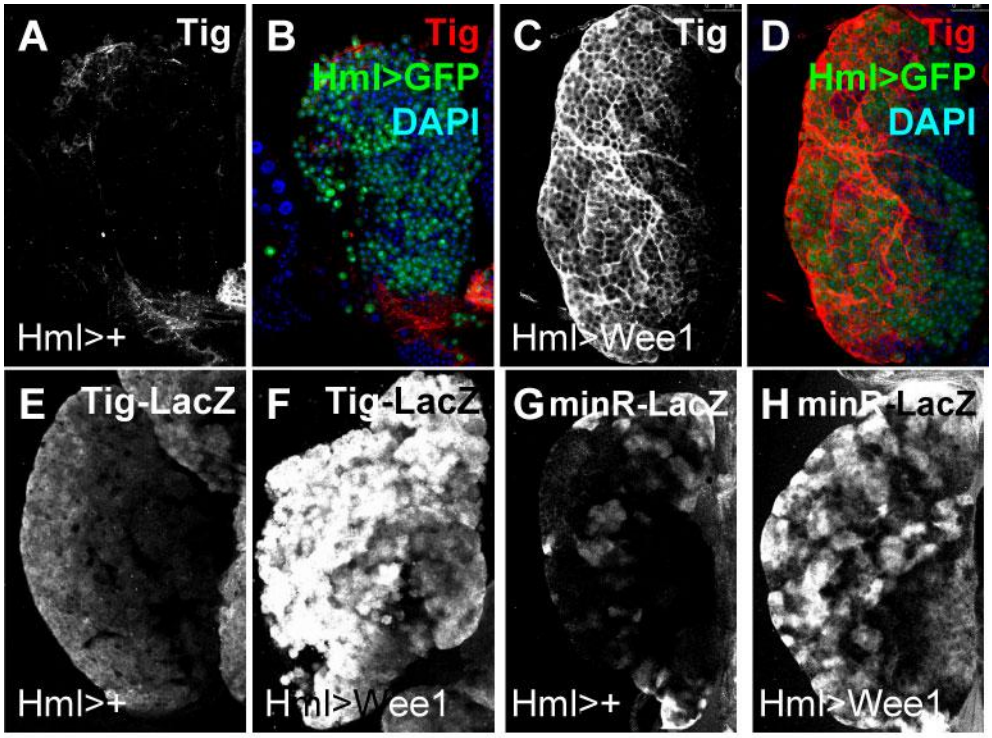


Figure 3.12. Wee1 activates Tig through transcription. Confocal images of LGs from mid/late 3rd instar larvae containing P[Hml-Gal4] and P[UAS-GFP] with or without P[UAS-Wee1]. Wee1 induces strong activation of Tig protein (compare **A** with **C**) in 50% of LGs examined. For Tig-LacZ, strong Wee1 dependent activation of the reporter (compare **E** with **G**) was observed in 75% of LGs. For minR-LacZ, strong induction (compare G with H) was observed in all LGs. Experiments were performed at 29˚C.

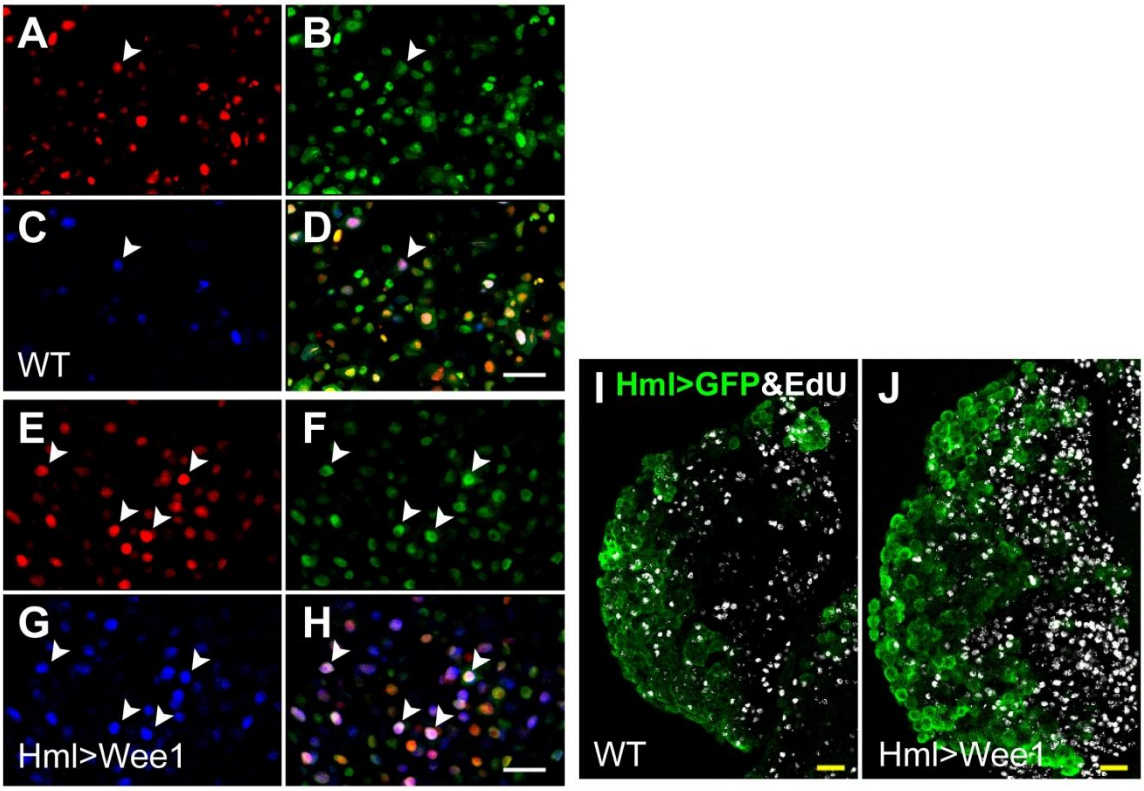


Figure 3.13. CZ-expression of Wee1 arrests CZ cells at G_2/M transition and induces MZ cell proliferation. Confocal images of mid/late-3rd instar larval LGs containing P[Hml-Gal4] and P[UAS-GFP] with or without P[UAS-Wee1] and labeled with the P[UAS-RGB] cell cycle stage reporter **(A-H)** or EdU **(I-J)**. The red, green and blue signals are from fluorescent proteins CycB$^{1-96}$-CycB$^{1-285}$-tdTomato, EGFP-PCNA and Cdt1$^{1-101}$- EBFP, respectively. There is an increased number of purple (red^{+}, green^{+}, blue^{+}) cells in Hml>Wee1 than control LGs (arrowheads in **A-H**), demonstrating that many cells in Hml>Wee1 are arrested at G_2 (Handke et al., 2014). **(I-J)** More S-phase cells (EdU^{+}) are found in the MZ (Hml>GFP^{-}) of Hml>Wee1 LGs.

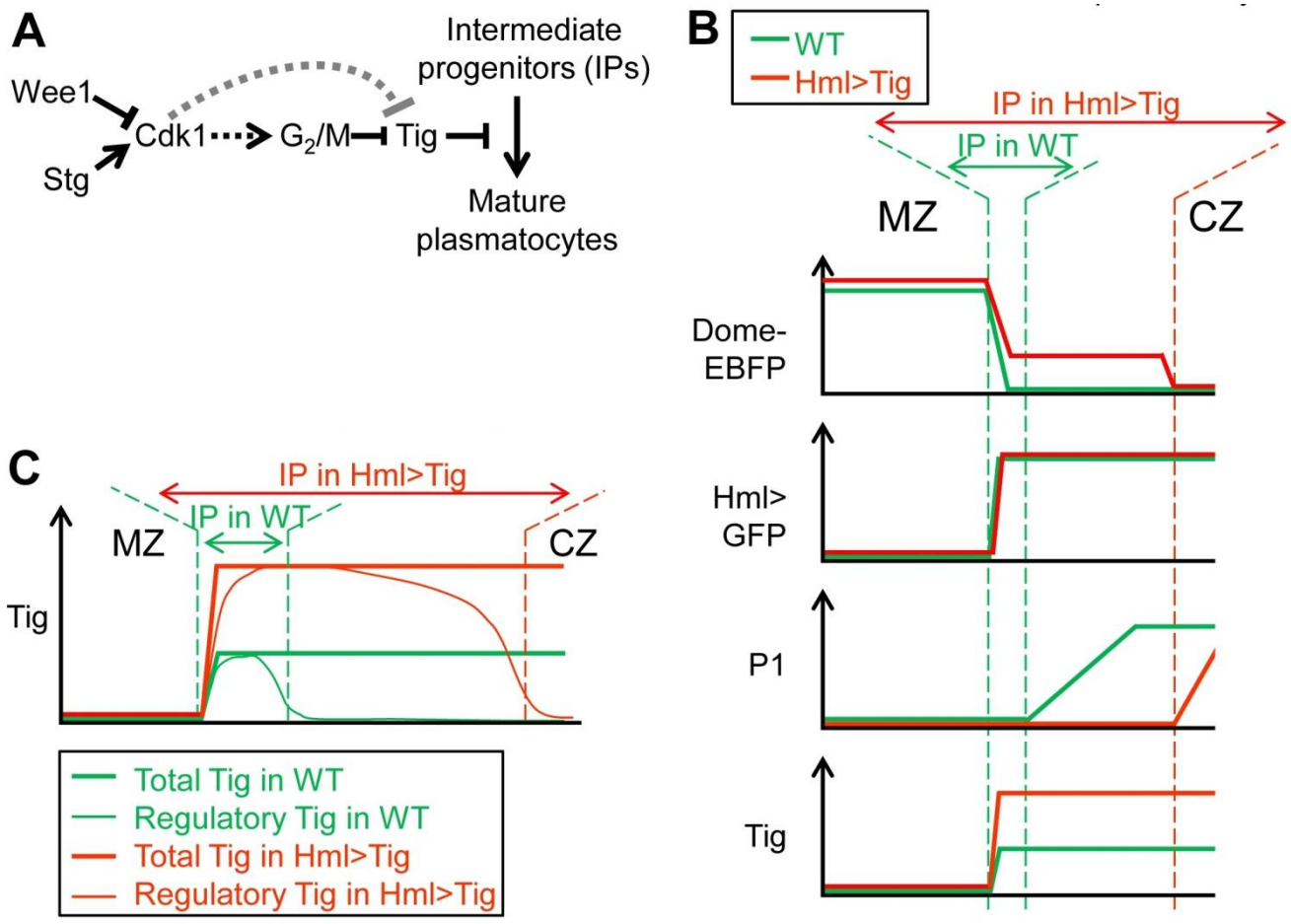


Figure 3.14. Working model of the regulation of plasmatocyte maturation by Tig and cell cycle regulators. **(A)** Genetic pathway controlling plasmatocyte differentiation. Tig and Wee1 inhibit the transition from IPs to plasmatocytes, while Stg accelerates it. Tig is epistatic to Wee1 and Wee1 activates Tig expression. Wee1 and Stg could affect Tig though their common target Cdk1. These cell cycle regulators may affect Tig expression by altering the G_2/M transition; alternatively, they could act on Tig transcription independent of the cell cycle. **(B)** Summary of the expression levels of different LG proteins across the MZ and CZ in wild-type and Hml>Tig LGs at the mid/late-3rd instar stage. There is a dramatic expansion of the domain containing IPs in Hml>Tig, i.e., cells that are $Dome^{+}/Hml^{+}/P1^{-}$. **(C)** A speculative model for a "regulatory" pool of Tig promoting the IP cell fate. Tig protein is detected at uniform levels throughout the CZ (Zhang et al., 2014), but Tig transcriptional reporters are expressed at the highest levels in cells with low P1, i.e., IPs and immature plasmatocytes. We propose that newly synthesized Tig protein forms the regulatory pool, which acts as a brake on plasmatocyte maturation, allowing the maintenance of the IP pool in the CZ. The cells actively expressing Tig is expanded in Hml>Tig, causing a concomitant expansion of IPs.

Experiment	Genotype	Offsprings without balancer	Offsprings with balancer	Percentage of escapers
GOF	♂Hml-Gal4, UAS-GFP × ♀UAS-Tig;+/SM5-Tm6	215	206	104
LOF 1	♂TigA1, UAS-Tig; +/SM5-TM6 × ♀TigX;+/SM5-TM6	5	248	4
LOF 2	♂TigA1;+/SM5-TM6 × ♀TigX;Hml-Gal4, UAS-GFP/SM5-TM6	0	224	0
Rescue	♂TigA1, UAS-Tig;+/SM5-TM6 × ♀TigX;Hml-Gal4, UAS-GFP/SM5-TM6	56	345	32.5

Table 3.1. CZ-expression of Tig partially rescues the pupal lethality of *Tig*$^{A1/X}$. Crosses were set up as indicated. Adult F1 flies were scored based on the Curly and Humeral markers on the SM5a-TM6B balancer chromosome. Data was combined from two to three vials for each cross. Experiments were performed at 25˚C, the same temperature as the LG rescue experiments where no detectable dominant effect was observed with Hml>Tig in an otherwise WT background (Figure 3.1, 3.8).

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Chapter IV:

Concluding Remarks

Summary of contributions

My thesis work covers two fields, gene regulation by Wnt/β-catenin signaling and *Drosophila* hematopoiesis. They are connected by the Tig gene, which is directly repressed by Wnt/β-catenin signaling in *Drosophila* cell culture and the larval hematopoietic system through non-traditional bipartite TCF binding sites, and regulates maturation of immune cells in the *Drosophila* larval lymph gland likely by responding to the cell cycle regulator Wee1. My thesis work provides an unusual example of direct repression of target gene expression by Wnt/β-catenin signaling, arguing that DNA sequence motifs are not just docking sites for transcription factors but can also profoundly influence their activity. Besides, my thesis work advances the understanding of the maturation of plasmatocytes in the LG, and elucidates connections between cell cycle regulators, the extracellular matrix and hematopoiesis.

Questions and future directions

Which other factors are involved in Wnt/TCF-mediated direct repression?

The current understanding of Wnt-mediated direct transcriptional repression (the reverse switch) is still at a starting point. Only two core factors, Arm and TCF, are known to be required for the repression. In addition, TCF is also required for the basal activation of targets of the reverse switch. However, it is still unclear whether there are other regulators of the Wnt-repressed W-CRMs and what transcriptional cofactors participate in this regulation.

GATA factors

GATA factors Serpent (Srp) and Pannier (Pnr) are important regulators of *Drosophila* hematopoiesis (Fossett et al., 2001; Mandal et al., 2004). Interestingly, the WGAWAWR sites look similar to GATA sites (Figure 4.1A) (Senger et al., 2004). This raised the possibility that GATA factors can also bind to the WGAWAWR sites. To test whether GATA factors regulates the Wnt-repressed targets, I knocked-down Srp in the whole LG using Srp>Srp RNAi. This caused a dramatic derepression of the minR reporter without any obvious change in the CZ size as marked by Cut (Figure 4.1B-G). Depression of minR and Tig reporters was also observed with Srp>Pnr RNAi, but the CZ size was not evaluated in those cases (data not shown). These data suggest that GATA factors counteract TCF in the CZ to repress the transcriptional reporters of reverse switch targets.

One important question is whether GATA factors regulate minR and Tig reporters through direct binding to the W-CRMs. Direct binding of GATA factors to the WGAWAWR sites can be tested in vitro using EMSA assay. Srp recombinant protein has been used in EMSA previously (Waltzer et al., 2002), but one need to test

the feasibility of making recombinant Pnr. If the GATA factors bind WGAWAWR sites in vitro, then one can use EMSA assays determine how GATA factors affects TCF-WGAWAW interaction (or vice versa), e.g. would GATAs and TCF compete for binding to the WGAWAW sites or would they collaborate? ChIP assay in cell culture will be needed to further establish this in a more endogenous context, but the experimental condition needs to be optimized as I found that Srp is necessary for the proper growth and survival of *Drosophila* Kc-167 cells.

Besides, the effect of GATA factors on *Tig* and *Ugt36Bc* expression need to be better established. Gain- and loss- of function of GATA factors will be carried out using Gal4 drivers specific for the MZ and CZ, and the expression of minR, Tig and Ugt36Bc reporters as well as the transcripts of *Tig* and *Ugt36Bc* will be assayed. Since both Srp and Pnr have different splicing isoforms that play similar or different roles depending on the context, it would be interesting to know how these isoforms affect the reverse switch targets. To reduce the contribution of indirect regulation through TCF, conditions where the TCF level stays similar to the WT will be favored. The state of plasmatocyte differentiation can also be examined upon manipulation of GATA factors.

It has been proposed that degenerate TF binding sites allow recognition by multiple TFs and more specific regulation (Vuong et al., 2015). Exploring the role of GATA factors in the regulation of reverse switch targets will not only promote our understanding of the reverse switch mechanism, but also provide good examples of how the degenerate WGAWAWR sites integrate inputs of multiple TFs.

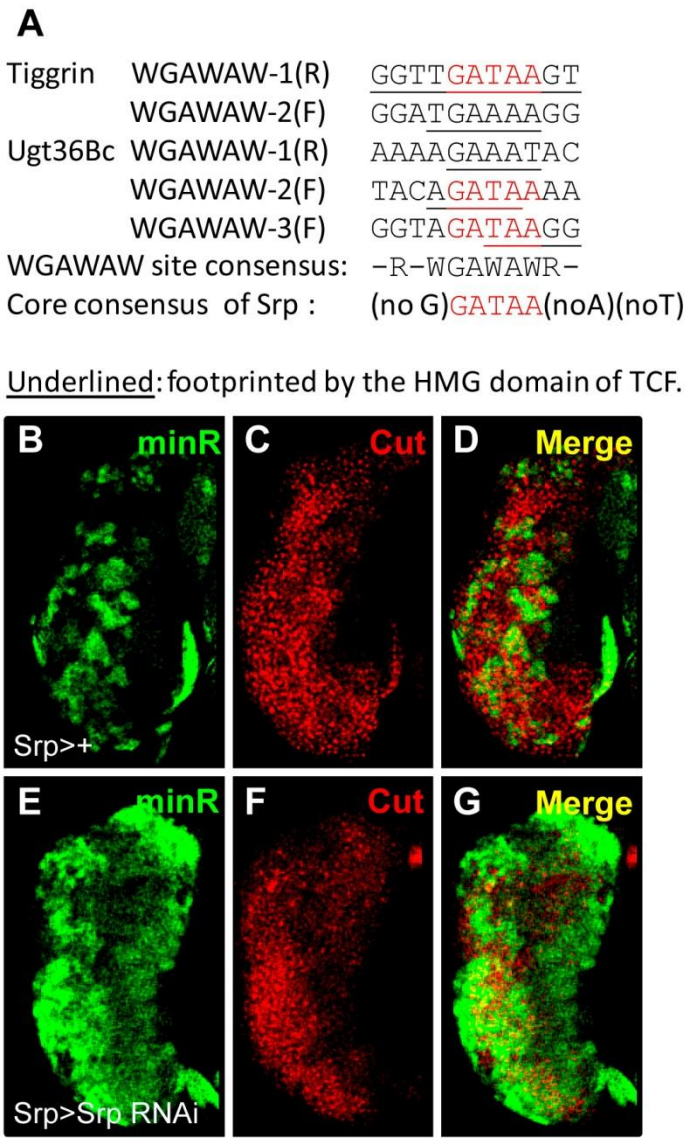


Figure 4.1. The GATA factor Srp is a potential repressor of Tig. (A) WGAWAWR sites are consistent with the GATA consensus. **(B-G)** Knock-down of Srp derepresses minR expression. Mid/late 3rd instar LGs containing P[Srp-Gal4], P[minR-LacZ] with or without P[UAS-SrpRNAi] are immunostained for LacZ (green) and the CZ marker Cut (red). In Srp>Srp RNAi LGs, there is no obvious change of the CZ size, but the LacZ reporter is expressed at higher level in the overall LG with the highest signal intensity in the CZ.

Screening for other transcriptional regulators of the reverse switch

While many cofactors have been characterized for Wnt-activated targets (Chapter I), what cofactors are used for the reverse switch remain unknown. Several specific attempts did not lead to interesting candidates. For example, my preliminary data showed that the chromatin methylation mark H3K27me3 is unregulated at the Tig locus upon Wnt activation (Axin RNA in Kc-167 cells), but inhibition of the major enzyme depositing this modification, Enhancer of Zeste (E(z)), reduced Tig transcription and reporter activity. Besides, inhibition of the common co-repressors Gro and CtBP in Kc-167 cells increases Tig reporter activity, but these effects are independent of TCF and activity of the Wnt pathway (data not shown).

Candidate-based screening can be carried out in both cell culture and the LG to search for more regulators of the reverse switch targets. Gene categories such as chromatin regulators, transcription factors and cofactors, proteins that interact with TCF or Arm will be prioritized for screening. RNAi libraries can be used in primary screening to allow broad and convenient analysis, and the minR reporter will be used because of its simplicity. Interesting candidates will be confirmed using standard genetic approaches such as overexpression, mutant analysis and complementation test; more readout such as reporters and transcripts of *Tig* and *Ugt36Bc* will be also examined. It is important to validate the functions of candidate genes in the LG. Depending on the nature of the candidate gene, further investigations may include their interactions with TCF/Arm, recruitment to the chromatin, and effect on chromatin modification and structure.

Is Wnt/TCF-mediated direct repression a general and important mechanism for Wnt-regulation?

The Wnt/TCF-mediated direct repression (reverse switch) was first found in *Drosophila* cell culture, and now has been extended to the *Drosophila* hematopoietic system. But the impact of this mechanism in other contexts has not been extensively investigated.

Improve the current understanding of repressive TCF binding sites

One reason to study individual W-CRMs is for the hope of summarizing the binding site consensus and applying it to target search in the genome. The current consensus of r-Helper is preliminary: it is only summarized from three binding sites and the consensus is degenerate (KCCSSNWW, K=T/G, S=G/C, W=A/T; Figure 2.4C). A SELEX type of experiment could potentially detect high affinity r-Helper sites in vitro. Recombinant HMG-C-clamp protein will be used to select high affinity DNA probes from a library containing a high quality WGAWAWR site fixed in the center of the probe. Sequences flanking the WGAWAWR site from enriched probes will likely represent a high quality r-Helper site. If results look promising, SELEX-seq can be used to survey probes enriched for less rounds to collect lower affinity sequences and get a more comprehensive consensus of r-Helper (Slattery et al., 2011). To avoid (or reduce) the selection of activating HMG sites, the probes can be pre-cleaned using HMG-only (and then C-clamp-only, if needed) recombinant protein fragments before used in the SELEX experiments.

Are there more targets of the reverse switch in Drosophila?

While the consensuses of repressive TCF sites so far are summarized from only two genes, my preliminary data indicate that they are slightly enriched in TCF ChIP-seq peaks from embryos, suggesting that these DNA sequences might help to recruit TCF in vitro. Using an algorithm developed from the lab, I searched for pairs of WGAWAW sites and r-Helpers in embryonic TCF ChIP-seq data (Archbold et al., 2014; Hallikas et al., 2006). Regardless of the orientation of binding site pairs, I observed a 2-fold enrichment of these sites in TCF peaks compared to randomly picked intronic and intergenic regions (data not shown). This algorithm has been used to successfully identify several Wnt-activated targets. The next step for Wnt-repressed targets is to raise the stringency (and use the most comprehensive binding site consensus) as see if there are interesting returns, e.g. conserved clusters of hits that aligns well with known repressive TCF sites.

Is the reverse switch mechanism also used in the vertebrate system?

To test if the reverse switch also functions in the vertebrate system, minR expression can be examined in different contexts, i.e. cell lines and different stages in transgenic mice. But it might be hard to find the right context where minR displays a basal activity.

As a general approach, combination of TCF ChIP-seq and RNA-seq data will help to narrow down direct targets that are repressed by Wnt signaling. For example, TCF ChIP-seq comparing WT and C-clamp mutated TCFs combined with nascent RNA-seq has been done in colorectal cancer cells (Hoverter et al., 2014). Interestingly, this study has identified TCF ChIP-seq peaks that are dependent on

C-clamp function but do not contain classic HMG sites. It would be interesting to search for the repressive TCF sites in these peaks.

There is other specific information in the literature that might help to further narrow down the search. In a study looking at TCF4 and GATA-3 targets in MCF-7 cells, the authors did RNA-seq with or without siRNA treatments of TCF4 or GATA-3 and ChIP-seq of both factors in addition to several chromatin marks (Frietze et al., 2012). Genes that are inhibited by TCF4 siRNA and are activated by GATA-3 siRNA are consistent with my observation that minR is depressed by Srp or Pnr RNAi in the *Drosophila* LG. Interestingly, peaks co-bound by TCF4 and GATA-3 in MCF-7 cells lack classic TCF sites. This was interpreted as TCF4 is recruited by GATA-3 to the chromatin, however, it is also possible that TCF4 recognizes untraditional WGAWAWR-like sequences that look similar to GATA sites. To test this, genes of the interesting group will be confirmed by RT-Q-PCR first. Those that are indeed activated by TCF4 and repressed by GATA-3 will be analyzed for tentative W-CRMs based on whether repressive TCF sites are found in the ChIP-seq peaks.

Is Wnt-mediated repression of Tig important and general for Wnt-regulation?

Wg signaling has been shown to promote MZ maintenance in the LG (Sinenko et al., 2009). However, several Wnt-activated reporters, synthetic or containing endogenous W-CRMs from *Naked* and *Notum* that are active in many other tissues, exhibit no detectable activity in the LG (Zhang et al., 2014). Is the reverse switch mechanism an important mediator of Wg's role in MZ maintenance? If Tig loss-of-function inhibits the MZ premature differentiation phenotype caused by

reduced Wg signaling, or if over-expression of Tig in the MZ promotes the pool of IP cells (expressing both MZ and CZ markers), it would argue that keeping Tig at low level is necessary for MZ maintenance. To avoid artificial high expression of Tig, one can also express dominant-negative TCF in the MZ as it brings Wg-repressed targets back to the expression level in the absence of Wg signaling in *Drosophila* cell culture (Blauwkamp et al., 2008 and my unpublished observation).

While it is possible that the MZ cell fate is sensitive to Tig expression level, Tig might not be the sole mediator of Wg function. This is because over-expression of Wg in the MZ reduces the CZ size, but there is no detectable Tig expression in the MZ to be further repressed by Wg (Sinenko et al., 2009). This suggests that Wg either activate targets through an uncommon mechanism, or regulates MZ cell fate beyond the transcriptional level.

Is the reverse switch mechanism used outside of the hematopoietic system? LacZ staining of minR and Tig reporters in 3rd instar stage larvae did not reveal obvious expression in other tissues except for the fat body (Zhang et al., 2014). However, Tig mRNA is detected in the imaginal discs, CNS and adult head, according to the modENCODE project (http://flybase.org/cgi-bin/gbrowse2/dmel/?Search=1;name=FBgn0011722). It is possible that Tig is regulated through unknown W-CRMs in these tissues. To test this, in situ or RT-PCR will be used to confirm Tig transcription. If Tig is indeed expressed, the next step is to test if known factors such as TCF and other mediators of the Wg pathway, GATA factors and Wee1 regulate Tig expression in these other

tissues.

How can Tig advance the current understanding of *Drosophila* hematopoiesis?

Tig over-expression allows analysis of the intermediate cell population

The transitory stage of cells is often difficult to study due to the rapid and continuous shift of cell fate in their small population (Zhao et al., 2016). I have found that overexpression of Tig in the CZ greatly enrich the IP cells, which provides a valuable chance to analyze this population. For example, expression profiling of the IP pool can be done in comparison to the pool of mature plasmatocytes. LGs expressing Hml>GFP with or without over-expression of Tig will be sorted for Hml>GFP+ cells via FACS and processed for RNA-seq. Including Eater-dsRed in the gating will help to further distinguish mature plasmatocytes, although the sorting might need to be further optimized for this additional channel.

Since Hml>Tig is not lethal, it can be temporarily kept and used for a secondary suppressor screening using RNAi stocks to verify repressors of the mature plasmatocyte cell fate functioning downstream of Tig. Otherwise, standard genetics approaches can be used to validate the function of interesting genes. Many genes might be differentially expressed between different cell fates. I will not focus on genes that look like the terminal effectors of the maturation process, e.g. factors involved in the phagocytosis pathway or in anti-microbial peptide synthesis. Instead, I will analyze potential regulators of the cell fate, such as transcriptional factors and cofactors, kinases and mediators of signaling pathways. One group of particular

interesting genes is transmembrane proteins and receptors, as it is still unclear how the level of the extra-cellular matrix protein Tig is sensed.

The role of Tig in other Drosophila hematopoietic tissues

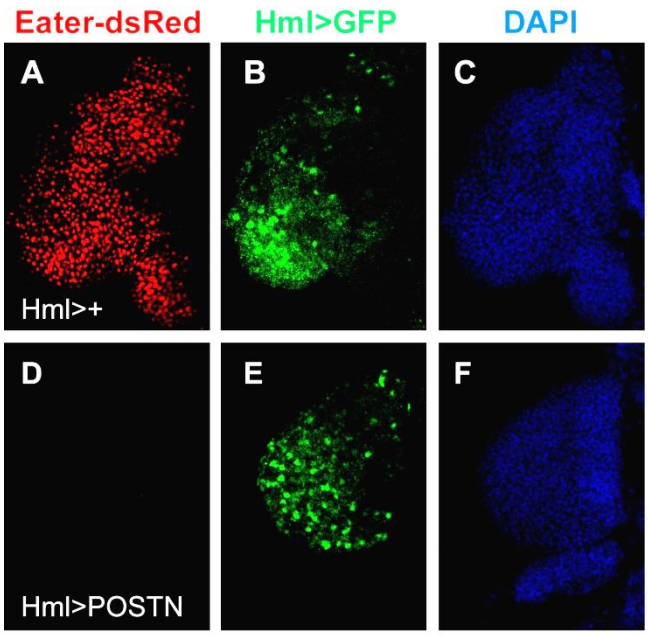
My data so far has been focused on plasmatocyte maturation in the LG, but are the regulatory mechanisms shared in the other hematopoietic tissues in *Drosophila*? This remains as an open question since many factors are expressed in both the LG cells and embryonic hemocytes but their roles could be general or tissue-specific (Evans et al., 2014). Tig mRNA and reporters are detected in embryonic hemocytes and larval circulating hemocytes (Blauwkamp et al., 2008; Zhang et al., 2014). In fact, the number of circulating hemocytes is reduced by half in Tig mutants, suggesting that Tig might be a positive regulator of embryonic hemocyte proliferation (data not shown). To test this possibility, the S-phase and mitotic indexes of the embryonic hemocytes from WT or Tig mutant animals will be compared at different developmental stages. There has not been an established IP population in circulating hemocytes, and it would be very interesting to examine whether over-expression of Tig or Wee1 will also cause an expansion of circulating hemocytes expressing no mature markers.

Is POSTN a functional counterpart of Tig in the vertebrate system?

In Chapter III, Tig has been characterized as an important regulator of *Drosophila* hematopoiesis. Can we apply the knowledge of Tig function to the vertebrate system? BLAST and conservation search did not reveal obvious

homolog of Tig outside of Diptera (blast.ncbi.nlm.nih.gov/; https://genome.ucsc.edu/). However, literature digging led us to a mammalian protein, Periostin (POSTN). POSTN has been connected to interesting biological functions such as cardiac repair and recruitment of tumor-associated macrophages from peripheral blood (Kuhn et al., 2007; Zhou et al., 2015). Similar as Tig, POSTN is an extra-cellular matrix protein that can promote cell proliferation in multiple cell types and is involved in cell adhesion and migration at least partially through functioning as an intergrin ligand (Gillan et al., 2002; Baril et al., 2007; Padial-Molina et al., 2014; Ishikawa et al., 2014; Taniguchi et al., 2014). Importantly, two short stretches of amino acids on Tig aligns with one region in the C-terminus of POSTN.

To test if POSTN functions as a mammalian counterpart of Tig, I first tested if POSTN can mimic the role of Tig in the *Drosophila* LG. Surprisingly, my preliminary data show that overexpression of POSTN in the CZ represses the mature plasmatocyte marker Eater-dsRed as strongly as overexpression of Tig (Figure 4.2). The next step is to test if the C-terminal region of POSTN is required for this regulation. There is an ETLK motif in the C-terminal region of POSTN that is aligned to the two Tig stretches aligned to POSTN, making it an interesting and surgical target of mutagenesis. The functional importance of the ETLK motif can be tested in either the *Drosophila* LG or mammalian cell culture, as assays have been established in cells such as human periodontal ligament fibroblasts and HEK 293T cells to examine the role of POSTN in cell proliferation, adhesion and migration.



G

Genotype \ Reporter	Eater-dsRed	
	High	Low
Hml>+	8	2
Hml>POSTN	2	12

Figure 4.2. Human Periostin represses Eater-dsRed in the *Drosophila* LG. Mid/late 3rd instar LGs containing P[Hml-Gal4], P[UAS-GFP], P[Eater-dsRed] with or without P[UAS-POSTN]. **(A-F)** Hml>POSTN strongly represses Eater-dsRed. **(G)** Summary of the phenotype. Experiments were carried out at 29 ℃.

If Tig and POSTN indeed share functional similarities through the ETLK motif in these contexts, it would be interesting to predict the tertiary structure of both proteins (the aligned regions) using softwares such as PHYRE2 and CPHModels to see if they also share structural properties. Although rare, it has been reported that proteins with limited similarity in their primary sequences could adapt similar structures and functions in different organisms (Kidd AR 3rd et al., 2005; unpublished data from Matthew Chapman's lab). If this is the case for Tig and POSTN, it would provide a dramatic example of how a *Drosophila* protein helps to understand the molecular function of its mammalian counterpart.

Materials and Methods

Fly Stocks used in chapter IV: UAS-Srp RNAi: BDSC #35813; UAS-Pnr RNAi: BDSC #28935, #33697; UAS-POSTN: the line used in Figure 4.2 is M02#3, a P-element insertion; Hml>GFP: BDSC #30140.

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