GENETIC AND MOLECULAR CHARACTERIZATION OF ISWI FAMILY CHROMATIN REMODELERS IN WINGLESS SIGNALING

by

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如入火聚，得清凉门。

Walking through the burning pyre
To reach the gateway of soothing coolness

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ii

LIST OF FIGURES...ix

CHAPTER I: GENERAL INTRODUCTION..1

ATP-dependent chromatin remodelers 2

ISWI family chromatin remodelers in transcription 6

Components of ISWI family complexes.......................................6

The *in vivo* functions of ISWI family complexes..........................9

Mechanisms of ISWI family complexes in chromatin remodeling... 14

Wnt/β-catenin signaling 20

Wnts in development and disease...20

Wnt/β-catenin signaling...23

Transcriptional repression in Wnt signaling..26

TCFs as transcriptional repressors...26

(a) TCFs as repressors 26

(b) Derepressable Wnt targets...29

Other transcriptional repressors in Wnt signaling........................33

(a) Factors that associate/modify TCF 33

(b) Factors that buffer β-cat/TCF binding 36

(c) Factors that bind to DNA 39

Transcriptional regulation of Wnt targets in chromatin 41

Summary of my work in this dissertation.. 43

References.. 46

CHAPTER II: TOUTATIS AND ACF1, TWO RELATED CHROMATIN REMODELERS, REDUNDANTLY ANTAGONIZE WINGLESS SIGNALING IN *DROSOPHILA* 63

Abstract... 63

Introduction... 64

Results 67

A misexpression screen in the *Drosophila* eye designed to identify antagonists of Wg signaling 67

Overexpression of Toutatis (Tou), a chromatin remodeling protein, blocks Wg signaling.. 69

tou null mutant reveals no obvious defect in Wg signaling 75

Double mutant of *tou* and its potential redundant gene *acf1* reveals no obvious defect in Wg signaling.. 79

Tou and ACF1 act redundantly to repress Wnt targets in fly and human cell lines 82

Discussion.. 85

The role of Tou and ACF1 in flies 85

The role of Tou and ACF1 in fly and human cells....................... 87

Mechanism of Tou and ACF1’s action....................................... 88

Materials and methods... 90

Drosophila strains... 90

Generation of *tou* and *acf1* deletions, and *tou*^{RNAi} lines 90

Immunostaining 91

Drosophila cell culture 92

Quantitative real-time PCR (q-PCR) 93

Human cell culture 93

Acknowledgements 94

References 95

CHAPTER III: THE CHROMATIN REMODELERS ISWI AND ACF1 DIRECTLY REPRESS WINGLESS TRANSCRIPTIONAL TARGETS 101

Abstract 101

Introduction 102

Results 106

ISWI/ACF1 represses wg targets in the absence of signaling in *Drosophila* cultured cells 106

ISWI specifically represses Wg targets in the developing wing. 109

ACF1 binds to broad regions of several Wg transcriptional targets 113

ISWI/ACF1 represses Wg targets independent of chromatin assembly 118

Wg signaling reduces ACF1's binding to Wg targets 120

Discussion 122

ISWI/ACF1 transcriptionally represses Wg target genes 122

Mechanism of ISWI/ACF1 regulation of Wg signaling 124

Regulation of ISWI/ACF1 by Wg signaling 126

Materials and methods 127

Drosophila genetics 127

Antibodies, immunoblot and immunostaining 128

Drosophila cell culture 129

Real-time quantitative PCR (Q-PCR) 130

Chromatin immunoprecipitation (ChIP) 130

Acknowledgements 131

References 132

CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS 138

ISWI/ACF1 specifically repress Wg targets 138

Widespread binding of ACF1 to Wg targets 141

Different activities between ISWI and ACF1 145

Action of ISWI/ACF1 with other repressors 148

Future perspectives 150

References 151

LIST OF FIGURES

Figure

Figure 1.1 Domain structure of four classes of ATPases in ATP-dependent chromatin remodeling complexes....4

Figure 1.2 Summery of components in ISWI-type chromatin remodeling complexes....7

Figure 1.3 Outline of the Wnt/β-catenin pathway....24

Figure 1.4 Summary of factors that repress Wnt targets expression in the nucleus 34

Figure 2.1 Misexpression of *tou* (via the A145 insertion) suppresses a Wg signaling-dependent small eye phenotype....68

Figure 2.2 Overexpression of *tou* (via the A428 insertion) blocks Wg signaling in the wing....71

Figure 2.3 All four GSV lines induce *tou* expression....72

Figure 2.4 Diagram of Tou and its related protein ACF1, and their human homologues.74

Figure 2.5 Depiction of two isoforms of *tou* and *tou* mutants....76

Figure 2.6 Depiction of *acf1* mutant....80

Figure 2.7 Depletion of *tou* and *acf1* activate Wg targets in fly and human cells....83

Figure 3.1 ISWI/ACF1 represses Wg targets in the absence of Wg signaling....108

Figure 3.2 Loss of *iswi* results in an expansion/derepression of Wg targets in wing imaginal discs....110

Figure 3.3 *iswi* mutant cells do not affect several non-Wg targets in wing imaginal discs 112

Figure 3.4 ACF1 binds to the broad regions of the *nkd* and *hth* genes 114

Figure 3.5 TCF preferentially binds to a specific site in the *hth* gene 116

Figure 3.6 ACF1 binds to *Notum* in a similar fashion as to *nkd* 117

Figure 3.7 ISWI/ACF1 represses Wg targets independent of post-mitotic chromatin assembly 119

Figure 3.8 ACF1 binding to Wg targets is modestly reduced by Wg signaling 121

Figure 4.1 Model of ISWI/ACF1's action in Wnt signaling 143

Figure 4.2 ISWI preferentially binds to the site where TCF binds in the *hth* locus........ 147

Figure 4.3 Ttk represses *nkd* expression and facilitates CtBP/TCF binding to *nkd*........ 149

CHAPTER I

GENERAL INTRODUCTION

In eukaryotic cells, DNA is packaged into a compact chromatin structure. The basic unit of chromatin, the nucleosome, comprises 146bp DNA wrapped around an octamer of four different core histones H2A, H2B, H3 and H4, with two copies for each type. Between nucleosomes are short linker DNAs where histone H1 binds. The crystal structure of the nucleosome core has been resolved, revealing that each histone has a short N-terminal domain (~15-40 amino acid) that protrudes from the core structure (Luger et al., 1997), making it accessible for a variety of post-translational modifications. Arrays of nucleosomes fold into a 30nm chromatin fiber, which is further condensed at least several hundred fold to form a higher-order chromosome structure (Tremethick, 2007). In humans, about 2 meters of DNA is eventually folded to fit into a nucleus about 10μm in diameter.

Intuitively, such a compact chromatin structure presents a formidable barrier to all processes involved in protein-DNA interactions, notably regulation of gene expression. The central question is how does chromatin serve as a DNA template that is readily

accessible for protein binding, while at the same time still maintaining a condensed genome in the nucleus?

In the field of transcription, the prevailing model is that chromatin structure undergoes dynamic alterations at the gene loci where transcription occurs to accommodate effective protein-DNA interactions. Two major classes of highly conserved factors are involved in this process. One class of factors covalently modify the amino acids in histone tails, including the acetylation of lysines, the methylation of lysines and arginines, the phosphorylation of serines and threonines, and the ubiquitination of lysines (Khorasanizadeh, 2004). Specific modifications have been correlated with distinct transcriptional events. For example, histone deacetylation is generally connected to transcriptional repression (Gallinari et al., 2007), while trimethylation of histone H3 at lysine 4 (H3K4me3) renders an almost universal marker for transcriptional activation (Santos-Rosa et al., 2002). It was hypothesized that the combination of multiple histone modifications presents a "histone code" for a specific transcriptional event (Strahl and Allis, 2000). Enormous effort has been spent on understanding how such a code is created and interpreted by various regulatory proteins to bring about downstream transcriptional events (Kouzarides, 2007).

ATP-dependent chromatin remodelers

In addition to enzymes that modify chromatin, a second class of factors, often referred to as chromatin remodelers, physically alter the position or/and structure of

nucleosomes by hydrolyzing ATP. These chromatin remodelers have been found to act in multi-subunit complexes, each of them containing a core enzyme with ATPase activity. These enzymes belong to the helicase superfamily 2 (SF2), as they harbor a characteristic ATPase domain with seven helicase motifs of the SF2 superfamily (Caruthers and McKay, 2002). However, they are different from the typical helicases since they do not have the ability to unwind the DNA duplex (Cote et al., 1998). Rather, they have been shown to catalyze the mobilization of nucleosomes around DNA in an ATP-dependent manner, either by translocating the DNA or relocating the histone octamer (Smith and Peterson, 2005).

Based on the additional motifs these enzymes contain, they are divided into at least four families: SWI/SNF family proteins harbor a bromodomain, ISWI family proteins contain SANT and SLIDE domains, CHD family members share a chromodomain, and INO80 family members have split ATPase domains (Fig. 1.1). In the following section, I will discuss the function of these chromatin remodeling proteins in transcription, with a particular focus on ISWI family members. As will be demonstrated in a number of examples below, a concerted cooperation between histone modification and chromatin remodeling has been observed in many transcriptional events.

There are at least two distinct and highly conserved SWI/SNF-type chromatin remodeling complexes identified in yeast, flies and humans (reviewed in Martens and Winston, 2003; Peterson and Workman, 2000). Besides the core ATPase, these two

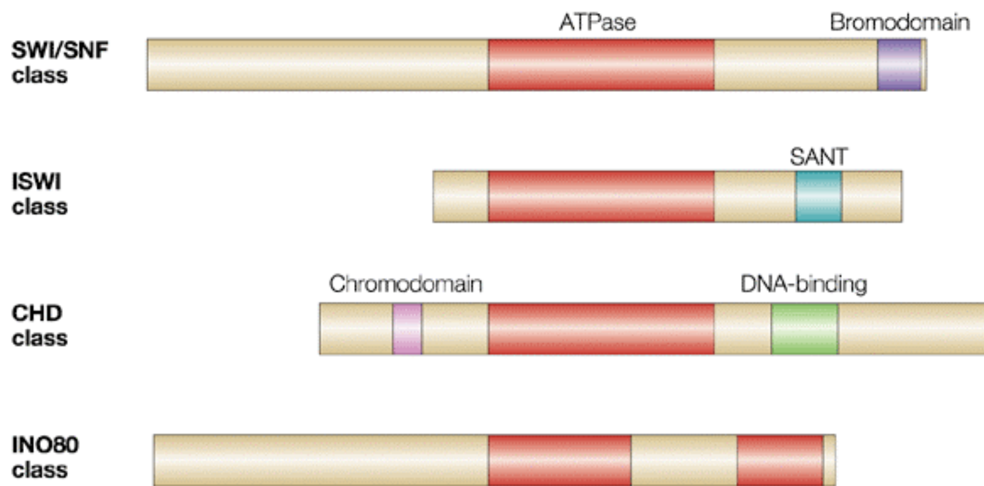

Figure 1.1. Domain structure of four classes of ATPases in ATP-dependent chromatin remodeling complexes. Besides the ATPase domain (shown in red), the first three classes of ATPases contain additional signature motifs such as the Bromodomain, the SANT domain, and the Chromodomain. INO80 contains characteristic split ATPase motifs. The cartoon is borrowed from a stellar review (Tsukiyama, 2002).

complexes contain overlapping as well as unique cofactors to achieve specific functions. SWI/SNF complexes are generally correlated to transcriptional activation, and they can do so by interacting with modified histones and basic transcriptional machinery (reviewed in Narlikar et al., 2002). One prominent example showed that through its bromodomain, yeast Swi/Snf can be recruited to an acetylated nucleosomal template and activate transcription (Hassan et al., 2002). In addition, there is convincing evidence supporting that Swi/Snf represses transcription. For example, it was shown that yeast Swi2/Snf2 binds to the enhancer of *SER3* gene and represses its activity (Martens and Winston, 2002).

Several CHD-type chromatin remodeling complexes have been purified from various organisms. All ATPases in the CHD family share a chromodomain. It was previously shown that the chromodomain in heterochromatin protein 1 (HP1) binds to histone H3 methylated at lysine 9, which is usually associated with silenced chromatin (Bannister et al., 2001). Interestingly, NURD, a CHD-type complex identified in higher eukaryotes, comprises histone deacetylases (HDACs) and methylated-DNA-binding proteins. Since both histone deacetylation and DNA methylation are correlated to transcriptional repression, and Mi-2, the ATPase in the complex, physically interacts with transcriptional repressors including Hunchback and Tramtrack in *Drosophila*, NURD provides a good example of coordinating gene silencing at multiple layers (reviewed in Tsukiyama, 2002).

INO80 protein contains a characteristic split ATPase domain, and how its

catalytic activity differs from other types of ATPases is unknown. Several research groups showed that SWR1, an INO80 type complex, can replace conventional histone 2A with a variant form H2A.Z., which is important to define the boundary between heterochromatin and euchromatin (Meneghini et al., 2003; Mizuguchi et al., 2004).

ISWI family chromatin remodelers in transcription

Components of ISWI family complexes

The core component in ISWI family complexes is the ATPase ISWI (Imitation Switch), which contains SANT and SLIDE domains at the C-terminus. Although there is no evidence that these domains can bind to modified histone tails as the bromodomain or chromodomain does, they are important for nucleosome recognition and remodeling *in vitro* (Grune et al., 2003). The ATPase domain of ISWI is highly related to that of SWI/SNF, yet is likely to bear an unique catalytic activity that makes ISWI remodel chromatin differently from SWI/SNF. Consistent with this notion, a report demonstrated that a chimeric BRG1 (a human SWI/SNF), with its ATPase domain replaced with that of SNF2H (a human ISWI), displays characteristic ISWI-like remodeling activity *in vitro* and fails to activate some BRG1-responsive genes *in vivo* (Fan et al., 2005).

Several ISWI-type complexes have been purified from *Drosophila* (Fig. 1.2). They are ACF (ATP-utilizing chromatin assembly and remodeling factor), CHRAC (chromatin accessibility complex) and NURF (nucleosome remodeling factor). As the

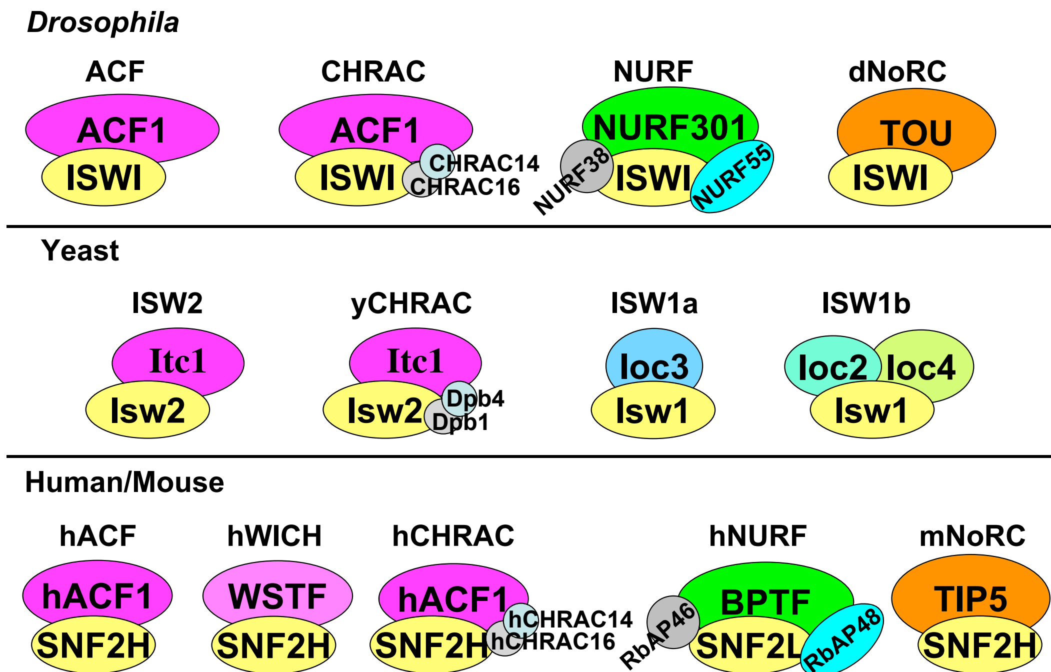

Figure 1.2. Summary of components in ISWI family chromatin remodeling complexes. ATPase ISWIs are well conserved from *Drosophila* to humans. While *Drosophila* has only one ISWI, yeast and mammals have two ISWI homologues, which are involved in distinct complexes. Many co-factors are conserved as well. Yeast Itc1 is the closest protein to ACF1, though it only shares N-terminal WAC and DDT motifs with ACF1 (Gelbart et al., 2001). Mammals have two ACF1 homologues, termed as hACF1 and WSTF respectively. Other complexes, such as CHRAC, NURF and NoRC, are also found in both *Drosophila* and mammals.

names imply, these complexes strongly catalyze chromatin assembly and nucleosome remodeling *in vitro.* Both ACF and CHRAC complexes contain ACF1, a protein with PHD fingers and a bromodomain at its C-terminus. The PHD fingers bind histones and are required for ACF to effectively mobilize nucleosomes *in vitro* (Eberharter et al., 2004). Besides ISWI and ACF1, CHRAC complex contains two additional histone-fold proteins CHRAC14 and CHRAC16, which facilitate ISWI/ACF1 mediated nucleosome sliding *in vitro* (Kukimoto et al., 2004). In the NURF complex, ISWI is associated with three other cofactors. The largest subunit, NURF301, shares structural domains with ACF1, and plays an important role in ISWI mediated nucleosome sliding (Xiao et al., 2001).

ISWI family complexes are highly conserved in eukaryotes (Fig. 1.2). There are two homologues of ISWI in yeast, Isw1 and Isw2. It appears that two complexes that Isw2 forms share certain similarity with fly ACF and CHRAC complexes (Gelbart et al., 2001; Iida and Araki, 2004). In mammals, two ISWI homologues, SNF2H and SNF2L, have been identified. Among several complexes that SNF2H forms are hACF, hCHRAC and hNURF (Barak et al., 2003; Poot et al., 2000). There exists a second ACF1 homologue in mammals called WSTF, which has been implicated in Williams syndrome, a developmental disorder (Lu et al., 1998). Another ISWI-type complex purified in mammals is NoRC (nucleolar remodeling complex), which contains TIP5 protein (also called BAZ2A) as a cofactor (Strohner et al., 2001). Interestingly, TIP5 is highly related to hACF1 and WSTF. Its fly homologue, Toutatis (Tou), has been shown

to physically interact with ISWI, indicating the existence of a similar complex in flies (Vanolst et al., 2005). In conclusion, ISWI is involved in multiple complexes that are highly conserved throughout eukaryotes, indicating their functional importance *in vivo*.

The *in vivo* functions of ISWI family complexes

ISWI-type complexes play important roles in a wide array of biological events including transcription, DNA replication, DNA repair and recombination (reviewed in Langst and Becker, 2001; Corona and Tamkun, 2004). For the purpose of my dissertation, I will focus on their roles in transcriptional regulation, especially transcriptional repression.

In yeast, Isw1 and Isw2 are not essential genes since *isw1/isw2* double mutant displays no evident phenotype at normal conditions (Tsukiyama et al., 1999). However, several reports strongly suggest that Isw1 and Isw2 are required for transcriptional repression in specific contexts. Tsukiyama and colleagues showed that Isw2 directly represses several early meiotic genes in cooperation with Rpd3-Sin3 histone deacetylase complex (Goldmark et al., 2000). Both Isw2 and Rpd3-Sin3 are recruited to target genes by DNA-binding protein Ume6. A later microarray analysis revealed that Isw2 and Rpd3-Sin3 act in parallel to repress a great number of gene expression, though it is unclear how many of them are directly regulated (Fazzio et al., 2001). Another report showed that Isw1 is recruited to *PHO8* promoter by the sequence-specific repressor Cbf1, and silences *PHO8* activity. Interestingly, increased binding of TBP (TATA-binding

protein) to the *PHO8* promoter was observed in *isw1* mutants, suggesting that Isw1 restricts chromatin accessibility for TBP binding to *PHO8* promoter, thereby preventing the basic transcriptional machinery from initiating transcription (Moreau et al., 2003).

In addition to repressing gene expression at the initiation stage, Isw1 was also found to coordinate RNA polymerase II (Pol II) complex mediated transcription elongation and termination (reviewed in Mellor and Morillon, 2004). Mellor and colleagues noted that Isw1 preferentially binds the *MET16* promoter in quiescent conditions, but is enriched in the coding region when the gene is induced. Loss of *isw1* causes an aberrant RNA termination of *MET16*, and the binding profile of Pol II across the *MET16* gene is significantly altered (Morillon et al., 2003). A follow-up study with a finer time course analysis indicates that Isw1 may play a role in delaying the release of Pol II complex for transcriptional elongation/termination to guarantee the timely histone modifications (Morillon et al., 2005).

It has long been proposed that chromatin modification/remodeling contributes to epigenetic transcriptional memory, though few convincing examples exist to support this argument. A recent study by Peterson and colleagues showed that *GAL1* induction is controlled by the prior expression state (Kundu et al., 2007). Up to 4 hours after shutdown of its expression, *GAL1* can be re-induced eight times faster than the prior activation (5 minutes vs. 40 minutes), suggesting that cells somehow “remember” that *GAL1* is previously transcribed. Such a transcriptional memory is not dependent on a variety of histone modifying enzymes, yet is “erased” in *SWI/SNF* mutants.

Furthermore, the lost memory in *SWI/SNF* mutants is restored upon loss of *isw1* or *isw2*. These observations suggest that SWI/SNF antagonizes ISWI to control the transcriptional memory of the *GAL1* gene.

Besides the examples given above, many other studies in yeast point to the role of ISWI in transcription, predominantly in transcriptional repression (van Vugt et al., 2007). In contrast, in higher eukaryotes, ISWI-type complexes have been shown to bear more diverse activities including transcriptional regulation, chromatin assembly during DNA replication, and global chromosome organization. In *Drosophila*, *iswi* mutants die at late larval stage (Deuring et al., 2000). Consistent with ISWI's role in transcriptional repression, ISWI and Pol II generally have no overlapping distribution pattern on polytene chromosome. Strikingly, the X chromosome in male *iswi* mutant is much shorter and broader than that in wild-type flies, indicating a defect in higher-order chromatin structure. A follow-up study showed that this phenotype can be significantly rescued upon mutation of a dosage compensation gene (Corona et al., 2002). Dosage compensation occurs in fly males to up-regulate gene expression on X chromosome by twofold, and this process involves the acetylation of histone H4 at lysine 16 (H4K16) (reviewed in Straub et al., 2005). Interestingly, H4K16 acetylation greatly reduces the ATPase activity of ISWI *in vitro* (Corona et al., 2002). These data suggest that dosage compensation complex counteracts ISWI's repression activity partly through H4 tail acetylation.

ISWI is highly expressed in fly embryos (Tsukiyama et al., 1995). Removal of

maternal ISWI leads to sterility, suggesting that ISWI is required for oogenesis (Deuring et al., 2000). A recent study showed that germline stem cells get lost rapidly in *iswi* mutants, indicating a role for ISWI in germline stem cell self-renewal (Xi and Xie, 2005). Two transcriptional targets of bone morphogenetic protein (BMP) signals are either derepressed or misregulated in *iswi* mutants, suggesting that ISWI regulates the proper expression of BMP targets in germline stem cells.

The mammalian genome encodes two ISWI homologues, SNF2H and SNF2L. These two proteins have distinct expression patterns in mice: SNF2H is ubiquitously expressed, while expression of SNF2L is more restricted to the central nervous system (Lazzaro and Picketts, 2001). Interestingly, overexpression of SNF2L promotes neurite outgrowth in mouse neuroblastoma cells, and the ATPase activity of SNF2L is required for this phenotype (Barak et al., 2003). Since Wnt-3a can induce neurite outgrowth in dorsal root ganglion (DRG) neurons (Lu et al., 2004), it is unclear whether the effect of SNF2L on neurite outgrowth is through Wnt signaling. SNF2H knockout mice were generated, and they died at early embryonic stage. SNF2H null blastocysts revealed phenotypes of growth arrest and cell death in both the trophectoderm and inner cell mass, suggesting that SNF2H is required for cell proliferation (Stopka and Skoultchi, 2003).

In contrast to the severe phenotypes *iswi* mutants display in flies and mice, ACF1 in *Drosophila* is not essential for viability (Fyodorov et al., 2004). Based on its strong ability to catalyze ATP-dependent chromatin assembly *in vitro*, Kadonaga and colleagues proposed that the ACF complex, containing ISWI and ACF1, participates in the

formation of repressive chromatin after DNA replication (Ito et al., 1999; Fyodorov and Kadonaga, 2002). Consistent with this notion, an acceleration of S phase progression in embryos and larval neuroblasts was observed in *acf1* mutants. Moreover, the bulk chromatin in *acf1* mutant has a shorter repeat length than that in wild-type, though the effect is very modest (Fyodorov et al., 2004). This defect could be due to an incomplete incorporation of histone H1 into the chromatin, since ACF1 has the unique activity to assemble H1-containing chromatin *in vitro* (Lusser et al., 2005).

Several studies in mammalian system showed that hACF1/WSTF and SNF2H are enriched in replicating heterochromatin in cultured cells, and their recruitment to replication foci is mediated by proliferating cell nuclear antigen (PCNA), an important cofactor for DNA synthesis (Moldovan et al., 2007). Depletion of hACF1/WSTF or SNF2H by RNAi impairs DNA replication in late S phage (Collins et al., 2002; Poot et al., 2004). These results indicate that the role of ACF complex in DNA replication is conserved.

Some ISWI family members are also implicated in transcriptional activation. The best evidence comes from the analysis of a unique subunit of NURF complex, Nurf301, in flies. NURF complex was initially purified in fly embryos by its activity to modulate chromatin structure at the *hsp70* promoter (Tsukiyama and Wu, 1995). Nurf301 can interact with the GAGA transcriptional factor and the heat-shock transcriptional factor (HSF) *in vitro* (Xiao et al., 2001). Consistently, the heat-shock induction of *hsp70* and *hsp26* is impaired in *nurf301* mutant flies (Badenhorst et al.,

2002). A later study showed that Nurf301 is required for the expression of a number of ecdysone target genes, and Nurf301 interacts with the ecdysone receptor (EcR) in an ecdysone-dependent manner (Badenhorst et al., 2005). These data suggest that Nurf301 directly activates transcriptional targets of ecdysteroid signaling. In addition, a study in human cells reported that hNurf301 (also called BPTF) binds to the enhancer of homeotic gene *engrailed* (*en*) and activates its expression (Barak et al., 2003). Different from other ISWI-type factors, Nurf301 may contain unique features to render NURF complex with the gene activation potential. How this is achieved will be discussed in the following section.

Mechanisms of ISWI family complexes in chromatin remodeling

It is evident that ISWI family complexes are involved in a wide array of biological events. However, what happens at the chromatin level for these events to occur is still poorly understood. Extensive biochemical studies have identified many characteristic activities of ISWI for chromatin remodeling *in vitro*. For example, unlike SWI/SNF, whose ATPase activity can be stimulated by either free or nucleosomal DNA, the ATPase activity of ISWI requires intact nucleosomes (Boyer et al., 2000). Moreover, the N-terminal tails of histone H4 are essential to stimulate nucleosome mobilization activity of a ISWI complex (Clapier et al., 2001; Eberharter et al., 2001). Cofactors also enhance the remodeling efficiency: ACF mobilizes nucleosomes ten fold more efficiently than ISWI alone. Interestingly, ACF1 alters the directionality of

nucleosome movement catalyzed by ISWI. While ISWI itself moves a mono-nucleosome from the center of a DNA fragment to the end, ACF moves it from the end to the center (Eberharter et al., 2001). The significance of such a directionality *in vivo* is unknown. Similarly, two histone fold subunits of CHRAC complex, CHRAC15 and CHRAC17, improve the efficiency of nucleosome mobilization by ACF (Kukimoto et al., 2004; Hartlepp et al., 2005).

Many ISWI complexes exhibit nucleosome sliding activity *in vitro*, which means the movement of histone octamers *in cis* (Hamiche et al., 1999; Langst et al., 1999). The first *in vivo* evidence of ISWI catalyzed nucleosome sliding came from a study of two yeast genes, *POT1* and *REC104*, which are repressed by Isw2. High-resolution mapping of the nucleosomes at these gene loci showed a clear shift of the nuclease insensitive area upon Isw2 induction, suggesting that Isw2 slides nucleosomes *in vivo* (Fazzio and Tsukiyama, 2003). To date, there is no evidence to support that ISWI-type complexes can remove histone octamers from DNA as SWI/SNF-type complexes do, or facilitate the histone variant exchange as INO80-type complexes do.

How do ISWI complexes slide nucleosomes? Two major mechanisms have been proposed. The first is “twist diffusion” model, which argues that a small DNA twist generates a DNA wave that propagates across the surface of the nucleosome (van Holde and Yager, 2003). Consistent with this model is the observation that ISWI can generate superhelical torsion *in vitro* (Havas et al., 2000). However, there is evidence that challenges this model. ISWI can still effectively induce nucleosome sliding on

nicked DNA, which presumably inhibits the propagation of a DNA twist (Langst and Becker, 2001). In addition, the ACF complex can still move nucleosomes bound to magnetic beads, which presents an obstacle for DNA twisting. Interestingly, the remodeling of nucleosomes by ACF generates a stretch of DNA long enough to be incorporated by ethidium bromide (EB) (Strohner et al., 2005). This result argues for a "loop recapture" model, in which ISWI complex induces the formation of a DNA loop at the nucleosome entry site that propagates across the surface of histone octamer. Readers who are interested in the details of these two models are recommended to peruse recent reviews (Langst and Becker, 2004; Saha et al., 2006).

Can DNA sequence influence nucleosome positioning? A recent *in vivo* study on a yeast Isw2 target, *POT1*, provided stimulating thought on this question (Whitehouse and Tsukiyama, 2006). A low-complexity, AT-rich DNA element was identified at the *POT1* promoter. This element poorly wraps into nucleosomes *in vitro*, and is refractory to nucleosome binding in *isw2* mutant. However, when Isw2 is present, the AT-rich element is occupied by a nucleosome. Replacing the endogenous AT element with an unbiased sequence (GATC) makes the chromatin less "open" over the *POT1* promoter. These results suggest that the AT element at the *POT1* promoter directs nucleosomes to thermodynamically stable positions accessible for transcriptional activators, while Isw2 repositions nucleosomes onto unfavorable DNA positions to shut down transcription. It is tempting to speculate that such a rule is not just limited to the AT sequence but can be extended to other cis-acting elements, though more gene loci need to be analyzed to

justify such a bold claim.

How are ISWI-type complexes targeted to chromatin for transcriptional regulation? At least two mechanisms have been identified. The first is the recruitment via sequence-specific transcriptional factors. As I described before, yeast Isw2 can be recruited to several early meiotic genes by the DNA-binding protein Ume6 to repress their expression (Goldmark et al., 2000). Yeast Isw1 can be recruited to *PHO8* locus via sequence-specific repressor Cbf1 (Moreau et al., 2003). Bdp1p, a component of the RNA polymerase III complex, is required for targeting Isw2 to tRNA genes (Bachman et al., 2005). Interestingly, only the catalytically inactive form of Isw2, not wide-type Isw2, is enriched at its target genes, suggesting that the recruitment of Isw2 is transient and dynamic (Gelbart et al., 2005).

There are also examples in mammals. Both hSNF2H and hACF1 can be targeted to the *IL-2Rα* (interleukin-2 receptor α) gene by SATB1 (special AT-rich sequence binding 1) to mediate repression. Interestingly, the ISWI complex appears to function over a long distance at *IL-2Rα* locus, as deletion of SATB1 causes alteration of nucleosome positioning over seven kilobases around *IL-2Rα* locus (Yasui et al., 2002). Another study showed that recruitment of hSNF2H to a thyroid hormone receptor (TR) regulated reporter is dependent on nuclear receptor co-repressor (N-CoR), though no physical interaction between hSNF2H and N-CoR was detected (Alenghat et al., 2006).

In contrast to the localized recruitment of ISWI to its targets, there is one example in yeast where Isw2 broadly associates with the DNA damage-inducible gene *RNR3*

(across ~4 kb region) and represses its expression. The binding of Isw2 even extends to the loci where the nucleosome positioning is not dependent on Isw2. The functional significance of this broad binding remains to be explored (Zhang and Reese, 2004).

The second mechanism involves interaction of ISWI factors with the histone proteins. I have already discussed the potential importance of histone H1/linker DNA in ACF mediated chromatin remodeling, which was supported by several *in vitro* studies (Kagalwala et al., 2004; Qian et al., 2005). In addition, acetylation of histone H4 at lysine 16 (H4K16Ac) inhibits the ATPase activity of ISWI and ACF-mediated nucleosome sliding *in vitro* (Corona et al., 2002; Shogren-Knaak et al., 2006). The genetic evidence that ISWI antagonizes H4K16Ac and the biochemical evidence that H4K16Ac inhibits the formation of chromatin fibers suggest that ISWI is required to maintain chromatin architecture by counteracting H4K16Ac activity. Interestingly, a recent report showed that TIP5, a subunit of the SNF2H-containing complex NoRC, binds to H4K16Ac through its bromodomain and this binding is important for NoRC mediated rDNA repression (Zhou and Grummt, 2005). These seemingly incongruous observations could be reconciled by the distinct contributions of unique cofactors (TIP5 vs. ACF1) for ISWI activity.

The targeting of ISWI complexes to activated genes has been connected to trimethylation of histone H3 at lysine 4 (H3K4me3), which marks the transcription initiation sites of many active genes (Santos-Rosa et al., 2002). Both hSNF2H and yeast Isw1 bind to trimethylated H3K4, and this histone modification is required for Isw1

targeting (Santos-Rosa et al., 2003). Similarly, the human NURF complex associates with H3K4me3 through a PHD finger of Nurf301, and this interaction facilitates NURF complex binding to its target genes. A point mutation in the PHD finger of Nurf301 that abolishes its ability to bind H3K4me3 fails to rescue *nurf301* mutant phenotypes in *Xenopus*, suggesting that the recognition of H3K4me3 is crucial for NURF function *in vivo* (Wysocka et al., 2006).

Finally, both sequence-specific factors and histone modifications can act in a coordinate manner to recruit ISWI complexes to target genes. This was demonstrated in an example of thyroid hormone receptor (TR) mediated repression. Both nuclear receptor co-repressor (N-CoR) and HDAC are required for recruitment of SNF2H to its target. Although no physical interaction was detected between SNF2H and N-CoR/HDAC complex, SNF2H preferentially binds to unacetylated H4, which is mediated by HDAC. A model was proposed where the recruitment of HDAC by nuclear receptor results in the local deacytelation of histone H4, which is a prerequisite to target SNF2H for target gene repression (Alenghat et al., 2006).

Since the identification of the first ISWI-type complex in yeast more than 20 years ago, our knowledge of the activities of ISWI family members has exploded, mainly driven by the elegant biochemical studies *in vitro*. Only recently did their physiological functions *in vivo* begin to be elucidated. Yet, these studies, especially in multicellular organisms, tend to focus on the phenotypic analysis of ISWI-type factors at the whole-animal level, with an insufficient exploration of the molecular mechanisms

underneath. By studying several ISWI-type factors in an important signaling pathway, namely the Wnt signaling pathway, in *Drosophila*, this dissertation attempts to enhance our understanding of the role of ISWI family chromatin remodeling proteins in transcriptional repression. In the following sections, I will briefly introduce Wnt signaling, and then discuss our current knowledge on how Wnt target genes are repressed.

Wnt/β-catenin signaling

Wnts in development and disease

Precise regulation of cell-cell communication is of paramount importance for proper development of multicellular organisms. It appears that several families of signaling molecules, including Hedgehogs, Wnts, and bone morphogenetic proteins (BMPs), play pivotal roles throughout animal development. Cells respond to these signaling molecules through highly conserved signal transduction cascades, which eventually lead to the transcriptional activation of various target genes. It is a general consensus that each signaling pathway displays tight control and high specificity for its transcriptional outcome. Devastating consequences, for example diseases in humans, often ensue when these pathways go awry.

Signaling molecules of the Wnt family are secreted lipid-modified glycoproteins that are highly conserved in all metozoans. The name derives from a fusion of *wingless*, the founding member in flies, and *int-1* (later renamed as *wnt1*), the first member

identified in mice (Nusse and Varmus, 1982). Defined by sequence, Wnt family encompasses a large group of proteins: at least 11 members in a cnidarian, 7 members in *Drosophila*, 5 members in *C. elegans*, and 19 members in mammals. How different Wnts execute their distinct functions to regulate animal development has been an intriguing question for Wnt researchers to tease out.

Wnts are required for a myriad of developmental processes including axis specification, cell identity determination, and pattern formation (Logan and Nusse, 2004). In *Drosophila*, the major Wnt gene *wingless (wg)* acquired its name from the phenotype of a hypomorphic allele wg^{1} , which displays missing wings in adult flies (Sharma, 1973). Later molecular analysis suggests that Wg acts as a gradient morphogen to regulate its target genes in the developing wing (Zecca et al., 1996). More severe alleles of *wg* were generated by the outstanding genetic screen performed by Eric Wieschaus and Christiane Nusslein-Volhard (Nusslein-Volhard and Wieschaus, 1980). *wg* null mutants cause embryonic lethality with severe segmentation defects, underscoring the essential role of Wg during embryogenesis (Bejsovec, 2006). In *Xenopus*, ectopic induction of Wnt1 in ventral blastomeres of 4-cell-stage embryos triggers a duplication of the body axis, and depletion of maternal Wnt11 causes embryos to lose dorsal axis identity (McMahon and Moon, 1989; Tao et al., 2005). The notion that Wnts are important for embryonic axis formation is also buttressed by studies in mice, where Wnt3 knockout mice lack the primitive streak before gastrulation (Liu et al., 1999). Many Wnt genes have been knocked out in mice, and a multitude of developmental

defects in various tissues have been observed. Readers are referred to a recent review for more detailed information (van Amerongen and Berns, 2006).

In addition to their essential requirement in many developmental events, Wnts also play important roles in adult tissue homeostasis. Examples include the maintenance of stem-cell fate in the intestinal epithelium, control of haematopoietic stem cell (HSC) self-renewal, establishment of the hair follicle, and regulation of bone mass formation (Reya and Clevers, 2005; Clevers, 2006). Recently, Wnts have also been implicated in tissue regeneration upon injury (Stoick-Cooper et al., 2007).

Given the importance of Wnts in animal development and adult tissue homeostasis, it is not surprising that misregulation of Wnt signaling leads to various diseases in humans (Logan and Nusse, 2004; Moon et al., 2004). For example, Wnt3 loss of function mutations are linked to Tetra-amelia, a genetic disorder characterized by complete loss of all limbs (Niemann et al., 2004). Loss of function mutations in LRP5, a co-receptor in Wnt signaling, leads to reduced bone mass as well as vasculature defects in the eye (Gong et al., 2001). Probably the most notable disease correlated to inappropriate activation of Wnt signaling is cancer, especially colorectal cancer (Polakis, 2007). Familial adenomatous polyposis (FAP), a hereditary predisposition to colorectal cancer, is caused by truncation mutations in APC, a negative regulator of the pathway (Kinzler et al., 1991; Nishisho et al., 1991). Strikingly, loss of APC has been found in most sporadic colorectal cancers (Kinzler and Vogelstein, 1996). Less frequent cases were also identified in other types of cancers when Wnt signaling is aberrantly activated,

including hepatocellular carcinoma, melanoma, and mammary carcinoma (Polakis, 2007). It has become increasingly popular to view cancer as a stem cell disease, since the molecular mechanisms that control normal stem cell self-renewal and inappropriate carcinogenesis are quite symmetrical (Taipale and Beachy, 2001). This is best illustrated in the case of intestine epithelia stem cell biology/pathology (Radtke and Clevers, 2005).

Wnt/β-catenin signaling

Since the discovery of the first Wnt in 1982, a combination of genetic and biochemical studies from a variety of organisms has depicted a highly conserved signaling pathway downstream of Wnts. The pathway has several branches, and here I only focus on the branch through β-catenin (β-cat), sometimes called the canonical Wnt pathway (Fig. 1.3). In this branch of signaling, Wnt binds to a seven-transmembrane receptor Frizzled (Fz) and a single-transmembrane coreceptor of the low density lipoprotein related protein family (LRP). The Fz/LRP complex, probably bridged by Wnt, transduces two disparate signals into the cytoplasm. The first signal is the phosphorylation of LRP by two kinases GSK3 and casein kinase I-γ (CKIγ), which then recruits Axin and leads to its inactivation/degradation. The second signal is the phosphorylation and activation of Dsh/Dvl through Fz, the mechanism of which is still mysterious, and the subsequent inhibition of a degradation complex comprising APC, Axin and GSK3. The net outcome of these two signals is the stabilization of β-cat in

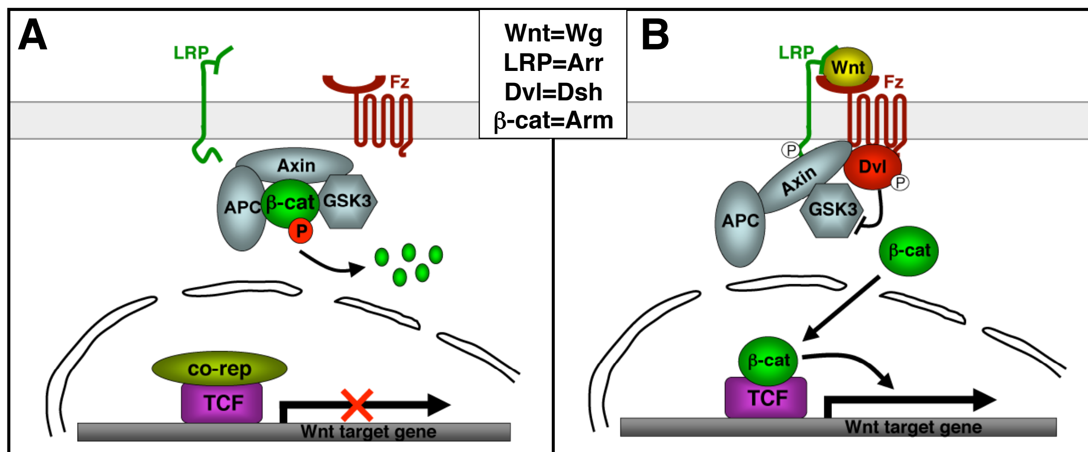

Figure 1.3. Outline of the Wnt/β-catenin pathway. (A) In the absence of Wnt, β-catenin (β-cat) is phosphorylated by a complex comprising APC, Axin and GSK3 in the cytosol, which targets it for proteasomal degradation. In the nucleus, TCF binds to Wnt regulated enhancer (WRE) and silences Wnt targets expression with the aid of co-repressors. (B) Upon Wnt binding to the Fz-LRP receptor complex, a combination of LRP-Axin interaction and Dvl activation leads to the inhibition of APC-Axin-GSK3 complex from phosphorylation of β-cat. β-cat is then stabilized and translocated into the nucleus, where it binds to TCF and converts it into a transcriptional activator. With other co-activators (not shown), they activate Wnt targets expression. Corresponding names of proteins in vertebrates and flies are listed in the inset. For a more elaborate description of Wnt signaling, check a recent review (Cadigan and Liu, 2006) and the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html.

the cytoplasm, which would have been rapidly degraded by the degradation complex in unstimulated cells (Cadigan and Liu, 2006).

Upon stabilization by Wnt signal, β-cat enters the nucleus and interacts with the DNA-binding T-cell factor/lymphoid-enhancing factor (TCF/LEF) family of HMG-box proteins to activate Wnt target genes. A variety of co-activators bind to β-cat, many of which negotiate with chromatin structure and the basal transcriptional machinery. The N-terminal β-cat recruits two essential proteins, Legless (BCL9) and Pygopus, while the C-terminal β-cat binds to a SWI/SNF-type protein Brg-1, histone acetyltransferase CBP/p300, and Hyrax/Parafibromin, a component of a Pol II interacting complex (Stadeli et al., 2006). The recruitment order of these co-activators onto β-cat and the mechanism of their action in transcriptional activation are yet to be elucidated.

What is the situation for Wnt targets in the absence of Wnt signaling? We can envision that for some genes, absence of co-activators is sufficient to keep them silenced, while for others, an active repression mechanism is required to prevent their basal transcription. A body of strong evidence suggests that when not bound to β-cat, TCFs act as repressors, probably in cooperation with co-repressors, to maintain the silent state of Wnt target genes (Fig 1.3). Thus far, our knowledge on the repression mechanism of Wnt targets is still incomplete. In the following sections, I will first discuss extensively the role of TCFs in repressing Wnt targets, followed by a comprehensive survey of all co-repressors implicated in Wnt signaling.

Transcriptional repression in Wnt signaling

TCFs as transcriptional repressors

Besides the widely regarded view that TCFs, when bound to β-cat, act as activators in Wnt signaling, there is accumulating evidence in the past decade that TCFs can also repress Wnt target expression in the absence of Wnt signaling. Therefore, TCFs serve as switches, silencing Wnt target genes until β-cat converts them into activators. The following section is a summary of the evidence from several model organisms that supports the repression activity of TCFs. The results can be divided into two categories: First, TCF mutant derepresses Wnt targets or shows phenotypes reflecting an activation of the pathway. Second, mutation of putative TCF binding sites in a Wnt regulated enhancer (WRE) leads to the derepression of the target.

(a) TCFs as repressors

Invertebrates such as *Hydra*, *Drosophila* and *C. elegans* only have one TCF ortholog, while vertebrates have four TCF members (TCF1, LEF1, TCF3 and TCF4). All TCFs contain the highly conserved N-terminal β-cat binding domains as well as the HMG DNA-binding domains. The central domains and the C-terminal tails are highly varied, which are thought to interact with distinct proteins. In addition, *Drosophila* and all vertebrate TCFs have alternative splicing forms, though their significance is not well understood (Arce et al., 2006).

In *Drosophila*, a careful analysis of *TCF* mutant phenotypes in embryos suggests

its role as a negative regulator in Wnt signaling. Either *TCF* mutants or *TCF/wg* double mutants display a less severe defect in Wg signaling in the embryonic epidermis than that of *wg* mutants (Cavallo et al., 1998). This difference can not be explained by the maternal contribution of TCF since embryos devoid of maternal and zygotic TCF show similar phenotypes as those of zygotic *TCF* mutant (Schweizer et al., 2003). These results strongly suggest that TCF acts as a repressor in Wnt signaling. However, *TCF* mutant embryos only reveal a partial activation of Wnt signaling, indicating that alleviation of TCF repression is not sufficient for Wnt target expression (Schweizer et al., 2003). A similar repressive activity was also observed for POP-1, the worm ortholog of TCF. Either *POP-1* single mutants or *POP-1/MOM-2* (a worm Wnt) double mutants result in embryonic phenotypes that are opposite to *MOM-2* mutants (Rocheleau et al., 1997; Thorpe et al., 1997). Later studies demonstrated that Wnt signaling regulates nuclear export of POP-1 to relieve its repression on target genes (Lo et al., 2004). It appears that in worms, POP-1 can act solely as a repressor in Wnt signaling, and antagonizing its repression activity is sufficient to activate Wnt target genes.

Things are complex in vertebrates where TCFs have evolved into divergent forms. An intriguing question is whether all vertebrate TCFs have activation/repression activities or some isoforms are more dedicated to activation or repression than others? The latter scenario seems to be the case as so far only the activation role has been identified for LEF1 and the repression role has been identified for TCF3, while TCF1 and TCF4 can do both. I will focus on the repressive role of vertebrate TCFs based on

loss-of-function results.

Abundant evidence exists for TCF3 as a repressor in Wnt signaling from various organisms. In *Xenopus*, depletion of *TCF3* by antisense oligos leads to an ectopic activation of several Wnt targets in ventral blastomeres, including a direct Wnt target *siamois*, while there is no significant reduction of these target expression in dorsal blastomeres (Houston et al., 2002). It would be interesting to see whether the induction of *siamois* by xTCF3 knockdown can be rescued by other TCFs. Another study dissected the non-redundant roles of xTCF3 and xTCF1/LEF1 during mesoderm induction (Liu et al., 2005). When xTCF3 was fused to VP16, a strong transcriptional activator, it could rescue either xLEF1 or xTCF1 knockdown phenotypes. In contrast, only a constitutive repressor form of xTCF3 with its N-terminal deletion could rescue the xTCF3 knockdown phenotypes. This result suggests that xTCF3 controls mesoderm development through its repression activity. A similar analysis for TCF3 was performed in zebrafish, where TCF3, also called Headless (Hdl), is essential for head development (Kim et al., 2000). The *headless* phenotype can be rescued by a constitutive repressor form of TCF3 (N-terminal deletion or fused to the repressor domain of the Engrailed protein), but can not be rescued by a TCF3-VP16 fusion. Again, this suggests that TCF3 acts as a repressor for zebrafish head formation.

TCF3 knockout mice reveal axis formation defects (e.g., expansion and duplication of node and notochord) that resemble those of the Axin or APC knockout mice, supporting TCF3's role as a repressor in Wnt signaling (Merrill et al., 2004). At

the molecular level, TCF3 directly binds to the regulatory region of *Nanog*, a Wnt-responsive gene important for embryonic stem cell self-renewal, and represses its expression (Pereira et al., 2006).

Do other TCFs have repression activity? Knockdown of xTCF1 leads to activation of several Wnt targets in ventral blastomeres. xTCF1 knockdown also decreases the expression of these targets in dorsal blastomeres, indicating that xTCF1 plays a dual role of activating and repressing Wnt targets in different developmental domains (Standley et al., 2006). In addition, *TCF1* knockout mice develop spontaneous tumors in the gut and mammary glands, suggesting an inappropriate activation of Wnt signaling. It was speculated that a truncated form of TCF1, serving as a naturally occurring dominant negative, is responsible for this phenotype (Roose et al., 1999). There are also reports for TCF4 acting as a repressor, but they are based on either overexpression analysis or a modest activation of a Wnt target upon *TCF4* RNAi in cultured cells (Lei et al., 2006; Shulewitz et al., 2006). More rigorous loss-of-function analysis for TCF4 *in vivo* is required to confirm its repressor activity.

(b) Derepressable Wnt targets

TCFs contain a single high mobility group (HMG) DNA-binding domain, which specifically bind to the minor groove of the DNA. *In vitro* analysis demonstrated that HMG domains of LEF1 and TCF1 recognize the sequence CCTTTGWW (W=A or T) with high affinity (Giese et al., 1991; van de Wetering et al., 1991). Such a TCF

binding site, commonly found in clusters, has been identified in many Wnt regulated enhancers (WREs). In many examples given below, mutating the TCF sites diminishes the activation of Wnt target genes, supporting a model where TCFs specifically bind to WREs and activate transcription.

Interestingly, mutating TCF sites in some WREs results in a clear derepression of Wnt targets. For all examples described below, the recurring theme is that mutation of TCF site(s) in a minimal WRE, presumably disrupting TCF binding, leads to the ectopic expression of the reporter, suggesting that TCF acts as a repressor to maintain Wnt target gene silenced in the absence of signaling.

In *Drosophila*, an enhancer element of *decapentaplegic (dpp*, a TGFβ family member*)* that controls its expression in visceral mesoderm was identified. Mutation of two TCF binding sites results in a clear expansion of the *dpp* reporter throughout the visceral mesoderm (Yang et al., 2000). A similar approach identified another minimal Wnt response sequence in visceral mesoderm, which controls expression of the homeotic gene *Ultrabithorax (Ubx)*. Mutation of the TCF site in this element causes a subtle expansion of the reporter (Riese et al., 1997). A more obvious expansion of this reporter was observed when repressors Osa or dCBP were absent, indicating a TCF-independent repression mechanism (Waltzer and Bienz, 1998; Collins and Treisman, 2000, see below for details). A third derepressable WRE in flies is from the pericardial WRE of *even-skipped (eve)*, where an obvious ectopic expression of the reporter can be detected once the TCF sites are destroyed (Knirr and Frasch, 2001).

In *C. elegans*, Wnt signaling is required to specify endoderm fate, which is achieved through alleviating POP-1 (the worm TCF) mediated repression on endoderm specific genes (Rocheleau et al., 1997; Thorpe et al., 1997). One of such genes is *end-1* (Calvo et al., 2001). Mutation of a TCF binding site in the *end-1* enhancer leads to an obvious ectopic expression of the reporter in MS cells that generate mesoderm (Shetty et al., 2005). A similar phenotype was observed in *pop-1* mutant worms. These results suggest that POP-1 represses the endoderm specific gene *end-1* in the mesoderm founder cells.

The best example for TCF's repression activity in vertebrates comes from the analysis of the enhancer of a homeobox gene *siamois*, which is a direct target of Wnt signaling and plays an important role in dorsal axis formation in *Xenopus*. Elimination several TCF sites in the minimal *siamois* enhancer leads to a robust activation (~20 fold) of the reporter in ventral bastomeres (Brannon et al., 1997). This result indicates that TCF silences the ventral expression of *siamois* to restrict its activity in dorsal blastomeres. Similar analysis was performed for another dorsal specification gene, *Xenopus Nodal-related 5* (*Xnr5)*, though the effect is milder. Mutation of two TCF sites in the minimal enhancer of *Xnr5* leads to ~3 fold activation of the reporter in ventral blastomeres (Hilton et al., 2003).

A recent study on a gene involved in ventral neural tube development, *Nkx2.2*, provided additional evidence for TCF's repression activity in mammals. *Nkx2.2* is expressed in a specific domain (p3) of the ventral neural tube in mouse embryos. When

two putative TCF sites were deleted in the minimal enhancer of *Nkx2.2*, ectopic reporter expression was observed throughout the whole ventral neural tube and even expanded into the dorsal spinal cord (Lei et al., 2006). The authors argue that TCF regulates the threshold response of *Nkx2.2* so it can be properly activated by Shh signaling in the p3 area of the ventral neural tube.

Finally, it is worthwhile mentioning that not all Wnt targets are repressed by TCFs, or to be more accurate, by TCFs alone. In *Drosophila*, *TCF* mutants result in loss of activation of two Wnt targets (Dll and Vg) in the wing, yet no detectable derepression of these targets can be observed in the region where Wg is absent (Schweizer et al., 2003). For several Wnt targets in vertebrates (*Xnr3*, *Brachyury* and *Delta-like 1*), mutation of TCF sites in the WREs clearly abolishes their expression, yet no derepression can be detected (McKendry et al., 1997; Yamaguchi et al., 1999; Galceran et al., 2004). Therefore, it is probably unwise to assume that the amount of repression is the same for every Wnt target gene. It is conceivable that only a subset of Wnt targets, which have low basal expression threshold, require the tight control of TCFs to maintain their silent state outside the Wnt expression domain. Alternatively, in certain biological contexts, some Wnt targets may employ TCF-independent repression mechanisms. Several examples of this will be recounted later.

Other transcriptional repressors in Wnt signaling

How are Wnt targets silenced in the absence of Wnt signaling? In addition to the discovery that TCFs play an important role, several other factors have been implicated in the transcriptional repression of Wnt targets (Fig. 1.4). I will discuss these factors in three categories: first, transcriptional co-repressors that either bind to TCF or covalently modify TCF; second, proteins that bind to β-cat and divert it from binding to TCF; third, DNA-binding proteins that repress Wnt targets independently of the TCF complex. The purpose of such a categorization is to facilitate the comprehension of the repression picture in Wnt signaling, and readers should not take it too rigidly. In fact, several factors that are to be described have been implicated in more than one group (e.g., CtBP, Kaiso, Sox).

(a) Factors that associate/modify TCF

The best characterized factor in this group is a WD-repeat containing protein called Groucho(Gro)/TLE that functions as a co-repressor in Wnt signaling. In *Drosophila*, similar to what was observed for the *TCF/wg* genetic interaction, *gro/wg* double mutants show less severe embryonic phenotypes than those of *wg* mutant alone, suggesting that Gro represses Wnt targets in embryos (Cavallo et al., 1998). Several TLEs bind to the central portion of TCF (Brantjes et al., 2001; Roose et al., 1998). A mutant form of TCF3 that lacks TLE-binding domain is unable to rescue the *TCF3* mutant phenotype in *Xenopus*, demonstrating the functional revevance of TLE-TCF

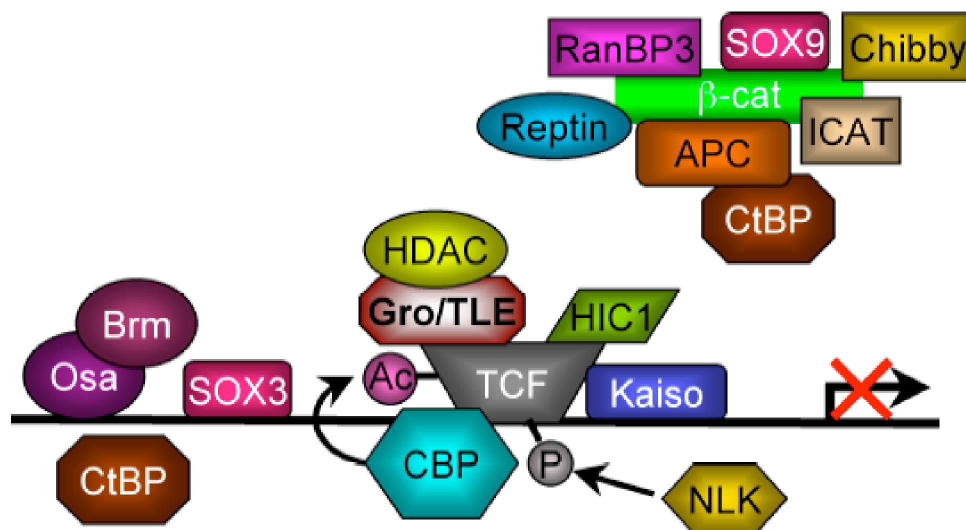

Figure 1.4. Summary of factors that repress Wnt target expression in the nucleus. These factors can be generally divided into three groups. Factors in the first group work through TCF. They either bind to it (e.g. Gro/TLE) or modify its activity (e.g. CBP) for repression. Factors in the second group bind to β-catenin and divert it from binding to TCF (e.g. Chibby). Factors in the third group act independently of TCF/β-catenin. They either bind to specific DNA sequence (e.g. Kaiso) or possibly work through such a binding (e.g. CtBP) to act in parallel with TCF for repression.

interaction (Liu et al., 2005). An *in vitro* study using highly purified proteins showed that β-cat directly displaces TLE from TCF (Daniels and Weis, 2005). Such a mechanism is likely to occur *in vivo*, since concomitant with an increase of β-cat binding, TLE's binding to Wnt targets is decreased upon the activation of the pathway (Sierra et al., 2006; Wang and Jones, 2006).

How does Gro/TLE mediate repression of Wnt targets? Part of the answer may reside in its recruitment of histone deacetylase (HDAC). Deacetylation of histone tails is generally associated with gene repression (Gallinari et al., 2007). In *Drosophila*, Gro directly binds to a HDAC protein Rpd3, and these two proteins genetically interact during embryonic development in a non-Wnt context (Chen et al., 1999). In human cultured cells, Wnt-responsive reporters are activated when HDAC activity is blocked by deacetylase inhibitor TSA (Billin et al., 2000). A more rigorous mutant analysis of HDAC for Wnt-related readouts is needed to confirm their role in Wnt signaling. In addition, although it is widely assumed that HDAC is recruited by Gro/TLE to Wnt targets, such a model has never been experimentally tested.

Several lines of evidence indicate that modification of TCF by acetylation, phosphorylation or sumoylation affects Wnt target expression. *Drosophila* histone acetyltransferase dCBP binds to TCF and acetylates it on K25 (in the Arm binding domain). Compared to wild-type TCF, acetylated TCF has a less binding affinity to Arm *in vitro*, suggesting that acetylation of TCF facilitates repression by disrupting TCF/Arm interaction (Waltzer and Bienz, 1998). Consistently, *CBP* mutants reveals

phenotypes in fly embryos reflecting an activation of Wg signaling. The repression role of CBP in Wg signaling appears to be conserved in vertebrates, as depletion of *CBP* leads to an activation of a Wnt reporter in human cultured cells (Li et al., 2007).

TCF can also be phosphorylated by MAP kinase-related nemo-like kinase (NLK). Phosphorylation of TCF by NLK inhibits the interaction of β-cat-TCF complex with DNA, thereby inhibits Wnt target activation (Ishitani et al., 1999). In *C elegans*, this phosphorylation is important for the nuclear export of POP-1 (the worm TCF) (Meneghini et al., 1999; Lo et al., 2004). In *Drosophila*, *nemo* is not an essential gene for animal development, and *nemo* mutants display a subtle derepression of a Wnt target in the wing (Zeng and Verheyen, 2004). Interestingly, knockdown of NLK in zebrafish reveals phenotypes indicative of blocking Wnt signaling, suggesting it also plays a positive role (Thorpe and Moon, 2004). This could be explained by NLK's specific interference with TCF3 to antagonize its repression activity in zebrafish.

TCF can also be sumoylated by a SUMO E3 ligase PIASy that is correlated to repression, though this effect is solely based on overexpression (Sachdev et al., 2001). PIASy knockout mice display no obvious Wnt defects, casting doubts on the physiological importance of it in Wnt signaling (Roth et al., 2004).

(b) Factors that buffer β-cat/TCF binding

Many factors in this group can bind to β-cat directly or indirectly, and prevent the β-cat-TCF complex from activating Wnt targets. Therefore, the function of these

"buffer" proteins is either to restrict the activity of trace amounts of nuclear β-cat in the absence of Wnt signaling or to set the threshold for the precise activation of Wnt targets.

The best example in this group, mainly due to the strong genetic evidence, is Chibby (Cby), a coiled-coil domain containing protein conserved from flies to humans (Takemaru et al., 2003). Endogenous Cby and β-cat interact in human cells, and Cby competes with TCF for binding to β-cat. Knockdown of *cby* by RNAi in fly embryos produced phenotypes resembling constitutive Wg activation. Importantly, in an *arm* mutant background, *cby* RNAi can no longer activate a Wnt target (Ubx-lacZ), yet *cby* RNAi can still activate this target in the *wg* mutant background. This data strongly suggest that unlike TCF or Gro, Cby mediates repression of Wnt targets in a β-cat dependent manner.

Another protein, called ICAT, can also bind to β-cat, and inhibits the interaction of β-cat with TCF4 (Tago et al., 2000). ICAT knockout mice showed phenotypes of neural fate posteriorization (Satoh et al., 2004), which are similar to those observed in *APC* mutant mice (Hasegawa et al., 2002) or mice embryos lacking Dickkopf1, an inhibitor of Wnt co-receptor (Mukhopadhyay et al., 2001). This result suggests that ICAT induce head formation by blocking Wnt signaling.

Reptin is another β-cat interacting protein, which was initially identified as a binding partner of Pontin (Bauer et al., 2000). These two related proteins are members of a highly conserved family of an ATP-dependent DNA helicase. Interestingly, Reptin and Pontin appear to play opposite roles in Wnt signaling. *reptin* mutation dominantly

suppresses the phenotypes caused by inactivation of Wg signaling in flies, suggesting that Reptin acts as a negative regulator in the pathway (Bauer et al., 2000). In zebrafish, a gain-of-function mutant of Reptin was identified, and it displays phenotypes in the developing heart similar to those of β-cat knockdown (Rottbauer et al., 2002). A rigorous loss-of-function study for Reptin is lacking. In addition, compared to Chibby and ICAT, much less is known about how it represses Wnt targets. A recent study showed that the histidine triad protein Hint1 interacts with Reptin/Pontin and disrupts their interaction to antagonize Wnt signaling (Weiske and Huber, 2005).

C-terminal binding protein (CtBP) is another repressor that plays an important role in silencing Wnt targets. Initial reports showed that CtBP binds to TCF and blocks Wnt signaling when overexpressed in *Xenopus* (Brannon et al., 1999; Valenta et al., 2003). However, later studies failed to detect any physical or functional interaction between CtBP and TCF in mammalian cells (Hamada and Bienz, 2004; Valenta et al., 2006). Instead, strong evidence shows that CtBP directly binds to APC (Hamada and Bienz, 2004; Sierra et al., 2006). Mutation of APC binding sites on CtBP reduces its ability to repress a Wnt reporter, underscoring the functional importance of such a binding. In addition, an increase binding between β-cat and TCF was observed in *CtBP* mutant cells (Hamada and Bienz, 2004). Therefore, similar to Chibby or ICAT, CtBP appears to divert β-cat from binding to TCF, probably through the adaptor APC, to antagonize Wnt signaling. Our laboratory recently showed that CtBP can also repress a Wnt target independently of β-cat-TCF complex in fly cells (Fang et al., 2006). It is

likely that different strategies are adopted by CtBP to repress Wnt targets in various biological contexts.

Several other proteins have also been shown to antagonize Wnt signaling by altering the subcellular localization of β-cat/TCF. For example, Ran binding protein 3 (RanBP3) binds to β-cat, and blocks Wnt signaling by stimulating its nuclear export (Hendriksen et al., 2005). HIC1, a BTB/POZ family member, binds to TCF4 and prevents TCF4/β-cat from associating with Wnt targets (Valenta et al., 2006). Duplin is another inhibitor of Wnt signaling that compete with TCF to bind to β-cat (Sakamoto et al., 2000). However, it might not be an indispensable factor in Wnt signaling since Duplin knockout mice displays no obvious Wnt defects (Nishiyama et al., 2004).

(c) Factors that bind to DNA

For this group of repressors, they have either specific or non-specific DNA binding capacity, and the prevailing model is that they cooperate with TCF to repress Wnt target genes. Three examples, Osa/Brahma, Kaiso and Sox family members, will be discussed in detail.

Drosophila osa encodes a protein containing an AT-rich interaction (ARID) domain. Osa is a cofactor in a fly SWI/SNF type chromatin remodeling complex. The core ATPase in this complex is Brahma (Brm). How the Osa/Brm complex is recruited to Wnt targets remains unknown as Osa does not specifically bind DNA *in vitro* (Collins et al., 1999). Loss-of function analysis in *Drosophila* revealed that Osa and Brm play

an important role in repressing several Wg targets in fly embryos and the developing wing (Collins and Treisman, 2000). Interestingly, *osa* and *gro/rpd3* (a fly HDAC) genetically interact to repress a Wg target in the wing, suggesting a cooperation between a chromatin remodeling complex and a histone deacytelation complex to repress Wg targets (Collins and Treisman, 2000). Further study is required to confirm the direct involvement of Osa/Brm in Wnt signaling.

In *Xenopus*, investigation of a BTB/POZ family member protein Kaiso has shed new light on the Wnt repression mechanism (Park et al., 2005). Unlike fly Osa, xKaiso contains sequence-specific binding capacity *in vitro* on a Wnt-responsive enhancer (*siamois*). Kaiso also directly binds to the *siamois* enhancer *in vivo*. Depletion of *Kaiso* by morpholino results in a derepression of several Wnt targets important for axis specification. Interestingly, Kaiso also physically binds to TCF3 and prevents it from binding to β-cat. These results argue that Kaiso has both TCF-dependent and TCF-independent activities to repress Wnt targets. Upon Wnt stimulation, Dsh stabilizes p120-catenin, which in turn binds to Kaiso and relieves its repression on Wnt targets (Park et al., 2006). Surprisingly, Kaiso knockout mice show no obvious abnormalities, drawing caution to extrapolate results from a single organism (Prokhortchouk et al., 2006).

Several Sox family members, which contain HMG domains, have also been implicated in Wnt signaling. Besides their DNA binding capacity, Sox proteins (xSox17 and xSox3) can also bind to β-cat, though the significance of this interaction is

elusive (Zorn et al., 1999). Loss-of-*Sox3* in *Xenopus* activates a Wnt target during early embryogenesis, and at least one amino acid in the HMG domain is important for its repression activity (Zhang et al., 2003). In mice, Sox9 interacts with β-cat, and conditional knockout of Sox9 in chondrocytes shows chondrodysplasia phenotype similar to that of constitutively activated β-cat (Akiyama et al., 2002; Akiyama et al., 2004). In *Drosophila*, *SoxNeuro (SoxN)* mutant reveals subtle gain-of-Wg-signaling phenotypes in embryos, though it clearly suppresses a *wg* mutant phenotype (Chao et al., 2007). There is no evidence that SoxN interacts with TCF or Arm, so it is unclear whether SoxN is a direct repressor in Wg signaling.

Finally, it is worth mentioning that several repressors described above (e.g. CtBP, CBP and Brm) are also involved in activating Wnt targets (Fang et al., 2006; Li et al., 2007; Barker et al., 2001). Possible scenarios include that activation or repression of Wnt targets is gene or tissue specific, or different forms of the protein (e.g. monomer or dimer) determine whether it activates or represses Wnt targets.

Transcriptional regulation of Wnt targets in chromatin

Increasing evidence in the past decade has shown that Wnt signaling regulates transcription in the chromatin context. A number of chromatin remodeling factors and histone modification factors have been implicated in the pathway, and much is to be explored for their role in transcriptional control of Wnt targets .

The importance of chromatin in β-cat/TCF mediated transcription was suggested

by a pioneering study which examined the transcription and binding activities of recombinant β-cat and LEF1 proteins on a chromatinized β-cat response element (BRE) *in vitro* (Tutter et al., 2001). Although LEF1 effectively binds naked DNA, it only weakly binds chromatized BRE. β-cat strongly enhances LEF1 binding to this chromatin. These data suggest that chromatin structure is crucial for β-cat/LEF1 mediated transcription *in vitro*. Interestingly, both a histone acetyltransferase p300 and a partially purified chromatin remodeling fraction enhance β-cat/LEF1 mediated transcription from a preassembled nucleosomal template, and inhibition of ubiquitination on chromatin template blocks the transcription, indicating the involvement of chromatin modification/remodeling proteins in "opening up" chromatin structure for β-cat/LEF1 to activate transcription (Tutter et al., 2001; Sierra et al., 2006).

A recent study provides *in vivo* evidence for some chromatin factors in regulating a direct Wnt target, c-Myc (Sierra et al., 2006). A C-terminal fragment of β-cat was used to purify its binding partners, and subunits from several chromatin modification/remodeling complexes were identified including the TRRAP/TIP60 histone acetyltransferase complex, ISWI chromatin remodeling complex, and SET1-type histone methyltransferase (HMT) complex. SET1-type complex mediates trimethylation of K4 on the N-terminal tail of histone H3 (H3K4Me3) that marks transcription initiation (Dehe and Geli, 2006). In agreement, association of several subunits in SET1 complex and trimethylation of H3K4 were observed on c-Myc enhancer concomitant with the β-cat binding. The recruitment of β-cat and SET complex to the c-Myc WRE is transient,

while elevated H3K4Me3 is more persistent. This prolonged elevation of H3K4Me3 was also observed for some activated genes in yeast, which was correlated to transcriptional memory (Ng et al., 2003). RNAi knockdown of MLL2, a subunit in SET1 complex, caused a modest reduction of c-Myc expression, suggesting that SET1 complex is functionally important for Wnt signaling.

In contrast to the limited knowledge we have gained for the role of chromatin factors in Wnt target activation, we know very little about the role of these proteins in Wnt target repression. I have extensively discussed the function of a variety of transcriptional repressors in Wnt signaling, and only a few of them (Brm/Osa, HDAC, Reptin) bear activities of chromatin modulation. Studies on these factors are either contradictory or incomplete so the definitive explanation of their roles in mediating repression is lacking. Further investigation is needed in this perspective.

Summary of my work in this dissertation

The work of my PhD study focuses on the genetic and molecular study of several ISWI family chromatin remodeling proteins in Wnt signaling, using *Drosophila* as a model organism. A series of analyses in both flies and cultured cells suggest that these proteins, including ISWI, ACF1 and Tou, are involved in transcriptional repression of Wnt target genes.

My interest in ISWI family chromatin remodelers came initially from the identification of *toutatis (tou)* in a misexpression screen for negative regulators of the

Wg pathway performed in the fly eye (Parker et al., 2002). *tou* encodes a fly homologue of TIP-5, a subunit of a ISWI-type complex in mammals (Strohner et al., 2001). Overexpression of *tou* blocks Wg signaling in the adult eye and developing wing. However, a null *tou* mutant, which is semi-lethal, displays no obvious Wg phenotypes. Considering the possibility that Tou acts redundantly with other protein(s), I began to study Tou's closest partner ACF1, which associates with at least two ISWI-type complexes (Langst and Becker, 2001). While *acf1* null mutants are viable, *tou*/*acf1* double mutants die at late pupal stage, suggesting that they act redundantly in animal development. Nevertheless, no obvious Wg defects were observed in *tou/acf1* mutant flies. I also carried out loss of function studies in both fly and human culture cells, and found that Tou and ACF1 act redundantly to repress Wnt targets. This data suggests a cell-specific role of these proteins in Wnt signaling. In chapter II, I will recapitulate results from the genetic analysis of *tou* and *acf1* in flies and cultured cells.

I was not satisfied with the weak phenotype revealed by *tou/acf1* mutant. At least four ISWI containing complexes have been identified in flies (Fig. 1.2), two of which contain Tou and ACF1 as cofactors, respectively. Depletion of the shared component ISWI will presumably cripple all four complexes, and likely give a more severe phenotype than that of *tou/acf1* mutant. Indeed, characterization of *iswi* mutant led to a breakthrough for this project: loss of *iswi* causes the expansion of several Wg targets in the developing wing, suggesting a role in repression of Wg targets. Loss of *iswi* and *acf1* also results in significant derepression of several Wg targets in fly cultured

cells, and a line of evidence argues that the derepression is not due to a defect of post-mitotic chromatin assembly. ACF1 broadly binds to several Wg targets, and the binding is reduced upon Wg stimulation, leading us to propose a model where Wg signaling activates target gene expression by overcoming the chromatin barrier maintained by ACF1. This story will be elaborated in chapter III.

In the last chapter, I will summarize all the results and propose future directions, mainly to extend the story in Chapter III, for a further understanding of the mechanism of ISWI/ACF1's action in Wg target repression.

References

Akiyama, H., Chaboissier, M.C., Martin, J.F., Schedl, A. and de Crombrugghe, B. (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev*, **16**, 2813-2828.

Akiyama, H., Lyons, J.P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., Deng, J.M., Taketo, M.M., Nakamura, T., Behringer, R.R., McCrea, P.D. and de Crombrugghe, B. (2004) Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev*, **18**, 1072-1087.

Alenghat, T., Yu, J. and Lazar, M.A. (2006) The N-CoR complex enables chromatin remodeler SNF2H to enhance repression by thyroid hormone receptor. *Embo J*, **25**, 3966-3974.

Bachman, N., Gelbart, M.E., Tsukiyama, T. and Boeke, J.D. (2005) TFIIIB subunit Bdp1p is required for periodic integration of the Ty1 retrotransposon and targeting of Isw2p to S. cerevisiae tDNAs. *Genes Dev*, **19**, 955-64.

Badenhorst, P., Voas, M., Rebay, I. and Wu, C. (2002) Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev*, **16**, 3186-3198.

Badenhorst, P., Xiao, H., Cherbas, L., Kwon, S.Y., Voas, M., Rebay, I., Cherbas, P. and Wu, C. (2005) The Drosophila nucleosome remodeling factor NURF is required for Ecdysteroid signaling and metamorphosis. *Genes Dev*, **19**, 2540-2545.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, **410**, 120-124.

Barak, O., Lazzaro, M.A., Lane, W.S., Speicher, D.W., Picketts, D.J. and Shiekhattar, R. (2003) Isolation of human NURF: a regulator of Engrailed gene expression. *Embo J*, **22**, 6089-6100.

Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M. and Clevers, H. (2001) The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *Embo J*, **20**, 4935-43.

Bauer, A., Chauvet, S., Huber, O., Usseglio, F., Rothbacher, U., Aragnol, D., Kemler, R. and Pradel, J. (2000) Pontin52 and reptin52 function as antagonistic regulators of

beta-catenin signalling activity. *Embo J*, **19**, 6121-6130.

Bejsovec, A. (2006) Flying at the head of the pack: Wnt biology in Drosophila. *Oncogene*, **25**, 7442-7449.

Billin, A.N., Thirlwell, H. and Ayer, D.E. (2000) Beta-catenin-histone deacetylase interactions regulate the transition of LEF1 from a transcriptional repressor to an activator. *Mol Cell Biol*, **20**, 6882-6890.

Boyer, L.A., Logie, C., Bonte, E., Becker, P.B., Wade, P.A., Wolffe, A.P., Wu, C., Imbalzano, A.N. and Peterson, C.L. (2000) Functional delineation of three groups of the ATP-dependent family of chromatin remodeling enzymes. *J Biol Chem*, **275**, 18864-18870.

Brannon, M., Brown, J.D., Bates, R., Kimelman, D. and Moon, R.T. (1999) XCtBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development*, **126**, 3159-3170.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R.T. and Kimelman, D. (1997) A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. *Genes Dev*, **11**, 2359-2370.

Brantjes, H., Roose, J., van De Wetering, M. and Clevers, H. (2001) All Tcf HMG box transcription factors interact with Groucho-related co-repressors. *Nucleic Acids Res*, **29**, 1410-1419.

Cadigan, K.M. and Liu, Y.I. (2006) Wnt signaling: complexity at the surface. *J Cell Sci*, **119**, 395-402.

Calvo, D., Victor, M., Gay, F., Sui, G., Luke, M.P., Dufourcq, P., Wen, G., Maduro, M., Rothman, J. and Shi, Y. (2001) A POP-1 repressor complex restricts inappropriate cell type-specific gene transcription during Caenorhabditis elegans embryogenesis. *Embo J*, **20**, 7197-7208.

Caruthers, J.M. and McKay, D.B. (2002) Helicase structure and mechanism. *Curr Opin Struct Biol*, **12**, 123-133.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M. and Bejsovec, A. (1998) Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature*, **395**, 604-608.

Chao, A.T., Jones, W.M. and Bejsovec, A. (2007) The HMG-box transcription factor SoxNeuro acts with Tcf to control Wg/Wnt signaling activity. *Development*, **134**, 989-997.

Chen, G., Fernandez, J., Mische, S. and Courey, A.J. (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes Dev*, **13**, 2218-2230.

Clapier, C.R., Langst, G., Corona, D.F., Becker, P.B. and Nightingale, K.P. (2001) Critical role for the histone H4 N terminus in nucleosome remodeling by ISWI. *Mol Cell Biol*, **21**, 875-883.

Clevers, H. (2006) Wnt/beta-catenin signaling in development and disease. *Cell*, **127**, 469-480.

Collins, N., Poot, R.A., Kukimoto, I., Garcia-Jimenez, C., Dellaire, G. and Varga-Weisz, P.D. (2002) An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. *Nat Genet*, **32**, 627-632.

Collins, R.T., Furukawa, T., Tanese, N. and Treisman, J.E. (1999) Osa associates with the Brahma chromatin remodeling complex and promotes the activation of some target genes. *Embo J*, **18**, 7029-7040.

Collins, R.T. and Treisman, J.E. (2000) Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. *Genes Dev*, **14**, 3140-3152.

Corona, D.F., Clapier, C.R., Becker, P.B. and Tamkun, J.W. (2002) Modulation of ISWI function by site-specific histone acetylation. *EMBO Rep*, **3**, 242-247.

Corona, D.F. and Tamkun, J.W. (2004) Multiple roles for ISWI in transcription, chromosome organization and DNA replication. *Biochim Biophys Acta*, **1677**, 113-119.

Cote, J., Peterson, C.L. and Workman, J.L. (1998) Perturbation of nucleosome core structure by the SWI/SNF complex persists after its detachment, enhancing subsequent transcription factor binding. *Proc Natl Acad Sci U S A*, **95**, 4947-4952.

Daniels, D.L. and Weis, W.I. (2005) Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol*, **12**, 364-371.

Dehe, P.M. and Geli, V. (2006) The multiple faces of Set1. *Biochem Cell Biol*, **84**, 536-548.

Deuring, R., Fanti, L., Armstrong, J.A., Sarte, M., Papoulas, O., Prestel, M., Daubresse, G., Verardo, M., Moseley, S.L., Berloco, M., Tsukiyama, T., Wu, C., Pimpinelli, S. and Tamkun, J.W. (2000) The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. *Mol Cell*, **5**, 355-365.

Eberharter, A., Ferrari, S., Langst, G., Straub, T., Imhof, A., Varga-Weisz, P., Wilm, M. and Becker, P.B. (2001) Acf1, the largest subunit of CHRAC, regulates ISWI-induced nucleosome remodelling. *Embo J*, **20**, 3781-3788.

Eberharter, A., Vetter, I., Ferreira, R. and Becker, P.B. (2004) ACF1 improves the effectiveness of nucleosome mobilization by ISWI through PHD-histone contacts. *Embo J*, **23**, 4029-4039.

Fan, H.Y., Trotter, K.W., Archer, T.K. and Kingston, R.E. (2005) Swapping function of two chromatin remodeling complexes. *Mol Cell*, **17**, 805-815.

Fang, M., Li, J., Blauwkamp, T., Bhambhani, C., Campbell, N. and Cadigan, K.M. (2006) C-terminal-binding protein directly activates and represses Wnt transcriptional targets in Drosophila. *Embo J*, **25**, 2735-2745.

Fazzio, T.G., Kooperberg, C., Goldmark, J.P., Neal, C., Basom, R., Delrow, J. and Tsukiyama, T. (2001) Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol*, **21**, 6450-6460.

Fazzio, T.G. and Tsukiyama, T. (2003) Chromatin remodeling in vivo: evidence for a nucleosome sliding mechanism. *Mol Cell*, **12**, 1333-1340.

Fyodorov, D.V., Blower, M.D., Karpen, G.H. and Kadonaga, J.T. (2004) Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. *Genes Dev*, **18**, 170-183.

Fyodorov, D.V. and Kadonaga, J.T. (2002) Dynamics of ATP-dependent chromatin assembly by ACF. *Nature*, **418**, 897-900.

Galceran, J., Sustmann, C., Hsu, S.C., Folberth, S. and Grosschedl, R. (2004)

LEF1-mediated regulation of Delta-like1 links Wnt and Notch signaling in somitogenesis. *Genes Dev*, **18**, 2718-23.

Gallinari, P., Di Marco, S., Jones, P., Pallaoro, M. and Steinkuhler, C. (2007) HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics. *Cell Res*, **17**, 195-211.

Gelbart, M.E., Rechsteiner, T., Richmond, T.J. and Tsukiyama, T. (2001) Interactions of Isw2 chromatin remodeling complex with nucleosomal arrays: analyses using recombinant yeast histones and immobilized templates. *Mol Cell Biol*, **21**, 2098-2106.

Gelbart, M.E., Bachman, N., Delrow, J., Boeke, J.D. and Tsukiyama, T. (2005) Genome-wide identification of Isw2 chromatin-remodeling targets by localization of a catalytically inactive mutant. *Genes Dev*, **19**, 942-54.

Giese, K., Amsterdam, A. and Grosschedl, R. (1991) DNA-binding properties of the HMG domain of the lymphoid-specific transcriptional regulator LEF-1. *Genes Dev*, **5**, 2567-78.

Goldmark, J.P., Fazzio, T.G., Estep, P.W., Church, G.M. and Tsukiyama, T. (2000) The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell*, **103**, 423-433.

Gong, Y., Slee, R.B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A.M., Wang, H., Cundy, T., Glorieux, F.H., Lev, D., Zacharin, M., Oexle, K., Marcelino, J., Suwairi, W., Heeger, S., Sabatakos, G., Apte, S., Adkins, W.N., Allgrove, J., Arslan-Kirchner, M. et al. (2001) LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell*, **107**, 513-523.

Grune, T., Brzeski, J., Eberharter, A., Clapier, C.R., Corona, D.F., Becker, P.B. and Muller, C.W. (2003) Crystal structure and functional analysis of a nucleosome recognition module of the remodeling factor ISWI. *Mol Cell*, **12**, 449-460.

Hamada, F. and Bienz, M. (2004) The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. *Dev Cell*, **7**, 677-685.

Hamiche, A., Sandaltzopoulos, R., Gdula, D.A. and Wu, C. (1999) ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF. *Cell*, **97**, 833-842.

Hartlepp, K.F., Fernandez-Tornero, C., Eberharter, A., Grune, T., Muller, C.W. and Becker, P.B. (2005) The histone fold subunits of Drosophila CHRAC facilitate nucleosome sliding through dynamic DNA interactions. *Mol Cell Biol*, **25**, 9886-9896.

Hasegawa, S., Sato, T., Akazawa, H., Okada, H., Maeno, A., Ito, M., Sugitani, Y., Shibata, H., Miyazaki Ji, J., Katsuki, M., Yamauchi, Y., Yamamura Ki, K., Katamine, S. and Noda, T. (2002) Apoptosis in neural crest cells by functional loss of APC tumor suppressor gene. *Proc Natl Acad Sci U S A*, **99**, 297-302.

Hassan, A.H., Prochasson, P., Neely, K.E., Galasinski, S.C., Chandy, M., Carrozza, M.J. and Workman, J.L. (2002) Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. *Cell*, **111**, 369-379.

Havas, K., Flaus, A., Phelan, M., Kingston, R., Wade, P.A., Lilley, D.M. and Owen-Hughes, T. (2000) Generation of superhelical torsion by ATP-dependent chromatin remodeling activities. *Cell*, **103**, 1133-1142.

Hendriksen, J., Fagotto, F., van der Velde, H., van Schie, M., Noordermeer, J. and Fornerod, M. (2005) RanBP3 enhances nuclear export of active (beta)-catenin independently of CRM1. *J Cell Biol*, **171**, 785-797.

Hilton, E., Rex, M. and Old, R. (2003) VegT activation of the early zygotic gene Xnr5 requires lifting of Tcf-mediated repression in the Xenopus blastula. *Mech Dev*, **120**, 1127-1138.

Houston, D.W., Kofron, M., Resnik, E., Langland, R., Destree, O., Wylie, C. and Heasman, J. (2002) Repression of organizer genes in dorsal and ventral Xenopus cells mediated by maternal XTcf3. *Development*, **129**, 4015-4025.

Iida, T. and Araki, H. (2004) Noncompetitive counteractions of DNA polymerase epsilon and ISW2/yCHRAC for epigenetic inheritance of telomere position effect in Saccharomyces cerevisiae. *Mol Cell Biol*, **24**, 217-227.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H. and Matsumoto, K. (1999) The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature*, **399**, 798-802.

Ito, T., Levenstein, M.E., Fyodorov, D.V., Kutach, A.K., Kobayashi, R. and Kadonaga,

J.T. (1999) ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. *Genes Dev*, **13**, 1529-1539.

Kagalwala, M.N., Glaus, B.J., Dang, W., Zofall, M. and Bartholomew, B. (2004) Topography of the ISW2-nucleosome complex: insights into nucleosome spacing and chromatin remodeling. *Embo J*, **23**, 2092-2104.

Khorasanizadeh, S. (2004) The nucleosome: from genomic organization to genomic regulation. *Cell*, **116**, 259-272.

Kim, C.H., Oda, T., Itoh, M., Jiang, D., Artinger, K.B., Chandrasekharappa, S.C., Driever, W. and Chitnis, A.B. (2000) Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature*, **407**, 913-916.

Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D. and et al. (1991) Identification of FAP locus genes from chromosome 5q21. *Science*, **253**, 661-665.

Kinzler, K.W. and Vogelstein, B. (1996) Lessons from hereditary colorectal cancer. *Cell*, **87**, 159-170.

Knirr, S. and Frasch, M. (2001) Molecular integration of inductive and mesoderm-intrinsic inputs governs even-skipped enhancer activity in a subset of pericardial and dorsal muscle progenitors. *Dev Biol*, **238**, 13-26.

Kouzarides, T. (2007) Chromatin modifications and their function. *Cell*, **128**, 693-705.

Kukimoto, I., Elderkin, S., Grimaldi, M., Oelgeschlager, T. and Varga-Weisz, P.D. (2004) The histone-fold protein complex CHRAC-15/17 enhances nucleosome sliding and assembly mediated by ACF. *Mol Cell*, **13**, 265-277.

Kundu, S., Horn, P.J. and Peterson, C.L. (2007) SWI/SNF is required for transcriptional memory at the yeast GAL gene cluster. *Genes Dev*, **21**, 997-1004.

Langst, G. and Becker, P.B. (2001) Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. *J Cell Sci*, **114**, 2561-2568.

Langst, G. and Becker, P.B. (2004) Nucleosome remodeling: one mechanism, many phenomena? *Biochim Biophys Acta*, **1677**, 58-63.

Langst, G., Bonte, E.J., Corona, D.F. and Becker, P.B. (1999) Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer. *Cell*, **97**, 843-852.

Lazzaro, M.A. and Picketts, D.J. (2001) Cloning and characterization of the murine Imitation Switch (ISWI) genes: differential expression patterns suggest distinct developmental roles for Snf2h and Snf2l. *J Neurochem*, **77**, 1145-1156.

Lei, Q., Jeong, Y., Misra, K., Li, S., Zelman, A.K., Epstein, D.J. and Matise, M.P. (2006) Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube. *Dev Cell*, **11**, 325-337.

Li, J., Sutter, C., Parker, D.S., Blauwkamp, T., Fang, M. and Cadigan, K.M. (2007) CBP/p300 are bimodal regulators of Wnt signaling. *EMBO J*, **26**, 2284-2294.

Arce, L., Yokoyama, N.N. and Waterman, M.L. (2006) Diversity of LEF/TCF action in development and disease. *Oncogene*, **25**, 7492-504.

Liu, F., van den Broek, O., Destree, O. and Hoppler, S. (2005) Distinct roles for Xenopus Tcf/Lef genes in mediating specific responses to Wnt/beta-catenin signalling in mesoderm development. *Development*, **132**, 5375-5385.

Liu, P., Wakamiya, M., Shea, M.J., Albrecht, U., Behringer, R.R. and Bradley, A. (1999) Requirement for Wnt3 in vertebrate axis formation. *Nat Genet*, **22**, 361-365.

Lo, M.C., Gay, F., Odom, R., Shi, Y. and Lin, R. (2004) Phosphorylation by the beta-catenin/MAPK complex promotes 14-3-3-mediated nuclear export of TCF/POP-1 in signal-responsive cells in C. elegans. *Cell*, **117**, 95-106.

Logan, C.Y. and Nusse, R. (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol*, **20**, 781-810.

Lu, W., Yamamoto, V., Ortega, B. and Baltimore, D. (2004) Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. *Cell*, **119**, 97-108.

Lu, X., Meng, X., Morris, C.A. and Keating, M.T. (1998) A novel human gene, WSTF, is deleted in Williams syndrome. *Genomics*, **54**, 241-249.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature*, **389**, 251-260.

Lusser, A., Urwin, D.L. and Kadonaga, J.T. (2005) Distinct activities of CHD1 and ACF in ATP-dependent chromatin assembly. *Nat Struct Mol Biol*, **12**, 160-166.

Martens, J.A. and Winston, F. (2002) Evidence that Swi/Snf directly represses transcription in S. cerevisiae. *Genes Dev*, **16**, 2231-2236.

Martens, J.A. and Winston, F. (2003) Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr Opin Genet Dev*, **13**, 136-142.

McKendry, R., Hsu, S.C., Harland, R.M. and Grosschedl, R. (1997) LEF-1/TCF proteins mediate wnt-inducible transcription from the Xenopus nodal-related 3 promoter. *Dev Biol*, **192**, 420-431.

McMahon, A.P. and Moon, R.T. (1989) Ectopic expression of the proto-oncogene int-1 in Xenopus embryos leads to duplication of the embryonic axis. *Cell*, **58**, 1075-1084.

Mellor, J. and Morillon, A. (2004) ISWI complexes in Saccharomyces cerevisiae. *Biochim Biophys Acta*, **1677**, 100-112.

Meneghini, M.D., Ishitani, T., Carter, J.C., Hisamoto, N., Ninomiya-Tsuji, J., Thorpe, C.J., Hamill, D.R., Matsumoto, K. and Bowerman, B. (1999) MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans. *Nature*, **399**, 793-797.

Meneghini, M.D., Wu, M. and Madhani, H.D. (2003) Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell*, **112**, 725-736.

Merrill, B.J., Pasolli, H.A., Polak, L., Rendl, M., Garcia-Garcia, M.J., Anderson, K.V. and Fuchs, E. (2004) Tcf3: a transcriptional regulator of axis induction in the early embryo. *Development*, **131**, 263-274.

Mizuguchi, G., Shen, X., Landry, J., Wu, W.H., Sen, S. and Wu, C. (2004) ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science*, **303**, 343-348.

Moldovan, G.L., Pfander, B. and Jentsch, S. (2007) PCNA, the maestro of the replication fork. *Cell*, **129**, 665-679.

Moon, R.T., Kohn, A.D., De Ferrari, G.V. and Kaykas, A. (2004) WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet*, **5**, 691-701.

Moreau, J.L., Lee, M., Mahachi, N., Vary, J., Mellor, J., Tsukiyama, T. and Goding, C.R. (2003) Regulated displacement of TBP from the PHO8 promoter in vivo requires Cbf1 and the Isw1 chromatin remodeling complex. *Mol Cell*, **11**, 1609-1620.

Morillon, A., Karabetsou, N., Nair, A. and Mellor, J. (2005) Dynamic lysine methylation on histone H3 defines the regulatory phase of gene transcription. *Mol Cell*, **18**, 723-734.

Morillon, A., Karabetsou, N., O'Sullivan, J., Kent, N., Proudfoot, N. and Mellor, J. (2003) Isw1 chromatin remodeling ATPase coordinates transcription elongation and termination by RNA polymerase II. *Cell*, **115**, 425-435.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D.W., Glinka, A., Grinberg, A., Huang, S.P., Niehrs, C., Belmonte, J.C. and Westphal, H. (2001) Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev Cell*, **1**, 423-434.

Narlikar, G.J., Fan, H.Y. and Kingston, R.E. (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell*, **108**, 475-487.

Ng, H.H., Robert, F., Young, R.A. and Struhl, K. (2003) Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol Cell*, **11**, 709-719.

Niemann, S., Zhao, C., Pascu, F., Stahl, U., Aulepp, U., Niswander, L., Weber, J.L. and Muller, U. (2004) Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am J Hum Genet*, **74**, 558-563.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S. and Hedge, P. (1991) Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science*, **253**, 665-669.

Nishiyama, M., Nakayama, K., Tsunematsu, R., Tsukiyama, T., Kikuchi, A. and Nakayama, K.I. (2004) Early embryonic death in mice lacking the beta-catenin-binding protein Duplin. *Mol Cell Biol*, **24**, 8386-8394.

Nusse, R. and Varmus, H.E. (1982) Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell*,

31, 99-109.

Nusslein-Volhard, C. and Wieschaus, E. (1980) Mutations affecting segment number and polarity in Drosophila. *Nature*, **287**, 795-801.

Park, J.I., Ji, H., Jun, S., Gu, D., Hikasa, H., Li, L., Sokol, S.Y. and McCrea, P.D. (2006) Frodo links Dishevelled to the p120-catenin/Kaiso pathway: distinct catenin subfamilies promote Wnt signals. *Dev Cell*, **11**, 683-695.

Park, J.I., Kim, S.W., Lyons, J.P., Ji, H., Nguyen, T.T., Cho, K., Barton, M.C., Deroo, T., Vleminckx, K., Moon, R.T. and McCrea, P.D. (2005) Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell*, **8**, 843-854.

Parker, D.S., Jemison, J. and Cadigan, K.M. (2002) Pygopus, a nuclear PHD-finger protein required for Wingless signaling in Drosophila. *Development*, **129**, 2565-2576.

Pereira, L., Yi, F. and Merrill, B.J. (2006) Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal. *Mol Cell Biol*, **26**, 7479-7491.

Peterson, C.L. and Workman, J.L. (2000) Promoter targeting and chromatin remodeling by the SWI/SNF complex. *Curr Opin Genet Dev*, **10**, 187-192.

Polakis, P. (2007) The many ways of Wnt in cancer. *Curr Opin Genet Dev*, **17**, 45-51.

Poot, R.A., Bozhenok, L., van den Berg, D.L., Steffensen, S., Ferreira, F., Grimaldi, M., Gilbert, N., Ferreira, J. and Varga-Weisz, P.D. (2004) The Williams syndrome transcription factor interacts with PCNA to target chromatin remodelling by ISWI to replication foci. *Nat Cell Biol*, **6**, 1236-1244.

Poot, R.A., Dellaire, G., Hulsmann, B.B., Grimaldi, M.A., Corona, D.F., Becker, P.B., Bickmore, W.A. and Varga-Weisz, P.D. (2000) HuCHRAC, a human ISWI chromatin remodelling complex contains hACF1 and two novel histone-fold proteins. *Embo J*, **19**, 3377-3387.

Prokhortchouk, A., Sansom, O., Selfridge, J., Caballero, I.M., Salozhin, S., Aithozhina, D., Cerchietti, L., Meng, F.G., Augenlicht, L.H., Mariadason, J.M., Hendrich, B., Melnick, A., Prokhortchouk, E., Clarke, A. and Bird, A. (2006) Kaiso-deficient mice show resistance to intestinal cancer. *Mol Cell Biol*, **26**, 199-208.

Qian, C., Zhang, Q., Li, S., Zeng, L., Walsh, M.J. and Zhou, M.M. (2005) Structure and chromosomal DNA binding of the SWIRM domain. *Nat Struct Mol Biol*, **12**, 1078-1085.

Radtke, F. and Clevers, H. (2005) Self-renewal and cancer of the gut: two sides of a coin. *Science*, **307**, 1904-1909.

Reya, T. and Clevers, H. (2005) Wnt signalling in stem cells and cancer. *Nature*, **434**, 843-850.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.C., Grosschedl, R. and Bienz, M. (1997) LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell*, **88**, 777-787.

Rocheleau, C.E., Downs, W.D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.H., Ali, M., Priess, J.R. and Mello, C.C. (1997) Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. *Cell*, **90**, 707-716.

Roose, J., Huls, G., van Beest, M., Moerer, P., van der Horn, K., Goldschmeding, R., Logtenberg, T. and Clevers, H. (1999) Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science*, **285**, 1923-1926.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature*, **395**, 608-612.

Roth, W., Sustmann, C., Kieslinger, M., Gilmozzi, A., Irmer, D., Kremmer, E., Turck, C. and Grosschedl, R. (2004) PIASy-deficient mice display modest defects in IFN and Wnt signaling. *J Immunol*, **173**, 6189-6199.

Rottbauer, W., Saurin, A.J., Lickert, H., Shen, X., Burns, C.G., Wo, Z.G., Kemler, R., Kingston, R., Wu, C. and Fishman, M. (2002) Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell*, **111**, 661-672.

Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F. and Grosschedl, R. (2001) PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev*, **15**, 3088-3103.

Saha, A., Wittmeyer, J. and Cairns, B.R. (2006) Chromatin remodelling: the industrial revolution of DNA around histones. *Nat Rev Mol Cell Biol*, **7**, 437-447.

Sakamoto, I., Kishida, S., Fukui, A., Kishida, M., Yamamoto, H., Hino, S., Michiue, T., Takada, S., Asashima, M. and Kikuchi, A. (2000) A novel beta-catenin-binding protein inhibits beta-catenin-dependent Tcf activation and axis formation. *J Biol Chem*, **275**, 32871-32878.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J. and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature*, **419**, 407-411.

Santos-Rosa, H., Schneider, R., Bernstein, B.E., Karabetsou, N., Morillon, A., Weise, C., Schreiber, S.L., Mellor, J. and Kouzarides, T. (2003) Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. *Mol Cell*, **12**, 1325-1332.

Satoh, K., Kasai, M., Ishidao, T., Tago, K., Ohwada, S., Hasegawa, Y., Senda, T., Takada, S., Nada, S., Nakamura, T. and Akiyama, T. (2004) Anteriorization of neural fate by inhibitor of beta-catenin and T cell factor (ICAT), a negative regulator of Wnt signaling. *Proc Natl Acad Sci U S A*, **101**, 8017-8021.

Schweizer, L., Nellen, D. and Basler, K. (2003) Requirement for Pangolin/dTCF in Drosophila Wingless signaling. *Proc Natl Acad Sci U S A*, **100**, 5846-5851.

Sharma, R.P. (1973) wingless, a new mutant in D. melanogaster. *Dros Inf Service,* **50**, 134.

Shetty, P., Lo, M.C., Robertson, S.M. and Lin, R. (2005) C. elegans TCF protein, POP-1, converts from repressor to activator as a result of Wnt-induced lowering of nuclear levels. *Dev Biol*, **285**, 584-592.

Shogren-Knaak, M., Ishii, H., Sun, J.M., Pazin, M.J., Davie, J.R. and Peterson, C.L. (2006) Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science*, **311**, 844-847.

Shulewitz, M., Soloviev, I., Wu, T., Koeppen, H., Polakis, P. and Sakanaka, C. (2006) Repressor roles for TCF-4 and Sfrp1 in Wnt signaling in breast cancer. *Oncogene*, **25**, 4361-4369.

Sierra, J., Yoshida, T., Joazeiro, C.A. and Jones, K.A. (2006) The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. *Genes Dev*, **20**, 586-600.

Smith, C.L., Peterson, C.L. (2005) ATP-dependent chromatin remodeling. *Curr Top Dev Biol*, **65**:115-48.

Stadeli, R., Hoffmans, R. and Basler, K. (2006) Transcription under the control of nuclear Arm/beta-catenin. *Curr Biol*, **16**, R378-385.

Standley, H.J., Destree, O., Kofron, M., Wylie, C. and Heasman, J. (2006) Maternal XTcf1 and XTcf4 have distinct roles in regulating Wnt target genes. *Dev Biol*, **289**, 318-328.

Stoick-Cooper, C.L., Moon, R.T. and Weidinger, G. (2007) Advances in signaling in vertebrate regeneration as a prelude to regenerative medicine. *Genes Dev*, **21**, 1292-1315.

Stopka, T. and Skoultchi, A.I. (2003) The ISWI ATPase Snf2h is required for early mouse development. *Proc Natl Acad Sci U S A*, **100**, 14097-14102.

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41-45.

Straub, T., Dahlsveen, I.K. and Becker, P.B. (2005) Dosage compensation in flies: mechanism, models, mystery. *FEBS Lett*, **579**, 3258-3263.

Strohner, R., Nemeth, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., Langst, G. and Grummt, I. (2001) NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. *Embo J*, **20**, 4892-4900.

Strohner, R., Wachsmuth, M., Dachauer, K., Mazurkiewicz, J., Hochstatter, J., Rippe, K. and Langst, G. (2005) A 'loop recapture' mechanism for ACF-dependent nucleosome remodeling. *Nat Struct Mol Biol*, **12**, 683-690.

Tago, K., Nakamura, T., Nishita, M., Hyodo, J., Nagai, S., Murata, Y., Adachi, S., Ohwada, S., Morishita, Y., Shibuya, H. and Akiyama, T. (2000) Inhibition of Wnt signaling by ICAT, a novel beta-catenin-interacting protein. *Genes Dev*, **14**, 1741-1749.

Taipale, J. and Beachy, P.A. (2001) The Hedgehog and Wnt signalling pathways in cancer. *Nature*, **411**, 349-354.

Takemaru, K., Yamaguchi, S., Lee, Y.S., Zhang, Y., Carthew, R.W. and Moon, R.T. (2003) Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless

pathway. *Nature*, **422**, 905-909.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C.C., Lin, X. and Heasman, J. (2005) Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell*, **120**, 857-871.

Thorpe, C.J. and Moon, R.T. (2004) nemo-like kinase is an essential co-activator of Wnt signaling during early zebrafish development. *Development*, **131**, 2899-2909.

Thorpe, C.J., Schlesinger, A., Carter, J.C. and Bowerman, B. (1997) Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. *Cell*, **90**, 695-705.

Tremethick, D.J. (2007) Higher-order structures of chromatin: the elusive 30 nm fiber. *Cell*, **128**, 651-654.

Tsukiyama, T. (2002) The in vivo functions of ATP-dependent chromatin-remodelling factors. *Nat Rev Mol Cell Biol*, **3**, 422-429.

Tsukiyama, T., Daniel, C., Tamkun, J. and Wu, C. (1995) ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell*, **83**, 1021-1026.

Tsukiyama, T., Palmer, J., Landel, C.C., Shiloach, J. and Wu, C. (1999) Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in Saccharomyces cerevisiae. *Genes Dev*, **13**, 686-697.

Tsukiyama, T. and Wu, C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell*, **83**, 1011-1020.

Tutter, A.V., Fryer, C.J. and Jones, K.A. (2001) Chromatin-specific regulation of LEF-1-beta-catenin transcription activation and inhibition in vitro. *Genes Dev*, **15**, 3342-3354.

Valenta, T., Lukas, J., Doubravska, L., Fafilek, B. and Korinek, V. (2006) HIC1 attenuates Wnt signaling by recruitment of TCF-4 and beta-catenin to the nuclear bodies. *Embo J*, **25**, 2326-2337.

Valenta, T., Lukas, J. and Korinek, V. (2003) HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target

Axin2/Conductin in human embryonic kidney cells. *Nucleic Acids Res*, **31**, 2369-2380.

van Amerongen, R. and Berns, A. (2006) Knockout mouse models to study Wnt signal transduction. *Trends Genet*, **22**, 678-689.

van de Wetering, M., Oosterwegel, M., Dooijes, D. and Clevers, H. (1991) Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *EMBO J,* **10**, 123-32.

van Holde, K. and Yager, T. (2003) Models for chromatin remodeling: a critical comparison. *Biochem Cell Biol*, **81**, 169-172.

van Vugt, J.J., Ranes, M., Campsteijn, C. and Logie, C. (2007) The ins and outs of ATP-dependent chromatin remodeling in budding yeast: biophysical and proteomic perspectives. *Biochim Biophys Acta*, **1769**, 153-171.

Vanolst, L., Fromental-Ramain, C. and Ramain, P. (2005) Toutatis, a TIP5-related protein, positively regulates Pannier function during Drosophila neural development. *Development*, **132**, 4327-4338.

Waltzer, L. and Bienz, M. (1998) Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature*, **395**, 521-525.

Wang, S. and Jones, K.A. (2006) CK2 controls the recruitment of Wnt regulators to target genes in vivo. *Curr Biol,* **16**,2239-44.

Weiske, J. and Huber, O. (2005) The histidine triad protein Hint1 interacts with Pontin and Reptin and inhibits TCF-beta-catenin-mediated transcription. *J Cell Sci*, **118**, 3117-3129.

Whitehouse, I. and Tsukiyama, T. (2006) Antagonistic forces that position nucleosomes in vivo. *Nat Struct Mol Biol*, **13**, 633-640.

Wysocka, J., Swigut, T., Xiao, H., Milne, T.A., Kwon, S.Y., Landry, J., Kauer, M., Tackett, A.J., Chait, B.T., Badenhorst, P., Wu, C. and Allis, C.D. (2006) A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature*, **442**, 86-90.

Xi, R. and Xie, T. (2005) Stem cell self-renewal controlled by chromatin remodeling factors. *Science*, **310**, 1487-1489.

Xiao, H., Sandaltzopoulos, R., Wang, H.M., Hamiche, A., Ranallo, R., Lee, K.M., Fu, D. and Wu, C. (2001) Dual functions of largest NURF subunit NURF301 in nucleosome sliding and transcription factor interactions. *Mol Cell*, **8**, 531-543.

Yamaguchi, T.P., Takada, S., Yoshikawa, Y., Wu, N. and McMahon, A.P. (1999) T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. *Genes Dev*, **13**, 3185-90.

Yang, X., van Beest, M., Clevers, H., Jones, T., Hursh, D.A. and Mortin, M.A. (2000) decapentaplegic is a direct target of dTcf repression in the Drosophila visceral mesoderm. *Development*, **127**, 3695-3702.

Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P. and Kohwi-Shigematsu, T. (2002) SATB1 targets chromatin remodelling to regulate genes over long distances. *Nature*, **419**, 641-645.

Zecca, M., Basler, K. and Struhl, G. (1996) Direct and long-range action of a wingless morphogen gradient. *Cell*, **87**, 833-844.

Zeng, Y.A. and Verheyen, E.M. (2004) Nemo is an inducible antagonist of Wingless signaling during Drosophila wing development. *Development*, **131**, 2911-2920.

Zhang, C., Basta, T., Jensen, E.D. and Klymkowsky, M.W. (2003) The beta-catenin/VegT-regulated early zygotic gene Xnr5 is a direct target of SOX3 regulation. *Development*, **130**, 5609-5624.

Zhang, Z. and Reese, J.C. (2004) Ssn6-Tup1 requires the ISW2 complex to position nucleosomes in Saccharomyces cerevisiae. *EMBO J,* **23**, 2246-57.

Zhou, Y. and Grummt, I. (2005) The PHD finger/bromodomain of NoRC interacts with acetylated histone H4K16 and is sufficient for rDNA silencing. *Curr Biol*, **15**, 1434-1438.

Zorn, A.M., Barish, G.D., Williams, B.O., Lavender, P., Klymkowsky, M.W. and Varmus, H.E. (1999) Regulation of Wnt signaling by Sox proteins: XSox17 alpha/beta and XSox3 physically interact with beta-catenin. *Mol Cell*, **4**, 487-498.

CHAPTER II

TOUTATIS AND ACF1, TWO RELATED CHROMATIN REMODELERS, REDUNDANTLY ANTAGONIZE WINGLESS SIGNALING IN *DROSOPHILA*

Abstract

Increasing evidence suggests that chromatin plays an important role in transcriptional regulation of eukaryotic genes. We have identified Tou and Acf1, two closely related proteins in ISWI family chromatin remodeling complexes, as novel antagonists of Wnt signaling. Overexpression of *tou* or *acf1* consistently blocks Wg signaling in fly eyes and wings. Loss of *tou* or *acf1* has subtle or no detectable phenotype, respectively. In contrast, *tou/acf1* double mutants have a more severe phenotype, indicating functional redundancy between these two genes. Consistent with the fly data, Tou and Acf1 redundantly repress Wnt targets expression in both fly and human cells. We conclude that Tou and Acf1 are not absolutely required for Wnt signaling, yet they might act as modulators in fine-tuning the pathway. The possibilities that a third protein acts redundantly with Tou/Acf1 in flies and that Tou and Acf1 repress Wnt targets in a tissue/cell specific manner are discussed.

Introduction

Eukaryotic DNA is packaged into a highly organized chromatin structure, which presents an inhibitory barrier for protein-DNA interactions (Khorasanizadeh, 2004). Regulation of chromatin influences many biological processes that require specific protein-DNA contacts, such as transcription. The "opening" and "closing" of chromatin have been linked to the transcriptional activation and repression, respectively. Previous studies showed that two major mechanisms, covalent modification of histone tails and ATP-dependent chromatin remodeling, play pivotal roles in altering the structure and position of nucleosomes to regulate transcription (Narlikar et al., 2002; Li et al., 2007).

ATP-dependent chromatin remodeling complexes utilize the energy of ATP hydrolysis to regulate the DNA accessibility to the interacting proteins (Becker and Horz, 2002). Based on the identity of their conserved ATPase subunit, they can be classified into several families, including SWI/SNF, ISWI, CHD and INO80. All family members have been implicated in transcriptional regulation, though the specific mechanisms of the regulation and the particular biological events in which they are involved differ between families (Bouazoune and Brehm, 2006).

Several ISWI family chromatin remodeling complexes have been identified in *Drosophila*, including ACF, CHRAC and NURF (Langst and Becker, 2001; Corona and Tamkun, 2004). Besides the shared ATPase ISWI, these complexes contain additional cofactors which endow them with unique biochemical activities. In the case of ATP-utilizing chromatin assembly and remodeling factor (ACF) which consists of ISWI

and ACF1, it has been shown that interaction of ACF1 with ISWI increases nucleosome sliding efficiency by an order of magnitude (Ito et al., 1999; Eberharter et al., 2001; Eberharter et al., 2004). This suggests that the ACF1 subunit significantly contributes to the chromatin remodeling activity of ISWI. The ACF complex was initially purified from fly embryos, and extensive biochemical analysis has revealed that it exhibits strong chromatin assembly and nucleosomal remodeling activities *in vitro* (Fyodorov and Kadonaga, 2002). The mammalian counterpart of ACF has been indicated in regulating heterochromatic DNA replication in several cell lines (Collins et al., 2002; Poot et al., 2004). Surprisingly, *acf1* mutant flies survive to adulthood, only displaying mild phenotypes (Fyodorov et al., 2004). This suggests that ACF1 act redundantly with at least one additional factor to mediate chromatin remodeling *in vivo*.

The closest protein to ACF1 in the fly genome is called Toutatis (Tou). *tou* was initially found in a genetic screen where a *tou* mutant suppresses the extra-sex-combs phenotype of *polyhomeotic (ph)*. Since *ph* is a gene in the polycomb group, whose function is thought to maintain the repression state of homeotic genes, *tou* was considered to be a new trithorax group gene, which presumably is involved in transcriptional activation of homeotic genes (Fauvarque et al., 2001). Recently, a more rigorous study of Tou has shown that it interacts with ISWI, and they cooperate with the GATA factor Pannier (Pnr) to positively regulate preneural gene expression *in vivo* (Vanolst et al., 2005). One human homologue of Tou is TIP5 (also called BAZ2A), which has been shown to be co-purifed with a human ISWI (SNF2h) in a complex termed NoRC

(nucleolar remodeling complex) (Strohner er al., 2001). Extensive studies of this complex revealed that NoRC remodels chromatin similarly as ACF *in vitro*, and that NoRC silences ribosomal genes transcription *in vivo* (Zhou et al., 2002; Strohner et al., 2004). The repression of rDNA is thought to be achieved through NoRC mediated histone H4 deacetylation, histone H3K9 dimethylation, and DNA methylation (Santoro et al., 2002). This provides a good example that covalent modification of DNA/histone tails and ATP-dependent chromatin remodeling coordinately regulate transcriptional repression.

These previous studies suggest that Tou/TIP5 are versatile proteins that might be involved in a wide array of biological processes. One context we are particularly interested in is the Wg/Wnt signaling pathway, which is important for many aspects of animal development (Logan and Nusse, 2004). The transcriptional regulation of Wnt targets revolves around the DNA-binding protein TCF/LEF-1 and its binding partner Armadillo//β-catenin (Arm/β-cat). In the quiescent state, TCF interacts with the co-repressor Groucho and represses Wnt target expression. Upon Wnt stimulation, Arm/β-cat becomes stabilized and enters the nucleus, where it complexes with TCF to activate Wnt targets by displacing Groucho and recruiting additional co-activators (Parker *et al.*, 2007). It has been shown that chromatin structure is important for these regulations, and several chromatin remodelers have been implicated in the pathway (Tutter *et al.*, 2001). These include the SWI/SNF type ATPase Brg-1/Brahma and its binding partner Osa, and the INO80 subfamily proteins Pontin52 and Reptin52 (Collins

and Treisman, 2000; Barker *et al.*, 2001; Rottbauer *et al.*, 2002). The mechanisms of these proteins' action in mediating Wnt targets expression remain elusive.

In this study, we implicate Tou and ACF1 as transcriptional repressors in Wg signaling in *Drosophila*. Overexpression of Tou or ACF1 antagonizes Wg signaling in the eye and wing. However, neither *tou* nor *acf1* mutants reveal misregulation of Wg targets. Interestingly, mutation of both *tou* and *acf1* exhibits a stronger phenotype in flies. In addition, depletion of these factors causes activation of Wnt target genes in both fly and mammalian cell culture. These results provide the first evidence that Tou and ACF1 act redundantly in fine-tuning the repression of Wnt targets.

Results

A misexpression screen in the *Drosophila* eye designed to identify antagonists of Wg signaling

Drosophila has a typical insect compound eye (Fig. 2.1A). When *wingless (wg)* is placed under the control of the eye-specific promoter *GMR*, a severe reduction in eye size is observed (Fig. 2.1B). To identify novel negative regulators in the Wg pathway, a misexpression screen was performed by crossing the GMR-Gal4/UAS-*wg* line to transposon insertion lines, which contain a single P-element transposon with UAS sites upstream of a proximal promoter (Rorth, et al., 1998). These transposons, called EP elements, are known to preferentially insert into the 5' promoter region of genes, placing them under Gal4 control. As a result, *wg* and the gene near the EP insertion are

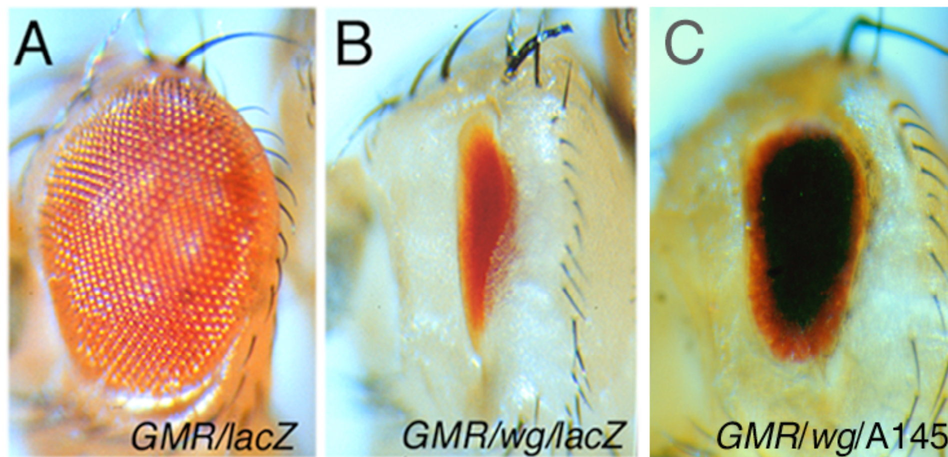

Figure 2.1 Misexpression of *tou* (via the A145 insertion) suppresses a Wg signaling-dependent small eye phenotype. Pictures are micrographs of adult *Drosophila* eyes. P[*GMR-Gal4*]; P[*UAS-lacZ*] eyes are identical to wild-type (A). P[*GMR-Gal4*], P[*UAS-wg*] eyes are greatly reduced (B), while P[*GMR-Gal4*], P[*UAS-wg*]; GSV[A145] eyes are significantly bigger (C). Overexpression of three other GSV insertions (A41, A80, A428) at *tou* locus also suppresses GMR-*wg* induced small eye (data not shown). All crosses were performed at 25°C. Figure (A) and (B) are courtesy from Parker et al., 2002.

co-overexpressed in the fly eye, and the EP lines with an obvious suppression of the small eye phenotype were selected.

The primary screen performed by Kenneth Cadigan and Jemileh Jemison identified about 100 lines that significantly increase the size of GMR/*wg* eyes. Several of these lines are inserted into known negative regulators in the pathway (e.g. Axin and GSK3). One insertion was in a novel gene called *pygopus* (*pygo*), which has since been shown to be an essential factor in Wg signaling (Parker et al., 2002; Kramps et al., 2002; Belenkaya et al., 2002; Thompson et al., 2002). Two other genes identified from this screen, the histone acetyltransferase Creb-binding protein (CBP) and C-terminal-binding protein (CtBP), also provided fruitful insights on the transcriptional mechanisms of Wg signaling (Fang et. al., 2006; Li et. al., 2007). There are four lines from the screen that are inserted into a gene called *toutatis* (*tou*). My work has been focusing on the characterization of its role in Wg signaling.

Overexpression of Toutatis (Tou), a chromatin remodeling protein, blocks Wg signaling

GSV lines are the derivatives of EP lines (Toba et al., 1999), and have UAS sites engineered onto both ends of P-element, allowing them to drive gene expression on both sides of P-element. Consistently, our lab has identified more positives from the primary screen with GSV lines than positive EP lines. Four GSV lines, A41, A145, A80 and A428 are inserted in the first intron of *tou* locus (Fig. 2.3A). Given the close proximity

of GSV insertions to *tou,* we considered *tou* as the most likely gene responsible for the overexpression phenotypes.

Overexpression of *tou* in the eye significantly suppresses the GMR/*wg* induced small eye phenotype (Fig. 2.1C), suggesting that it antagonizes Wg signaling. Overexpression of *tou* also suppresses the small eye phenotype induced by a stabilized form of Arm, implying that Tou acts downstream of Arm stabilization in Wg signaling (data not shown). In addition, *tou* overexpression in the wing imaginal disc gives phenotypes consistent with a loss of Wg signaling. Before pupation, the *Drosophila* wing imaginal disc is a flat columnar epithelia sheet with *wg* expressed in a stripe at the dorsal/ventral boundary (Cadigan, 2002; Fig. 2.2A). Wg signaling represses its own expression in this region (Rulifson et al., 1996; Cadigan et al., 1998) and activates the proneural gene *senseless* (*sens*) on either side of the Wg stripe (Parker et al., 2002; Fig. 2.2B). Loss of Wg signaling leads to a derepression of Wg and loss of Sens. This is exactly what was observed when *tou* was misexpressed in the posterior half of the wing disc using the *Engrailed-Gal4* (*En-Gal4*) driver (Fig. 2.2A-C). Taken together, analysis of three Wg readouts in the eye and wing suggests that when overexpressed, Tou blocks Wg signaling.

A cartoon of the *tou* locus including several adjacent genes is depicted in Fig. 2.3. Since enhancers can act over long distances and independently of orientation, it is possible that some other gene(s) around the GSV insertion sites could be responsible for the phenotypes. The fact that the GSV lines we used are bi-directional increases the

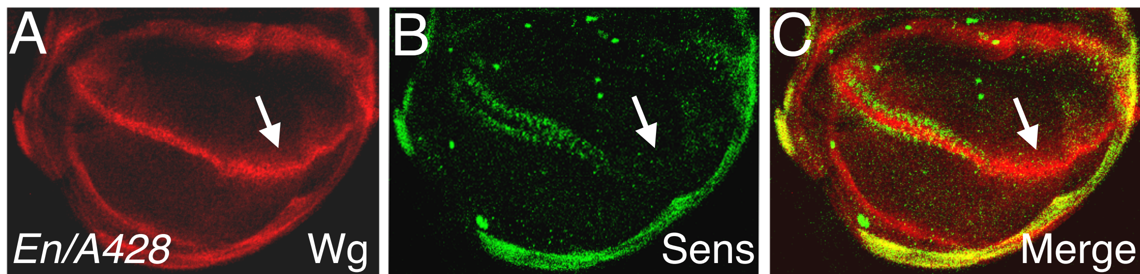

Figure 2.2 Overexpression of *tou* (via the A428 insertion) blocks Wg signaling in the wing. Wg is expressed in a stripe pattern at the dorsal/ventral boundary (A). Sens is activated by Wg signaling on either side of the D/V boundary (B). Tou is overexpressed in the posterior part of the wing imaginal disc (anterior is to the left) using *En-Gal4*. Compared to the left half, Wg stripe (red) is expanded (A) and Sens expression (green) is greatly reduced (B). Overexpression of three other GSV lines (A41/A80/A145) at the *tou* locus results in similar phenotypes. All crosses were performed at 25°C.

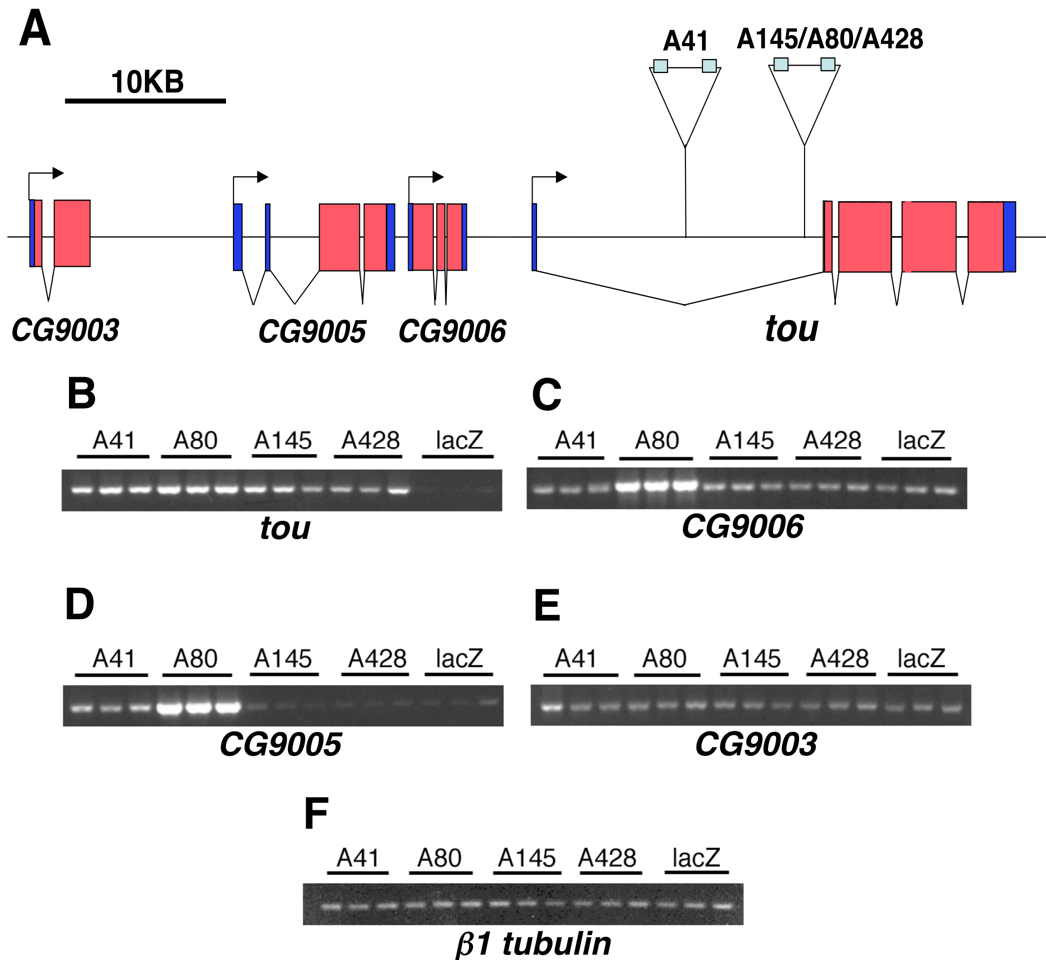

Figure 2.3 All four GSV lines induce *tou* expression. (A) Schematic diagram of the *tou* locus with three upstream genes included. The closest annotated gene downstream of *tou* is *engrailed (en)*, which is 50KB away (not shown). The insertion sites of four GSV lines are also illustrated, and the light blue boxes on the P-elements denote UAS sites. Although A145, A80 and A428 are drawn together for simplicity, their insertion sites are not exactly the same (within 100bp distance). Arrows represents the transcriptional start sites of respective genes. The red and blue boxes represent ORFs and UTRs of respective genes. For simplicity, small introns of these genes are not shown. (B-F) Each GSV line was crossed to heat-shock-Gal4, and the progenies at the third instar larva stage were subject to heat shock stimulation before the RNA was isolated for RT-PCR analysis. UAS-lacZ line serves as a control for heat shock induction, and *β1 tubulin* serves as a loading control for PCR. A series dilution of cDNA pool was tested to ascertain that PCR products were within the linear range (data not shown). All data were collected in triplicate. Evidently, only *tou* is induced by all four GSV line.

likelihood of such possibility. I used heat-shock-Gal4 to induce each of the GSV lines in the *tou* locus, and examined which gene(s) are induced by semi-quantitative RT-PCR. As shown in Fig 2.3, *tou* is significantly induced in all four GSV lines. Interestingly, one GSV line, A80, also induces the expression of two nearby genes (CG9006 and CG9005), supporting the notion that the UASs in P[GSV] can act over long distances (more than 30 kb) to drive gene expression. Nevertheless, the observation that all four GSV lines give a qualitatively similar phenotypes in the eye and wing strongly suggests that *tou* is the responsible gene for these phenotypes. Additional support came from the observation that when unidirectional EP lines inserted in the *tou* locus were used for phenotypic analysis, only those lines with the correct orientation for *tou* overexpression suppressed the GMR/*wg* induced small eye (data not shown).

Gene finding programs and the EST database predict four isoforms of *tou*. The largest isoform and one of the small isoforms are depicted in Fig. 2.5. The largest isoform encodes a large protein (3109 amino acids) with several motifs consistent with a function in chromatin remodeling. DDT and PHD domains are protein-protein interaction domains often found in transcription factors or chromatin remodeling molecules (Aasland et al., 1995; Doerks et al., 2001). Proteins containing BROMO domains can bind acetylated histone tails (Zeng and Zhou, 2002). The MBD domain can bind methylated DNA (Rountree et al., 2001), which is often related to gene silencing. The three small isoforms of *tou* encode proteins with the PHD and BROMO domains only. The closest relative of *tou* in the fly genome is *acf1*, which encodes a protein

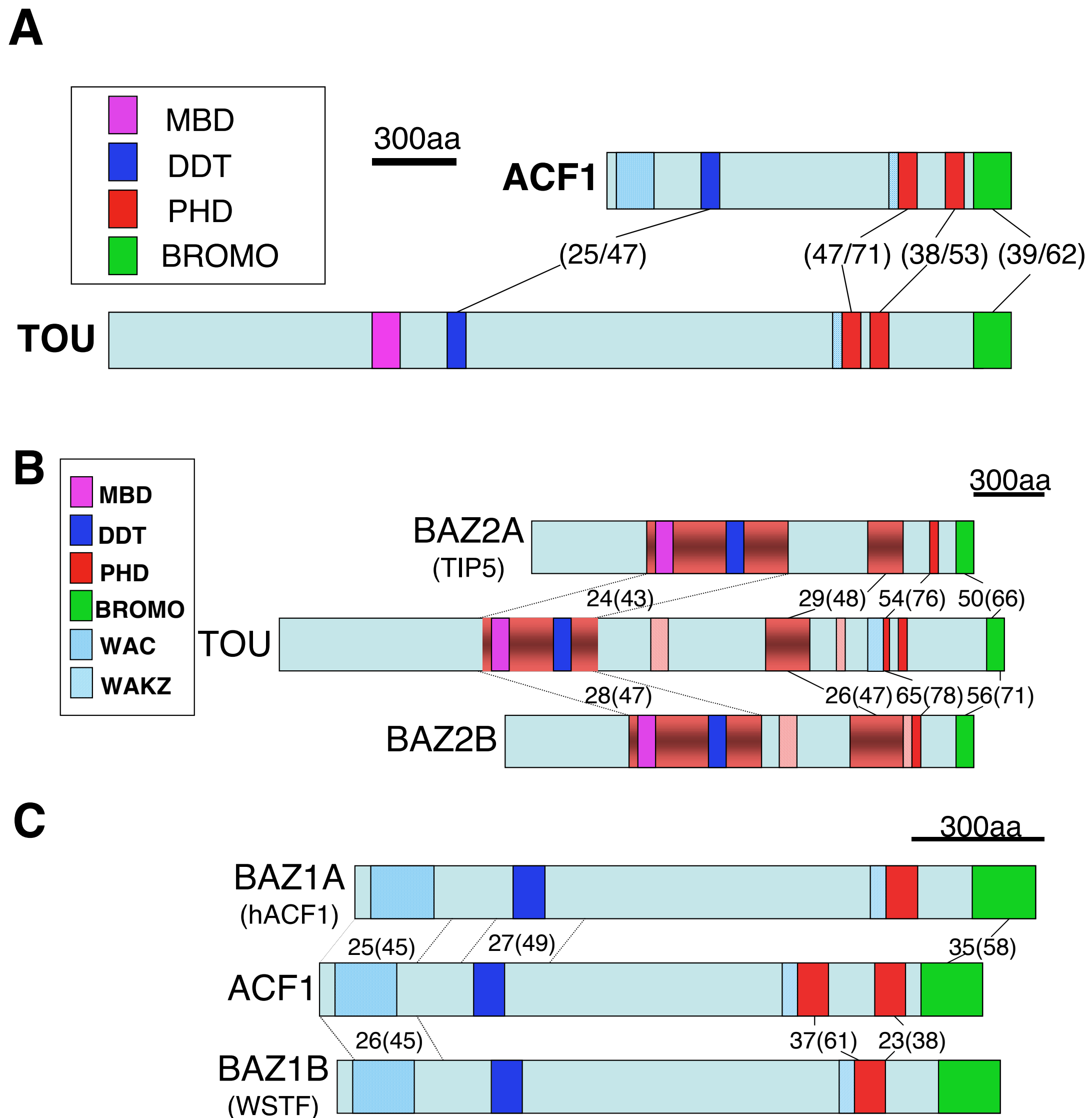

Figure 2.4 Diagram of Tou and its related protein ACF1, and their human homologues. (A) Alignment of Tou and ACF1 proteins. The numbers represent percentage of identity/similarity. The two hatched boxes are two novel conserved regions termed WAC (blue checkers) and WAKZ (blue stripes), respectively (Ito et al., 1999). (B) Human homologues of Tou. The two large dark-red boxes are regions conserved between all three proteins. There are two smaller red hatched boxes conserved only between Tou and BAZ2B. (C) Human homologues of ACF1. Both WAC and WAKZ domains are conserved between all three proteins.

containing DDT, PHD and BROMO domains (Fig. 2.4A). ACF1 was identified biochemically as a subunit of the ACF and CHRAC chromatin-remodeling complexes, which efficiently catalyze ATP-dependent chromatin assembly and nucleosomal sliding *in vitro* (Ito et al., 1999; Eberharter et al., 2001; Fyodorov and Kadonaga, 2002).

***tou* null mutants reveal no obvious defect in Wg signaling.**

When overexpressed, Tou is a potent inhibitor of Wg signaling in the eye and wing. However, the physiological significance of Tou in Wg signaling can best be determined by examining its loss-of-function phenotypes. The original *tou* allele is a transposon insertion line ~900bp upstream of the *tou* transcriptional start site (Fauvarque et al., 2001). It is homozygous viable with a subtle downward wing phenotype which may or may not be due to a change in Wg signaling. To obtain stronger or null alleles of *tou*, I carried out an imprecise excision screen in flies. The underlying principle is that when a transposon is mobilized, random deletions surrounding its insertion site can be generated (Robertson et al., 1988). Using this strategy, I successfully obtained several deletions that take away the ATG of the largest Tou isoform. The biggest deletion ($tou^{20\text{-}4}$) reaches 6.6kB downstream of ATG, and both MBD and DDT domains are deleted (Fig. 2.5). A smaller deletion ($tou^{4\text{-}10}$), which reaches 3kB downstream of ATG, was also used for later analysis (Fig. 2.5).

When $tou^{20\text{-}4}$ flies were crossed to $tou^{4\text{-}10}$ flies or several deficiencies which presumably remove the entire *tou* region (*Df(SFX31), Df(enA), Df(enB)*), the transheterozygous flies are semi-lethal with subtle downward or shriveled wing

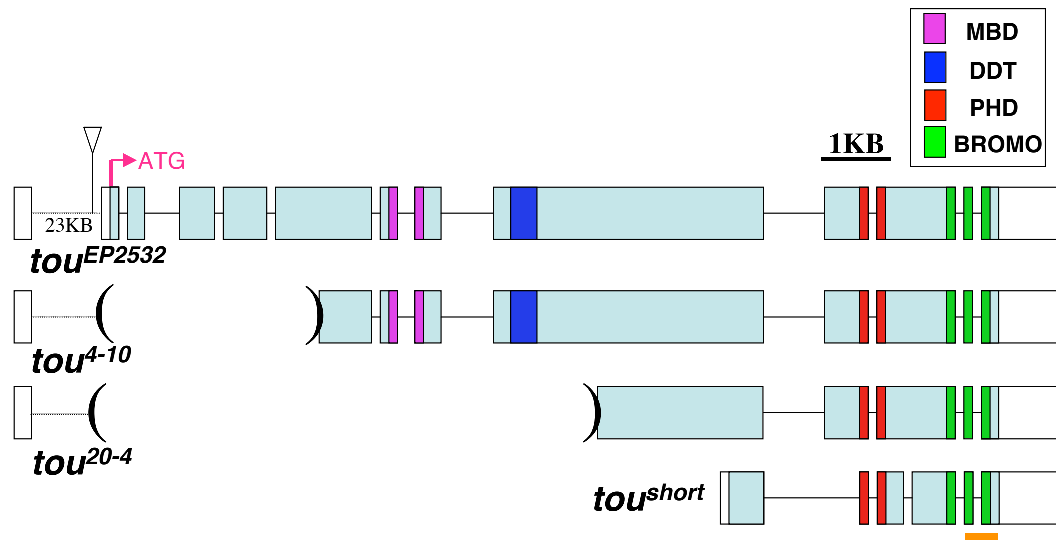

Figure 2.5 Depiction of two isoforms of *tou* and *tou* mutants. The white region and the light-blue region represent the UTR and ORF of *tou*, respectively. The other colored boxes represent the indicated protein domains. The first intron of *tou* is not drawn in proportion. The insertion site of EP2532, used for imprecise excision of *tou*, is indicated in the first intron. $tou^{4\text{-}10}$ and $tou^{20\text{-}4}$ have a 3.0kB and a 6.6kB deletion of *tou*, respectively. Neither of these deletions removes the potential short isoforms of *tou*. The short yellow line at the 3' end of *tou* ORF indicates the region targeted by *tou* RNAi.

<table>
<tr><th></th><th></th><th>Downward wings</th><th>Shriveled wings</th><th>Percentage of lethality</th></tr>
<tr><td rowspan="4">tou$^{20-4}$</td><td>Df(SFX31)</td><td>√</td><td></td><td>42%</td></tr>
<tr><td>Df(enA)</td><td></td><td>√</td><td>8%</td></tr>
<tr><td>Df(enB)</td><td></td><td>√</td><td>4%</td></tr>
<tr><td>tou$^{4-10}$</td><td>√</td><td></td><td>24%</td></tr>
<tr><td rowspan="3">tou$^{4-10}$</td><td>Df(SFX31)</td><td>√</td><td></td><td>52%</td></tr>
<tr><td>Df(enA)</td><td colspan="2">√</td><td>35%</td></tr>
<tr><td>Df(enB)</td><td colspan="2">√</td><td>36%</td></tr>
</table>

Table 2.1 Summary of phenotypes of *tou* mutant. Based on the cytogenetic information from flybase, all three deficiencies delete *tou* locus. *Df(enA)* and *Df(enB)* gives stronger wing phenotype, possible due to the deletion of additional genes in these two deficiencies. To calculate the percentage of lethality, the number of transheterozygote flies was counted, and was divided by the total number of F1 progenies. The resulting ratio was then normalized to that from a control cross, where a precise excision allele of *tou* from the same jumpout screen was used. Compared to the control, *tou* mutant flies suffer at least 50% decrease of viability. For each cross, 200-300 F1 progenies were counted for calculation. All crosses were performed at 25°C.

phenotypes. Table 2.1. summarizes *tou* phenotypes that I observed. Firstly, *tou* mutants are semi-lethal. When tou^{20-4} or tou^{4-10} were crossed to various deficiencies or to each other, I found that more than half of the progeny are dead (Table 2.1). The penetrance of lethality varies between crosses, probably due to different genetic backgrounds of deficiency lines or various residual Tou activities in *tou* mutants. Secondly, for those *tou* mutant flies that survive to adulthood, their wings bend downward and are held out, and in some extreme cases, their wings are deformed and shriveled. It is unclear what causes this phenotype, though it is not indicative of a major disruption of Wg signaling. Finally, I also carried out a backcross using transheterozygote female flies of the *tou* mutants, and did not observe a more severe phenotype in F2 progenies. This result suggests that depletion of maternal *tou* does not significantly enhance the *tou* mutant phenotype.

We suspect that tou^{20-4} may not be a null allele of *tou*, since it only deletes 5'-half of the largest transcript, and does not remove any sequences of the three predicted smaller transcripts. RT-PCR experiments indicate that these messages (and/or a truncated form of the larger transcript) are still expressed in tou^{20-4}/*Df(tou)* transheterozygotes (data not shown). Those smaller messages still include PHD and BROMO domains and could contribute to its function. To address this intragenic redundancy issue, I employed RNA interference under the control of inducible UAS promoter to conditionally knock down *tou* activity in flies (Lee and Carthew, 2003). A sequence specific for 3' portion of *tou* is used to target all four isoforms (indicated in Fig.

2.5). I crossed UAS- *tou*-RNAi transgenic lines to different Gal4 drivers, and examined various Wg readouts in fly embryos, eye and wing. For all tissues examined, no obvious Wg phenotypes were detected even when four strong lines of UAS-*tou*-RNAi were combined to boost the *tou* knockdown potential. Furthermore, a combination of $tou^{20\text{-}4}$ and *tou*-RNAi does not reveal more severe defects than the phenotype seen in $tou^{20\text{-}4}$ mutants. Collectively, these results suggest that $tou^{20\text{-}4}$ is a null allele, and disruption of *tou* itself does not result in manifest defect in Wg signaling.

Double mutant of *tou* and its potential redundant gene *acf1* reveals no obvious defect in Wg signaling

The lack of an obvious Wg signaling defect in *tou* mutants could be due to redundancy with another gene. The closest relative of *tou* in the fly genome is *acf1*, which encodes a protein containing DDT, PHD and BROMO domains (Fig. 2.4A). To address this intergenic redundancy issue, I performed both gain-of-function and loss-of-function analyses for *acf1*.

Overexpression of *acf1* under the control of the eye-specific promoter GMR suppresses the reduced eye phenotype caused by a stabilized form of Arm, suggesting that ACF1 plays an antagonistic role in Wg signaling, acting downstream of Arm stabilization (data not shown). There is one EP line (EP1181), which is inserted in the first intron of *acf1* gene downstream of ATG site (Fig 2.6). By adopting the similar imprecise excision strategy used for making *tou* mutants, I generated several *acf1*

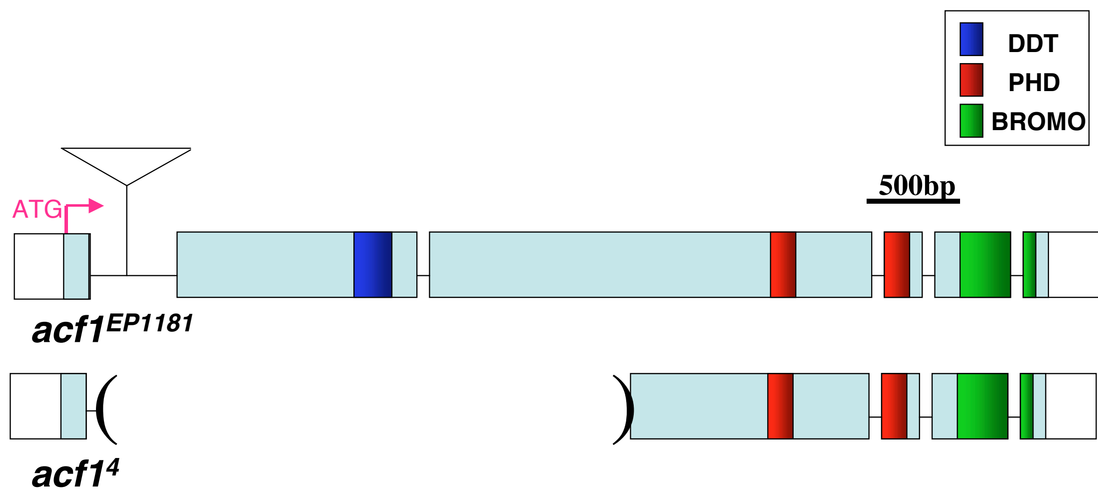

Figure 2.6 Depiction of *acf1* mutant. The white region and the light-blue region represent the UTR and ORF of *acf1* respectively. Other colored boxes are annotated domains of ACF1. The insertion site of EP1181, used for imprecise excision of *acf1*, is indicated in the first intron. *$acf1^4$* has a 2.8kB deletion of *acf1*.

deletions via mobilizing EP1181. The biggest deletion (*acf1*^{4}) excises 5'-half of the gene and is likely to be a null allele (Fig. 2.6).

The *acf1*^{4} homozygous flies survive to adulthood without any obvious phenotypes. Around the same time, another laboratory also generated several *acf1* mutants using the same strategy, and reported that *acf1* mutants are semi-lethal (Fyodorov et al., 2004). Since all their *acf1* deletions are smaller than *acf1*^{4} and their *acf1* deletions are likely to be null alleles based on the Western blot, the discrepancy between two labs' observation for *acf1* mutant phenotype could be due to the different genetic backgrounds. In either case, the *acf1* mutant itself reveals only subtle phenotypes (Fyodorov et al., 2004).

To examine whether *tou* and *acf1* act redundantly, I generated *tou/acf1* double mutant flies. Interestingly, we observed that *tou*$^{20\text{-}4}$/*acf1*^{4} double homozygotes are lethal at late pupal stage, which is more severe than the phenotype seen in *tou*$^{20\text{-}4}$ or *acf1*^{4} flies. This result suggests that Tou and ACF1 act redundantly during animal development. It is unclear what biological process is disturbed in *tou/acf1* mutant for such a generic phenotype. When the double mutant flies were dissected out from the pupal cases, we did not observe evident pattern defects for different tissues including the wing, the eye and the leg. In addition, immunostaining of several known Wg targets (Sens, Wg and Dll) in the developing wing displays normal expression patterns in *tou/acf1* mutants. We observed similar results when the maternal contributions of both *tou* and *acf1* were removed (data not shown). These data indicate that Wg signaling is not obviously disrupted in *tou/acf1* mutants.

Tou and ACF1 act redundantly to repress Wnt targets in fly and human cell lines

The relatively subtle phenotype of *tou/acf1* mutants implies that Tou and ACF1 are not essential factors in Wg signaling. Some nuclear repressors in the Wnt pathway have been shown to act in a tissue/cell-specific manner or as modulators for fine-tuning of the pathway (Parker et al., 2007). It is possible that Tou and ACF1 act in a similar fashion, and analysis of *tou/acf1* mutant from a whole animal perspective may obscure a more subtle defect in Wnt signaling in certain tissues or cells. Exploration of a *Drosophila* cell culture system might be helpful for us to understand the role of Tou and ACF1 in Wg signaling as well as the underlying mechanisms of their action.

Previous work in our laboratory has shown that *Drosophila* embryonic Kc167 (Kc) cells have a strong transcriptional response to Wg signaling, including several endogenous targets (Fang et al., 2006; Li et al., 2007). One of these targets is *naked cuticle (nkd)*, which has been shown to be a Wg induced antagonist in flies (Zeng et al., 2000). In addition, RNAi works robustly in *Drosophila* cultured cells (Clemons et al., 2000), which greatly facilitates the loss-of-function analysis. To explore whether Tou and ACF1 play a role in Wg signaling in Kc cells, I knocked down the activity of *tou*, *acf1* or *tou/acf1* by RNAi, and observed a consistent derepression of *nkd* in the absence of Wg signaling revealed by quantitative RT-PCR (Fig.2.7A). *acf1* RNAi itself leads to a six-fold derepression of *nkd*, while *tou* RNAi itself has no obvious effect. Interestingly, *acf1/tou* RNAi results in a highest derepression of *nkd* (more than 10 fold), suggesting that they act redundantly. These results are consistent with the *tou* or *acf1*

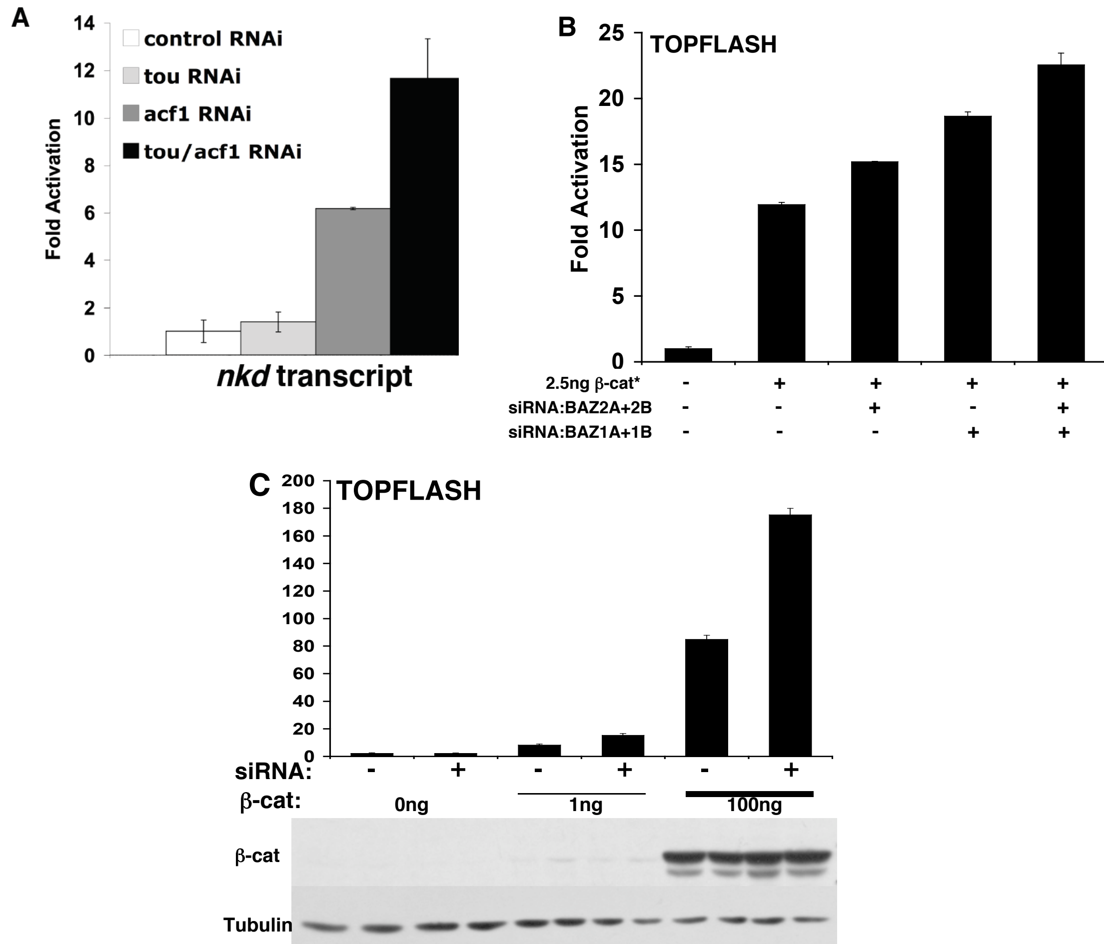

Figure 2.7 Depletion of *tou* and *acf1* activate Wg targets in fly and human cells. (A) *tou* and *acf1* RNAi activates Wg target gene *naked cuticle (nkd)* in fly Kc cells. Kc cells were treated with the indicated dsRNAs as described in Materials and methods. The transcript level for *nkd* was monitored by real-time PCR and normalized to the *β-tubulin56D* control. Experiments were done in duplicate. (B) Knockdown of *tou/acf1* homologues in human cells activates Wnt signaling. Human 293 cells were transiently transfected with pSUPER plasmids to inhibit different human *tou/acf1* homologues, and the Wnt response was detected by TOPFLASH assay. When 2.5ng β-cat was co-transfected, siRNA for all four human *tou/acf1* homologs gave the highest activation of the Wnt reporter. Each bar represents the result in duplicate with the standard deviation. The experiments were done at least five separate times, and similar results were observed. (C) β-cat level is not affected when human *tou/acf1* homologues are inhibited by siRNA. 293 cells were transfected with siRNA constructs for all four human homologues of *tou/acf1*. When no β-cat was co-transfected, no activation of TOPFLASH reporter could be observed. When 1ng or 100ng β-cat was co-transfected, a consistent two-fold further activation of TOPFLASH was observed. For all doses of β-cat tesed, no obvious change of its protein level was detected upon siRNA treatment.

overexpression phenotypes and the synthetic lethality phenotype of *tou/acf1* mutant in flies.

There are two genes in the human genome that appear to be homologues of *tou*. They have been called *BAZ2A* and *BAZ2B* and were identified in a database search (Jones et al., 2000; Fig 2.4B). There are also two genes called *BAZ1A* (*hACF1*) and *BAZ1B* (*WSTF*) that are homologues of *acf1* (Poot et al., 2000; Lu et al., 1998; Fig 2.4C). To explore whether the role of Tou and ACF1 in Wnt signaling is conserved in humans, I targeted the mRNAs of these *tou/acf1* human homologues for degradation by transfecting human embryonic kidney 293 cells with pSUPER plasmids expressing short RNA hairpins (McManus and Sharp, 2002). The effects on Wnt signaling were assayed using the TOPFLASH reporter, which contains multiple TCF binding sites upstream of the c-fos proximal promoter and luciferase ORF (Korinek et al., 1997). When a slight amount of β-catenin is co-transfected, reporter activation can be consistently observed when *tou/acf1* homologues are knocked down (Fig. 2.7B). The *acf1* homologues appear to have more contribution for repression, and the activation is highest when all four mRNAs are inhibited, indicating functional redundancy. Similar results were observed when a different Wnt-responsive reporter, cyclinD1-luc, was used (Tetsu and McCormick, 1999; data not shown). In contrast, the FOPFLASH reporter, in which TCF sites are mutated, is not regulated by the same siRNA treatment, arguing that there is certain specificity for the action of *tou/acf1* homologues (data not shown). In sum, these results suggest that the role of Tou/ACF1 as negative regulators in Wnt signaling is conserved between flies

and humans.

One possible mechanism that Tou/ACF1 homologues act to attenuate Wnt signaling in 293 cells is that they down-regulate β-catenin stability. To test this possibility, Western blot for β-catenin was performed in cells where all four mRNAs of *tou/acf1* homologues were inhibited by RNAi. We did not observe a significant change of β-catenin protein level in RNAi treated cells, suggesting that the human homologues of Tou/ACF1 antagonize Wnt signaling independently of β-catenin stability (Fig. 2.7C).

Discussion

The role of Tou and ACF1 in flies

Through an misexpression genetic screen for antagonists in Wg signaling, we identified a chromatin remodeling protein called Toutatis (Tou). Overexpression of Tou consistently blocks several Wg readouts in the adult eye and developing wing, suggesting it acts as a repressor in the Wg pathway. In contrast, loss of *tou* only reveals subtle phenotypes. We believe that our *tou* mutant ($tou^{20\text{-}4}$), which deletes N-terminal half of Tou protein, is likely to be a null allele based on two reasons. Firstly, a RNAi construct targeting the 3'-end of *tou* does not enhance the $tou^{20\text{-}4}$ phenotype. Secondly, a different *tou* mutant ($tou^{E44.1}$) that deletes C-terminal of Tou protein including PHD fingers and BROMO domain does not reveal a more severe phenotype than that of $tou^{20\text{-}4}$ (Vanolst et al., 2005).

Mutants of $tou^{20\text{-}4}$ are semi-lethal with more than half of flies dying at a late pupal

stage. For those flies that survive to adulthood, they exhibit downward or shriveled wing phenotype, which could reflect a modest defect in Wg signaling. Interestingly, previous report demonstrated that *tou* mutant flies have decreased number of dorsocentral (DC) bristles on notum (Vanolst et al., 2005). The formation of DC bristles is controlled by Wg signaling (Phillips and Whittle, 1993). Therefore, Tou could act as a tissue-specific regulator of Wg signaling to mediate DV sensory organ development.

By all means, these subtle phenotypes of *tou* mutant do not indicate a major perturbation of Wg signaling, suggesting the existence of redundant gene(s). When both *tou* and its closest gene *acf1* are disrupted, a more severe phenotype (i.e. late pupal lethal) is observed, supporting that Tou and ACF1 act redundantly in certain biological processes. The fact that no evident Wg phenotypes can be detected in *tou/acf1* mutant flies made us suspect that a third protein act redundantly with Tou/Acf1. This could be Nurf301, the second closest protein to Tou in the fly genome. Consistent with this notion, we found that *tou/acf1/nurf301* triple mutant flies die earlier than either *tou/acf1* mutant or *nurf301* mutant (data not shown). It has been shown that Nurf301 interacts with ISWI, and regulates transcription in specific contexts (Xiao et al., 2001; Badenhorst et al., 2002). Since Tou, ACF1 and Nurf301 all associate with ISWI, we reasoned that disruption of the shared component ISWI might result in the equivalent effect as disruption of Tou/ACF1/Nurf301 altogether, while technique-wise it is much easier to achieve the former one. In fact, loss of *iswi* in the wing leads to derepression or further activation of several Wg targets, suggesting that ISWI is required to repress Wg targets *in*

vivo. Details on the characterization of ISWI's role in Wg signaling will be elaborated in Chapter III.

The role of Tou and ACF1 in fly and human cells

We also performed loss-of-function analysis for *tou* and *acf1* in both fly and human cell lines. Consistent with what was observed in flies, we found that Tou and ACF1 redundantly repress Wg targets in both systems. These results indicate that the role of Tou and ACF1 is evolutionarily conserved. We also noticed an interesting disparity regarding the contribution of Tou or ACF1 in repressing Wg targets between flies and cultured cells. In flies, Tou appears to play a predominant role as overexpression of *tou* blocks several Wg readouts and *tou* mutant causes semi-lethality and abnormal wing, while *acf1* mutant has no manifest phenotype. In contrast,depletion of *acf1* results in a greater activation of Wg targets than depletion of *tou* in culture cells (Fig. 2.7). It is important not to overemphasize this discrepancy because the incomplete knockdown of gene's activity by RNAi thwarts a clear interpretation of results in cell culture and *tou* mutant phenotypes are not strongly correlated to Wg signaling defect. Despite these caveats, it is still suggestive that requirement of Tou and ACF1 differs in different tissues.

We also observed a difference between fly and human cell culture systems. In fly Kc cells, activation of Wg target *nkd* upon *tou/acf1* depletion occurs both in the absence and presence of Wg stimulation (Fig 2.7A, data not shown). In human 293 cells, only when the Wnt pathway is activated can a further activation of Wnt reporter be

observed upon depletion of *tou/acf1* homologues (Fig 2.7C). This disparity can be reconciled since different readouts, an endogenous gene vs. an engineered reporter, are used in the two systems. We suspect that TOPFLASH reporter might be too "naïve" to be derepressed especially considering that ACF1 can repress Wg targets independent of TCF (Chapter III). We favor a model where Tou and ACF1 are required for both maintaining the silent state of Wnt targets and "buffering" the activation of Wnt targets once the signal is received.

Mechanism of Tou and ACF1's action

Our genetic studies from both flies and cell culture have implicated Tou and ACF1 as negative regulators in Wg signaling. Then, are these two proteins directly involved in the pathway? Several lines of evidence suggest that they are. Firstly, overexpression of *tou* or *acf1* suppresses the small eye phenotype induced by an activated form of Arm, excluding the possibility that Tou/ACF1 activates a Wg antagonist upstream or at the level of Arm stabilization (e.g. Axin) to indirectly block Wg signaling. Secondly, RNAi of *tou/acf1* homologues in 293 cells activates TOPFLASH reporter, which only contains TCF binding sites and therefore is highly Wnt-specific. This result indicates that Tou/ACF1 are directly involved in the pathway, at least through regulating the TCF-dependent process. Finally, we have some convincing data from chromatin immunoprecipitation analysis (ChIP) that ACF1 directly binds to the chromatin of *nkd* locus (Chapter III). Given the sequence similarity between Tou and ACF1, We

speculate that Tou may directly bind to Wg targets as well. Several peptide antibodies for Tou have been generated in the lab, and it is tempting to perform ChIP analysis for Tou to test such a hypothesis.

Previous studies have shown that ACF1 plays an important role in chromatin assembly (Fyodorov and Kadonaga, 2002). However, two pieces of evidence argue against that the defect we saw in Wnt signaling is the non-specific effect of chromatin organization. Firstly, two genes adjacent to *nkd* locus are not derepressed upon *tou/acf1* knockdown, suggesting that there is no widespread chromatin disorganization around *nkd* locus when *tou* and *acf1* are depleted. Secondly, neither a FOPFLASH reporter where TCF sites are mutated nor a reporter responsive to insulin signaling is affected by knockdown of *tou/acf1* homologues in 293 cells (data not shown). This result suggests that there exists certain specificity in Tou/ACF1's action.

On a whole, this project is somewhat handicapped by a modest phenotype of *tou/acf1* mutant in flies. One possibility is redundancy, which we have proposed in the preceding section. It appears that this is not the only example where mechanism of redundancy is employed by the negative regulators in the pathway. A recent report shows that p66, a component in the NURD chromatin-remodeling/histone deacetylase complex, may have redundant partner(s) for its repression of Wg targets, since *p66* mutant has no detectable Wg phenotype (Kon et al., 2005).

Another possibility is that Tou/ACF1 are not "essential" components in Wg signaling, meaning they are not required in every tissue where Wg is expressed. Their

action could be tissue or cell specific, and the effect could be too subtle, not necessarily unimportant, to affect the whole animal biology. Our results from cell culture system definitely support this idea. A similar example has been reported previously for Ryk, a Wnt co-receptor. While Ryk is certainly an important factor in Wnt signaling in 293 cells and is required for Wnt induced neurite outgrowth in dorsal root ganglion (DRG) neurons, Ryk knockouts in mice or worms do not have dramatic defects in Wnt signaling (Lu et al., 2004; Halford et al., 2000; Inoue et al., 2004). Therefore, both Ryk and Tou/ACF1 could fall in the category of tissue/cell specific regulators of Wnt signaling.

Materials and methods

Drosophila strains

The P[GMR-Gal4] stock was provided by M. Freeman (Freeman, 1996). The P[UAS-*wg*] stock was provided by H. Krause. The bidirectional P[GSV] (Toba et al., 1999) was mobilized by transposase P[Δ2-3] and the subsequent insertions were screened for suppression of P[GMR-Gal4]P[UAS-*wg*] induced small eye. The insertion sites of A41, A80, A145 and A428 were mapped by using inverse PCR (http://www.fruitfly.org/about/methods/inverse.pcr.html). EnGal4 and UAS-lacZ lines were from the Bloomington Stock Center.

Generation of *tou* and *acf1* deletions, and *tou*^{RNAi} lines

Deletion mutants of *tou* and *acf1* were generated by imprecise excision of EP(2)2532 and EP(3)1181, respectively. A modified approach to screen deletions was

adopted compared to what was employed previously (Parker et al., 2002). The male w^- jumpout flies from dysgenic crosses were pooled in 30-40s and crossed to wild-type female flies. DNA was isolated from the progenies (at the embryonic stage) and subject to PCR characterization for deletions. Once a positive pool was identified, the parental jumpout males were separated and isogenic crosses were set up. A second round of PCR was performed to identify the particular jumpout event in that pool. Phenotypic analysis of the positive jumpout events followed the molecular characterization of them. For *tou*, about 1500 jumpout flies were screened, and 7 deletion alleles were identified. For *acf1*, about 1600 jumpout flies were screened, and 4 deletion alleles were identified. A detailed protocol of this modified screen for imprecise excision is available upon request.

The UAS-*tou*^{RNAi} transgene was generated by cloning an inverted repeat of a 700bp sequence at the 3' end of *tou*, including the BROMO domain and 3'UTR, into the pWIZ vector (Lee and Carthew, 2003) . The sequence is shared by all four predicted *tou* isoforms. Primers used for PCR this 700bp fragment are 5'GCTCATGAAGGAG CT-GGCTGTCTGCAA3' and 5'GGTCTTTACCGACGTAGGACGTTGAA3'. Transgenic flies were generated by using the standard methodology. More than 20 independent lines were obtained. Four of the strongest lines were used for phenotypic analysis.

Immunostaining

Immunostaining of wing imaginal discs was as described previously (Cadigan et

al., 1998). Guinea pig anti-Sens was described elsewhere (Fang et al., 2006). Mouse anti-Wg (4D4) was from the Developmental Studies Hybridoma Bank at the University of Iowa. The dilution factors were: anti-Sens (1:1000) and anti-Wg (1:100). Cy3 and Alexa488 conjugated secondary antibodies were obtained from Jackson Immunochemicals and Molecular Probes, respectively. All fluorescent pictures were generated with a Zeiss Axiophot coupled to a Zeiss LSM510 confocal apparatus.

***Drosophila* cell culture**

Drosophila embryonic Kc167 (Kc) cells were cultured at room temperature in the Schneider's *Drosophila* media (invitrogen) containing 5% FBS and Penicillin/ Streptomycin antibiotics. RNAi mediated knockdowns of genes' activities were achieved as described elsewhere (Clemens *et al.*, 2000) with modifications. Briefly, when Kc cells approximated confluent status after 4-5 days of culture (~8×10^6/ml), they were resuspended at 1×10^6/ml in standard media and seeded onto 12-well plates (1ml/well). dsRNA for each targeted gene was then added at a concentration of 9μg/ml. After 4 days, cells were resuspended, diluted into 1×10^6/ml with fresh media and reseeded onto 12-well plates. Cells were harvested after 2 additional days incubation. dsRNAs with a typical length of 500-700 bp were synthesized using the MEGAscript T7 *in vitro* transcription kit (Ambion). The sequences of the PCR primers for the dsRNA synthesis are: *tou* (5'GCTCATGAAGGAGCTGGCTGTCTGCAA3' and 5'GGTCTTTACCGACGTAGGACGTTGAA3') *acf1* (5'-CGACCACGTAACTCTTTG CGCCTATCTA-3' and 5'-GCGTGTGCTGAACTTAGAACTGACAT-3'). The

sequences of primers for control dsRNAs have been published previously (Fang *et al.*, 2006).

Quantitative real-time PCR (q-PCR)

Samples were analyzed using an iCycler iQ real-time PCR detection system. For RT-PCR, Trizol (Invitrogen) was used to extract total RNA from 1-5×10^6 cells. Reverse transcription was performed using Stratascript reverse transcriptase (Stratagene) followed by q-PCR analysis. *β-tubulin56D* was used to normalize the transcripts. q-PCR primers for *nkd* and *β-tubulin56D* were designed by using the online program Primer3 (http://frodo.wi.mit.edu/), and their sequences have been published previously (Fang et al., 2006).

Human cell culture

293HK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C. For transient transfection, 1 million 293HK cells were seeded onto 12-well plates 24 hours prior to transfection. Lipofectimin 2000 was used as the transfection reagent according to the protocol provided by Invitrogen.

The 64-nucleotide oligos containing the 19-nucleotide targeting sequence for each *tou/acf1* homolog were synthesized and annealed *in vitro* before cloned into the pSUPER vector provided by Duan's lab (Brummelkamp et al., 2002). As suggested, the 19nt target sequence is flanked in the mRNA with AA at the 5' and TT at the 3', and is in the coding region 100-300bp from the termination codon (targeting BROMO domain of respective genes). The sequences are as following: BAZ2B (5'CTCATGAG

GATGCATGGCC3'), BAZ2A (5'TCCCATGATGCAGCCTGGC3'), BAZ1A (5'TACAACCCTCGTAACACAA3'), BAZ1B (5'CAGTGTCTAGTGGCTCTGT3').

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References

Aasland R, Gibson TJ, Stewart AF (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci* **20**:56-9

Badenhorst P, Voas M, Rebay I, Wu C (2002) Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev* **16**:3186-98

Barker N, Hurlstone A, Musisi H, Miles A, Bienz M, Clevers H (2001) The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. *EMBO J* **20**:4935-43

Becker PB, Horz W (2002) ATP-dependent nucleosome remodeling. *Annu Rev Biochem* **71**:247-73

Belenkaya TY, Han C, Standley HJ, Lin X, Houston DW, Heasman J, Lin X (2002) pygopus Encodes a nuclear protein essential for wingless/Wnt signaling. *Development* **129**:4089-101

Bouazoune K, Brehm A (2006) ATP-dependent chromatin remodeling complexes in *Drosophila*. *Chromosome Res* **14**:433-49

Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**:550-3

Cadigan KM, Fish MP, Rulifson EJ, Nusse R (1998) Wingless repression of Drosophila frizzled 2 expression shapes the Wingless morphogen gradient in the wing. *Cell* **93**:767-77

Cadigan KM, Jou AD, Nusse R (2002) Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless. *Development* **129**:3393-402

Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, Dixon JE (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci USA* **97**:6499-503

Collins RT, Treisman JE (2000) Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. *Genes Dev* **14**:3140-52

Collins N, Poot RA, Kukimoto I, Garcia-Jimenez C, Dellaire G, Varga-Weisz PD (2002) An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. *Nat Genet* **32**:627-32

Corona DF, Tamkun JW (2004) Multiple roles for ISWI in transcription, chromosome organization and DNA replication. *Biochim Biophys Acta* 1677:113-9

Doerks T, Copley R, Bork P (2001) DDT -- a novel domain in different transcription and chromosome remodeling factors. *Trends Biochem Sci* **26**:145-6

Eberharter A, Ferrari S, Langst G, Straub T, Imhof A, Varga-Weisz P, Wilm M, Becker PB (2001) Acf1, the largest subunit of CHRAC, regulates ISWI-induced nucleosome remodelling. *EMBO J* **20**:3781-8

Eberharter A, Vetter I, Ferreira R, Becker PB (2004) ACF1 improves the effectiveness of nucleosome mobilization by ISWI through PHD-histone contacts. *EMBO J* **23**:4029-39

Fang M, Li J, Blauwkamp T, Bhambhani C, Campbell N, Cadigan KM (2006) C-terminal-binding protein directly activates and represses Wnt transcriptional targets in *Drosophila*. *EMBO J* **25**:2735-45

Fauvarque MO, Laurenti P, Boivin A, Bloyer S, Griffin-Shea R, Bourbon HM, Dura JM (2001) Dominant modifiers of the polyhomeotic extra-sex-combs phenotype induced by marked P element insertional mutagenesis in Drosophila. *Genet Res* **78**:137-48

Freeman M (1996) Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**:651-60

Fyodorov DV, Kadonaga JT (2002) Dynamics of ATP-dependent chromatin assembly by ACF. *Nature* **418**:897-900

Fyodorov DV, Blower MD, Karpen GH, Kadonaga JT (2004) Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. *Genes Dev* **18**:170-83

Halford MM, Armes J, Buchert M, Meskenaite V, Grail D, Hibbs ML, Wilks AF, Farlie PG, Newgreen DF, Hovens CM, Stacker SA (2000) Ryk-deficient mice exhibit craniofacial defects associated with perturbed Eph receptor crosstalk. *Nat Genet* **25**:414-8

Inoue T, Oz HS, Wiland D, Gharib S, Deshpande R, Hill RJ, Katz WS, Sternberg PW (2004) C. elegans LIN-18 is a Ryk ortholog and functions in parallel to LIN-17/Frizzled in Wnt signaling. *Cell* **118**:795-806

Ito T, Levenstein ME, Fyodorov DV, Kutach AK, Kobayashi R, Kadonaga JT (1999) ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. *Genes Dev* **13**:1529-39

Jones MH, Hamana N, Nezu J, Shimane M (2000) A novel family of bromodomain genes. *Genomics* **63**: 40-5

Khorasanizadeh S (2004) The nucleosome: from genomic organization to genomic regulation. *Cell* **116**:259-72

Kon C, Cadigan KM, da Silva SL, Nusse R (2005) Developmental roles of the Mi-2/NURD-associated protein p66 in Drosophila. *Genetics* **169**:2087-100

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**:1784-7

Kramps T, Peter O, Brunner E, Nellen D, Froesch B, Chatterjee S, Murone M, Zullig S, Basler K (2002) Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* **109**:47-60

Langst G, Becker PB (2001) Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. *J Cell Sci* **114**:2561-8

Lee YS, Carthew RW (2003) Making a better RNAi vector for Drosophila: use of intron spacers. *Methods* **30**:322-9

Li B, Carey M, Workman JL (2007) The role of chromatin during transcription. *Cell* **128**:707-19

Li J, Sutter C, Parker DS, Blauwkamp T, Fang M, Cadigan KM (2007) CBP/p300 are bimodal regulators of Wnt signaling. *EMBO J* **26**:2284-94

Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**:781-810

Lu X, Meng X, Morris CA and Keating MT (1998) A novel human gene, WSTF, is deleted in Williams syndrome. *Genomics* **54**:241-9

Lu W, Yamamoto V, Ortega B, Baltimore D (2004) Mammalian Ryk is a Wnt coreceptor required for stimulation of neurite outgrowth. *Cell* **119**:97-108

McManus MT, Sharp PA (2002) Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* **3**:737-47

Narlikar GJ, Fan HY, Kingston RE (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**:475-87

Parker DS, Jemison J, Cadigan KM (2002) Pygopus, a nuclear PHD-finger protein required for Wingless signaling in *Drosophila*. *Development* **129**:2565-76

Parker DS, Blauwkamp T, Cadigan KM (2007) Wnt-mediated transcriptional regulation. In *Wnt Signaling in Embryonic Development, Sokol S (ed), Advances in Developmental Biology*, Wassarman PM (ed) Vol 17, pp 1-61. San Diego: Elsevier

Phillips RG, Whittle JR (1993) wingless expression mediates determination of peripheral nervous system elements in late stages of Drosophila wing disc development. *Development* **118**:427-38

Poot RA, Dellaire G, Hulsmann BB, Grimaldi MA, Corona DF, Becker PB, Bickmore WA, Varga-Weisz PD (2000) HuCHRAC, a human ISWI chromatin remodeling complex contains hACF1 and two novel histone-fold proteins. *EMBO J* **19**:3377-87

Poot RA, Bozhenok L, van den Berg DL, Steffensen S, Ferreira F, Grimaldi M, Gilbert N, Ferreira J, Varga-Weisz PD (2004) The Williams syndrome transcription factor interacts with PCNA to target chromatin remodelling by ISWI to replication foci. *Nat Cell Biol* **6**:1236-44

Robertson HM, Preston CR, Phillis RW, Johnson-Schlitz DM, Benz WK, Engels WR (1988) A stable genomic source of P element transposase in Drosophila melanogaster. *Genetics* **118**:461-70

Rorth P, Szabo K, Bailey A, Laverty T, Rehm J, Rubin GM, Weigmann K, Milan M, Benes V, Ansorge W, Cohen SM (1998) Systematic gain-of-function genetics in *Drosophila*. *Development* **125**:1049-57

Rottbauer W, Saurin AJ, Lickert H, Shen X, Burns CG, Wo ZG, Kemler R, Kingston R, Wu C, Fishman M (2002) Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell* **111**:661-72

Rountree MR, Bachman KE, Herman JG, Baylin SB (2001) DNA methylation, chromatin inheritance, and cancer. *Oncogene* **20**:3156-65

Rulifson EJ, Micchelli CA., Axelrod JD, Perrimon N, Blair SS (1996) wingless refines its own expression domain on the Drosophila wing margin. *Nature* **384**:72-4

Santoro R, Li J, Grummt I (2002) The nucleolar remodeling complex NoRC mediates heterochromatin formation and silencing of ribosomal gene transcription. *Nat Genet* **32**:393-6

Strohner R, Nemeth A, Jansa P, Hofmann-Rohrer U, Santoro R, Langst G, Grummt I (2001) NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. *EMBO J* **20**:4892-900

Strohner R, Nemeth A, Nightingale KP, Grummt I, Becker PB, Langst G (2004) Recruitment of the nucleolar remodeling complex NoRC establishes ribosomal DNA silencing in chromatin. *Mol Cell Biol* **24**:1791-8

Tetsu O, McCormick F (1999) Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**:422-6

Toba G, Ohsako T, Miyata N, Ohtsuka T, Seong KH, Aigaki T (1999) The gene search system. A method for efficient detection and rapid molecular identification of genes in Drosophila melanogaster. *Genetics* **151**:725-37

Thompson B, Townsley F, Rosin-Arbesfeld R, Musisi H, Bienz M (2002) A new nuclear component of the Wnt signalling pathway. *Nat Cell Biol* **4**:367-73

Tutter AV, Fryer CJ, Jones KA (2001) Chromatin-specific regulation of LEF-1-beta-catenin transcription activation and inhibition in vitro. *Genes Dev* **15**:3342-54

Vanolst L, Fromental-Ramain C, Ramain P (2005) Toutatis, a TIP5-related protein, positively regulates Pannier function during *Drosophila* neural development. *Development* **132**:4327-38

Xiao H, Sandaltzopoulos R, Wang HM, Hamiche A, Ranallo R, Lee KM, Fu D, Wu C

(2001) Dual functions of largest NURF subunit NURF301 in nucleosome sliding and transcription factor interactions. *Mol Cell* **8**:531-43

Zeng W, Wharton KA Jr, Mack JA, Wang K, Gadbaw M, Suyama K, Klein PS, Scott MP (2000) naked cuticle encodes an inducible antagonist of Wnt signalling. *Nature* **403**:789-95

Zeng L, Zhou MM (2002) Bromodomain: an acetyl-lysine binding domain. *FEBS Lett* **513**:124-8

Zhou Y, Santoro R, Grummt I. (2002) The chromatin remodeling complex NoRC targets HDAC1 to the ribosomal gene promoter and represses RNA polymerase I transcription. *EMBO J* **21**:4632-40

CHAPTER III

THE CHROMATIN REMODELERS ISWI AND ACF1 DIRECTLY REPRESS WINGLESS TRANSCRIPTIONAL TARGETS

Abstract

The highly conserved Wingless (Wg)/Wnt signaling pathway controls many developmental processes by regulating the expression of target genes, most often through members of the TCF family of DNA-binding proteins. In the absence of signaling, many of these targets are silenced, by mechanisms involving TCFs that are not fully understood. Here we report that the chromatin remodeling proteins ISWI and ACF1 are required for basal repression of Wg target genes in *Drosophila*. This regulation is not due to global repression by ISWI/ACF1 and is distinct from their previously reported role in chromatin assembly. We found that ACF1 binds to broad regions of several Wg targets and regulates TCF binding to chromatin, while a TCF-independent role of ISWI/ACF1 in repression was also observed. Finally, we showed that Wg signaling reduces ACF1 binding to Wg targets, and ISWI/ACF1 regulates repression by antagonizing histone H4 acetylation. Our results argue that Wg signaling activates target genes expression partly by overcoming the chromatin barrier maintained by ISWI/ACF1.

Introduction

The Wnt/β-catenin pathway is an evolutionarily conserved signaling cascade that controls a large array of processes in animal development, including cell specification, proliferation and apoptosis, as well as stem cell fate maintenance in adult tissues (Logan and Nusse, 2004). Misregulation of the pathway has been causally linked to several human cancers and osteoporosis (Clevers, 2006). Further insights into how Wnt/β-catenin signaling specifically regulates its transcriptional targets are crucial for our understanding of its role in development and disease.

In unstimulated cells, β-catenin has a short half-life due to phosphorylation and subsequent degradation by the proteasome (Daniels *et al*, 2001; Ding and Dale, 2002). Binding of Wnt to a cell surface receptor complex blocks β-catenin phosphorylation, leading to its accumulation in the cytoplasm (Cadigan and Liu, 2006). This stabilized β-catenin then translocates to the nucleus, where it can bind to members of the TCF family of specific DNA-binding proteins to activate target gene expression (Städeli *et al*, 2006; Parker *et al*, 2007a).

In the absence of β-catenin, TCFs are thought to mediate transcriptional repression. This silencing activity is important in several development contexts. In invertebrates, these include patterning of the embryonic epidermis of *Drosophila* (Cavallo *et al*, 1998) and mesodermal cell fate specification in *C. elegans* embryos (Rocheleau *et al*, 1997; Thorpe *et al*, 1997). In amphibians, TCF repression is important for inhibiting dorsal cell fate in ventral blastomeres (Houston *et al*, 2002; Standley *et al*, 2006) as well as mesoderm induction (Liu *et al*, 2005). In fish and mice, repression by TCF3 is important for anterior structure specification and AP axis formation (Kim *et al*,

2000; Merrill *et al*, 2004). Loss of TCF1 in mice causes spontaneous tumors in the intestine and mammary glands, consistent with inappropriate activation of Wnt/β-catenin targets (Roose *et al*, 1999). Combined with the abundant evidence of TCFs acting as transcriptional activators, these findings suggest a model where TCFs act as switches, silencing Wnt target gene expression until β-catenin converts them to transcriptional activators (Parker *et al*, 2007a).

Although many co-activators have been identified which are recruited to Wnt regulated enhancers (WREs) by β-catenin (Städeli *et al*, 2006; Parker *et al,* 2007a), not as much is known about the factors that mediate repression of Wnt targets in the absence of signaling. Transcriptional co-repressors of the Groucho (Gro)/TLE family can bind to TCFs and antagonize their ability to activate Wnt-responsive reporter genes (Cavallo *et al*, 1998; Roose *et al*, 1998). Consistent with this, loss of *gro* leads to derepression of Wingless (Wg, a fly Wnt) targets in the absence of signaling (Cavallo *et al*, 1998; Fang *et al*, 2006). β-catenin binds competitively with TLE to TCFs (Daniels and Weis, 2005), suggesting that β-catenin displaces this co-repressor from WREs upon pathway activation (Sierra *et al*, 2006; Wang and Jones, 2006).

While Gro/TLE is recruited to WREs through direct binding to TCFs, other factors act in parallel with TCFs to repress target gene expression. Kaiso, a protein containing BTB/POZ and zinc finger domains, represses several Wnt targets in *Xenopus* by binding to TCF and specific sites in WREs (Park *et al*, 2005). In *Drosophila* cell culture, the co-repressor C-terminal binding protein (CtBP) is recruited to WREs independently of TCF, where it represses expression in parallel with TCF/Gro (Fang *et al*,

2006). Whether these factors act in a general or gene-specific manner in repressing Wnt targets remains to be determined.

The regulation of eukaryotic transcription is considered inextricably connected to chromatin structure, which is tightly controlled by chromatin modification and remodeling factors (Li *et al*, 2007a). Therefore, it is likely that some of these factors are involved in the repression of Wnt targets. For example, two subunits of a SWI/SNF-like chromatin remodeling complex, Brahma and Osa, have been shown to repress Wg targets *in vivo* (Collins and Treisman, 2000). However, it is not clear if this regulation is direct. In this report, we explore the role of two other chromatin remodelers, ISWI and ACF1, in repressing Wg targets.

ISWI and ACF1 belong to the ISWI family of ATP-dependent chromatin remodelers, which have been implicated in a variety of biological processes including transcription regulation, DNA replication and chromosome organization (Corona and Tamkun, 2004). ISWI and ACF1 form the ACF complex, while the CHRAC complex consists of ISWI, ACF1 and two additional subunits (Langst and Becker, 2001). Both ACF and CHRAC exhibit chromatin assembly and nucleosome sliding activity *in vitro* (Langst and Becker, 2001).

Genetic studies also reveal a role for ISWI in regulating chromosome architecture. Flies genetically null for *acf1* are semi-viable with several chromatin defects (Fyodorov *et al*, 2004), while *iswi* mutant flies die as larvae and display decondensation of the entire male X chromosome (Deuring *et al*, 2000). This *iswi* phenotype is dependent on the activity of the dosage compensation complex, with ISWI possibly acting antagonistically

to the acetylation of histone H4 at lysine 16 (AcH4K16) to mediate global gene repression and chromatin compaction (Corona *et al*, 2002; Shogren-Knaak *et al*, 2006).

ISWI has also been suggested to act in more localized gene repression in flies based on the observation that the distributions of ISWI and RNA polymerase II on polytene chromosomes do not generally overlap (Deuring *et al*, 2000). Consistent with this, the mammalian ISWI homologue SNF2H is required for repression of thyroid hormone receptor targets in the absence of ligand (Alenghat *et al,* 2006). In addition, there are several reports demonstrating that the yeast ISWI homologues, Isw1 and Isw2 are directly involved in transcriptional repression (Goldmark *et al*, 2000; Moreau *et al*, 2003; Zhang and Reese, 2004).

In this report, we implicate ISWI and ACF1 as important repressors in Wg signaling in *Drosophila*. Loss of *iswi*/*acf1* causes derepression or further activation of several Wg transcriptional targets in both cultured cells and fly wings. The derepression is still observed in non-dividing cells, ruling out that the effect is due to a post-mitotic chromatin assembly defect. *iswi*/*acf1* is required for maximal TCF binding to WREs and to antagonize histone acetylation in the absence of signaling. ACF1 is directly associated with broad regions of several Wg target loci and this binding is reduced upon activation of Wg signaling. These results are consistent with a model where ISWI/ACF1 silences target gene expression in unstimulated cells and modulates the switch to transcriptional activation by Wg signaling

Results

ISWI/ACF1 represses Wg targets in the absence of signaling in *Drosophila* cultured cells

Wg signaling can be studied in cell culture using *Drosophila* Kc167 (Kc) cells, which we have previously shown to be responsive to Wg signaling (Fang *et al*, 2006; Li *et al*, 2007b). Microarrays were used to identify genes whose expression increased upon stimulation of the pathway (T. Blauwkamp and K. Cadigan, unpublished data). Three activated targets, *naked cuticle (nkd)*, *Notum* and *homothorax* (*hth)*, were chosen for further study. *nkd* and *Notum* are feedback antagonists induced by Wg signaling in most fly tissues (Zeng *et al*, 2000; Giraldez *et al*, 2002; Gerlitz and Basler, 2002), while *hth* is activated only in select tissues (Azpiazu and Morata, 2000; Casares and Mann, 2000; Wernet *et al*, 2003). In Kc cells, treatment with Wg conditioned media (Wg-CM) significantly induced the transcript levels of all three genes (Fig. 3.1A).

To examine whether ISWI/ACF1 plays a role in regulating Wg targets, cells were depleted of these factors via RNA interference (RNAi). Both ISWI and ACF1 are expressed in Kc cells, and their expression can be efficiently inhibited by the respective dsRNA (Fig. 3.1B). In general, inhibition of *iswi* or *acf1* caused an increased expression of *nkd*, *Notum* and *hth* in unstimulated cells, while simultaneous knockdown of both *iswi* and *acf1* led to even higher levels of derepression (Fig. 3.1C). Two dsRNAs targeted to different portions of the *iswi* and *acf1* transcripts were used, with similar results obtained (data not shown).

For individual Wg targets, differences are observed in the *iswi* and *acf1* single RNAi treatments. ISWI and ACF1 equally contribute to the repression of *nkd*, while

ACF1 plays a greater role in inhibiting *Notum*. In contrast, repression of *hth* mainly depends on ACF1 (Fig. 3.1C). It is unclear whether these results reflect real mechanistic differences between the functions of these factors or threshold effects of the RNAi depletion.

To explore whether ISWI and ACF1 specifically repress Wg targets, three genes that are not responsive to Wg signaling were examined. Two of these, *Mkp3 and CG18135*, are located upstream and downstream of *nkd*, respectively, and a third one, *p53*, was picked from the non-Wg-target pool of the aforementioned microarray analysis. Depletion of *iswi* and/or *acf1* had no effect on these genes (Fig. 3.1D). In addition to demonstrating specificity towards Wg targets, these results argue against the increase in *nkd* expression being caused by a general loosening of the chromatin, since the genes adjacent to this locus are not affected.

Several negative regulators of the Wg/Wnt pathway act through antagonizing Arm/β-catenin binding to TCF (Parker *et al*, 2007a). ISWI/ACF1 represses Wg targets in the absence of exogenously added Wg, suggesting that they do not act by this mechanism. This was confirmed by the finding that the derepression of *nkd* expression caused by *iswi*/*acf1* depletion remained unchanged with the additional knockdown of *arm* (Fig. 3.1E). It was confirmed that *arm* transcript levels were effectively knocked down in both *arm* single RNAi and *iswi*/*acf1*/*arm* triple RNAi cells (data not shown). Thus, ISWI/ACF1 is acting as a *bona fide* silencer of Wg targets in the absence of signaling.

Since ISWI/ACF1 represses the steady state levels of target gene transcripts, it is possible that they regulate RNA stability. In this scenario, the elevated expression of Wg targets upon *iswi*/*acf1* RNAi is due to the decreased turnover of transcripts rather than the

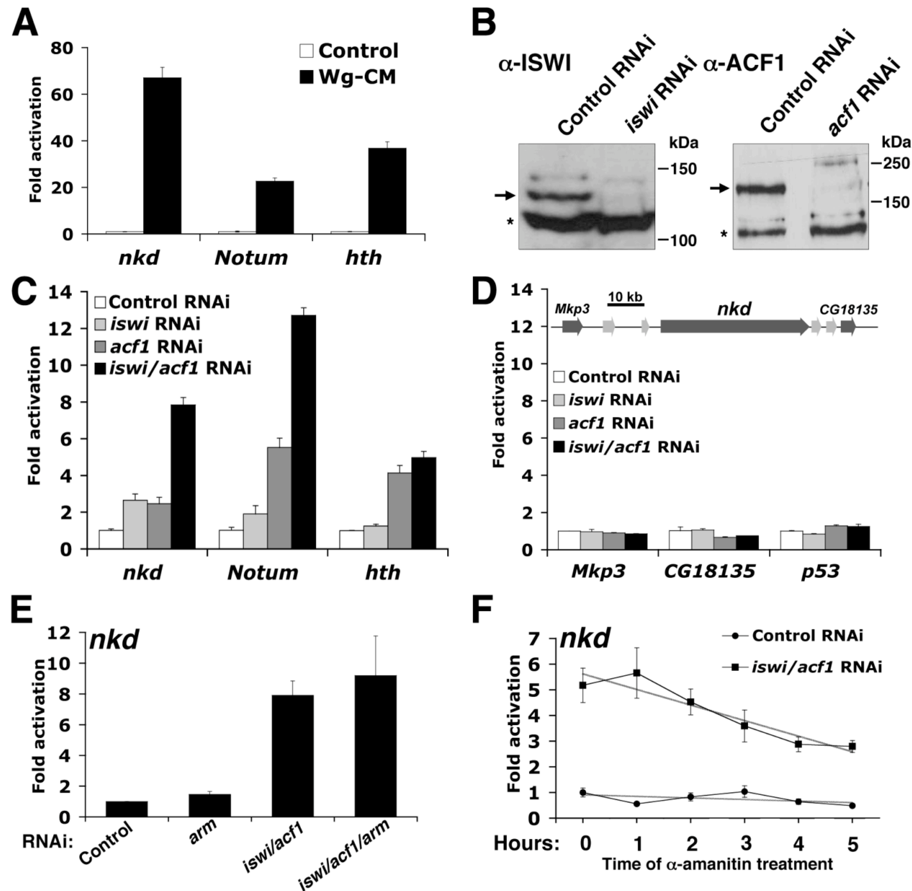


Figure 3.1. ISWI/ACF1 represses Wg targets in the absence of Wg signaling. (A) Kc cells were treated with control media or Wg-CM for 5 hours. Transcript levels of *nkd*, *Notum* and *hth* were measured by Q-RT-PCR and results were normalized to *β-tubulin56D* expression. Wg-CM significantly induced the expression of all three genes. **(B)** Western blot analysis of ISWI and ACF1 in control or corresponding RNAi-treated cells. *iswi* RNAi and *acf1* RNAi dramatically reduced ISWI and ACF1 expression, respectively. Arrows indicate the positions of ISWI and ACF1 proteins, and asterisks indicate nonspecific bands. **(C)** Derepression of *nkd*, *Notum* and *hth* by *iswi* or/and *acf1* RNAi. Kc cells were treated with the indicated dsRNAs as described in Materials and methods, and transcripts of Wg targets were measured by Q-RT-PCR. Results were normalized to the average of *β-tubulin56D, arm* and *TCF* expression. Each bar represents the mean (± S.E.) of duplicate cultures with duplicate Q-PCR reactions. **(D)** Two genes adjacent to *nkd* locus, *Mkp3* and *CG18135*, and *p53* were not derepressed by *iswi/acf1* RNAi. The same normalization strategy was used as in (**C**). **(E)** *arm* RNAi did not affect the derepression of *nkd* by *iswi/acf1* RNAi. **(F)** Kc cells incubated with control RNAi or *iswi/acf1* RNAi for six days were treated with α-amanitin (10ug/ml) for an additional 0-5 hours, and cells were harvested and *nkd* expression was analyzed without normalization. *nkd* transcripts in *iswi/acf1* RNAi treated cells had a faster turnover than that of control cells ($P<0.01$). Data shown here were the means of triplicates (± S.E.), and the lines were deduced from linear regression analysis. All experiments have been performed at least three separate times with similar results.

increased transcription. To test this hypothesis, the half-life of *nkd* transcripts was determined in control or *iswi*/*acf1* depleted cells where transcription was blocked with α-amanitin. Knockdown of *iswi*/*acf1* did not decrease *nkd* turnover (Fig. 3.1F). Rather, it significantly decreased *nkd* stability ($t_{1/2}$=5.2 hrs compared to control $t_{1/2}$=9.3 hrs, $p<0.01$). Therefore, we conclude that ISWI/ACF1 regulates Wg target gene expression at the level of transcription.

ISWI specifically represses Wg targets in the developing wing

To determine whether ISWI or ACF1 plays a physiological role in Wg signaling in flies, we analyzed the phenotype of wing imaginal disc cells lacking *iswi* or *acf1*. In this tissue, Wg is expressed in a stripe at the Dorsal/Ventral (D/V) boundary of the disc (Phillips and Whittle, 1993). Wg diffusing from the D/V stripe activates several genes, including Senseless (Sens; Parker *et al*, 2002), *nkd* (Zeng *et al*, 2000), *Notum* (Giraldez *et al*, 2002; Gerlitz and Basler, 2002) and *Dfz3* (Sivasankaran *et al*, 2000). The expression of these targets could be monitored by immunostaining (Sens), an enhancer trap (*Dfz3*) or lacZ reporters (*nkd* and *Notum*; see Materials and methods for more details).

Wing discs from flies homozygous for several null alleles of *acf1* were examined with no detectable misregulation of the Wg targets mentioned above (Chapter II). Because *iswi* homozygous mutants died during early to mid third instar, clones of a molecular null allele (*iswi*^{1}; Deuring *et al*, 2000) were generated by mitotic recombination (Xu and Rubin, 1993). Two thirds of the clones had no detectable effect on the expression of Wg at the D/V stripe (Fig. 3.2C) and no ectopic Wg was found in clones removed from the D/V boundary (Fig. 3.2G, K, O). In one third of clones

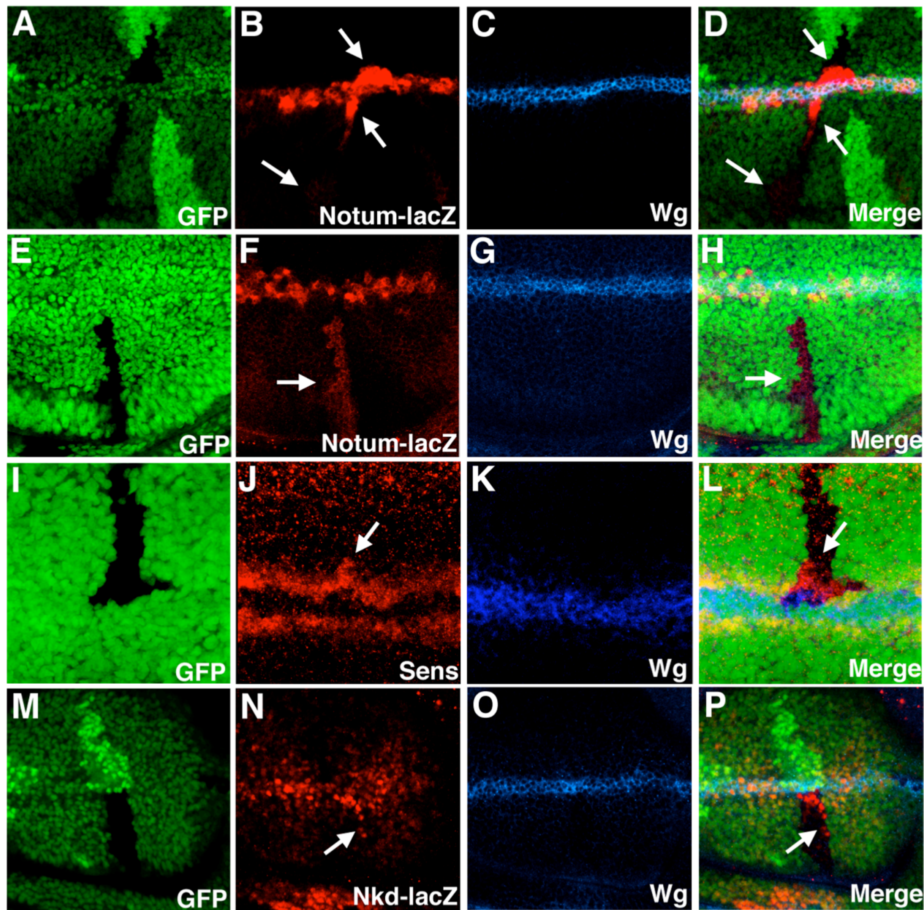


Figure 3.2. Loss of *iswi* results in an expansion/derepression of Wg targets in wing imaginal discs. (A-P) Confocal images of wing imaginal discs of late third instar larva stained for Wg (**C, G, K, O**), Notum-lacZ (**B, F**), Sens (**J**) and Nkd-lacZ (**N**). Mitotic clones of $iswi^1$ were marked by the absence of GFP (green). Notum-lacZ expression is expanded in clones along the Wg expression domain (**A-D**, 95% penetrance, n=39), and is derepressed in clones far away from the D/V boundary (**E-H**, 64% penetrance, n=22). Ectopic expression of Sens and Nkd-lacZ was observed in some clones along the D/V boundary (**I-L**, 46% penetrance, n=13; **M-P**, 43% penetrance, n=7).

at the D/V boundary, the Wg stripe was kinked (data not shown), and these clones were not included in our analysis.

The removal of *iswi* gene activity resulted in a dramatic increase in Notum-lacZ expression in the developing wing. In *iswi* mutant clones near the D/V border, the Notum reporter was expanded (Fig. 3.2A-D). In clones further away from the Wg stripe, ectopic expression of Notum-lacZ was observed in the majority of the clones (Fig. 3.2E-H), consistent with derepression of this Wg target.

Ectopic expression of other Wg targets were also seen in *iswi* mutant clones, though the level of expansion and penetrance is not as great as for Notum-lacZ. In approximately half of the clones that are close to the Wg D/V stripe, a modest activation of Sens and Nkd-lacZ can be seen (Fig. 3.2I-P). Similar results were also observed for another Wg target, Dfz3-lacZ (data not shown). Taken together, the *iswi* clonal analysis suggests that ISWI represses several Wg targets in the wing.

Since Wg is a target of the Notch signaling pathway in the wing (Diaz-Benjumea and Cohen, 1995), our finding that Wg expression was not affected in *iswi* clones (Fig. 3.2C, G, K, O) indicates some degree of specificity for genes activated by Wg signaling. To extend this analysis, several genes not regulated by Wg were examined in mutant clones. Engrailed (En)/Invected (Inv) is expressed in the posterior compartment of the wing (Patel *et al*. 1989). Decapentaplegic (Dpp)-lacZ is activated by Hedgehog (Hh) signaling in a stripe on the anterior side of the Anterior/Posterior (A/P) boundary, while Spalt is activated by Dpp signaling in a broad region surrounding the A/P boundary (Tabata, 2001). No significant alteration of the expression of any of these genes was observed in *iswi* mutant clones (Fig. 3.3), except for an occasional (<10%) reduction of

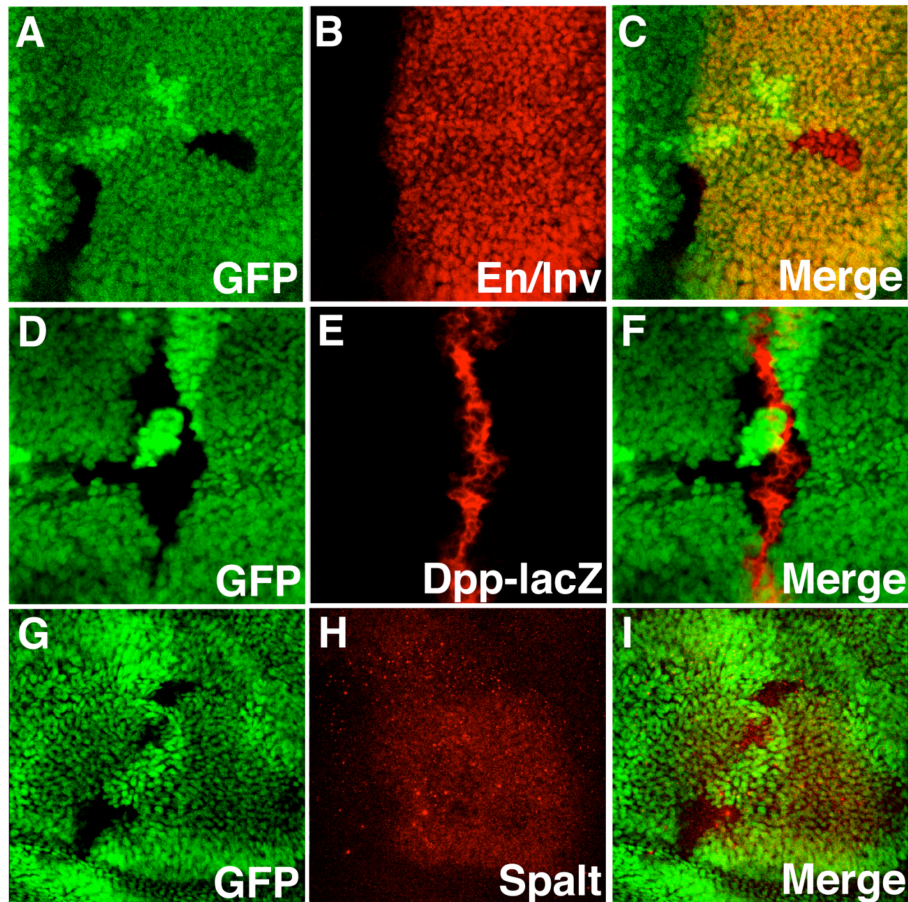


Figure 3.3. ***iswi*** **mutant cells do not affect several non-Wg targets in wing imaginal discs.** (A-I) Confocal images of wing imaginal discs of late third instar larva stained for En/Inv (B), Dpp-lacZ (E) and Spalt (H). The majority of *iswi* clones created in the posterior area displayed unchanged En/Inv expression (A-C, 91% penetrance, n=23), while 9% of the clones showed decrease/loss of En/Inv expression (data not shown). No derepression of En/Inv was ever seen in the anterior area (n>20). All *iswi* clones examined show normal Dpp-lacZ and Spalt expression (D-F, n=24; G-I, n=24).

En/Inv levels. These results demonstrate that ISWI is not a general repressor of gene expression,displaying substantial specificity for Wg targets.

ACF1 binds to broad regions of several Wg transcriptional targets

The *nkd* and *Notum* loci appear to be direct targets of the pathway, as judged by analysis of TCF binding using chromatin immunoprecipitation (ChIP). There is a major peak of TCF binding in a WRE in the first intron of *nkd*, approximately 5 kb downstream of the transcriptional start site (TSS; Fang *et al*, 2006). This intronic *nkd* WRE contains several TCF binding sites required for its induction by Wg signaling (Li *et al*, 2007b). In addition, there is an additional area bound by TCF 10 kb upstream of the *nkd* TSS (Parker *et al*, 2007b) that corresponds to the Nkd-lacZ reporter used in the wing imaginal disc (Fig. 3.2N). TCF binds to two areas in the *Notum* locus (Parker *et al*, 2007b). One site is 4 kb upstream of the TSS, corresponding to the Notum-lacZ construct activated by Wg in the wing imaginal disc (Fig. 3.2B, F) and cultured cells (Städeli *et al*, 2005). The second is in the first intron, about 6 kb downstream of the TSS.

In contrast to the binding by TCF to specific areas of the *nkd* and *Notum* genes, ACF1 was found more broadly across these loci. ACF1 binding was observed over the entire *nkd* locus, including the two aforementioned WRE sites (Fig. 3.4C). The ACF1 ChIP signal was significantly reduced by *acf1* RNAi, indicating that it was specfic for ACF1 (Fig. 3.4C). Interestingly, two genes adjacent to *nkd*, *Mkp3* and *CG18135*, were also bound by ACF1, although they were not regulated by ACF1 (Fig. 3.1D). Similar to the binding profile of *nkd*, ACF1 was also bound to the two WREs in *Notum*, as well to chromatin 15 kb downstream of the *Notum* TSS (Fig. 3.6B) , a region not bound by TCF

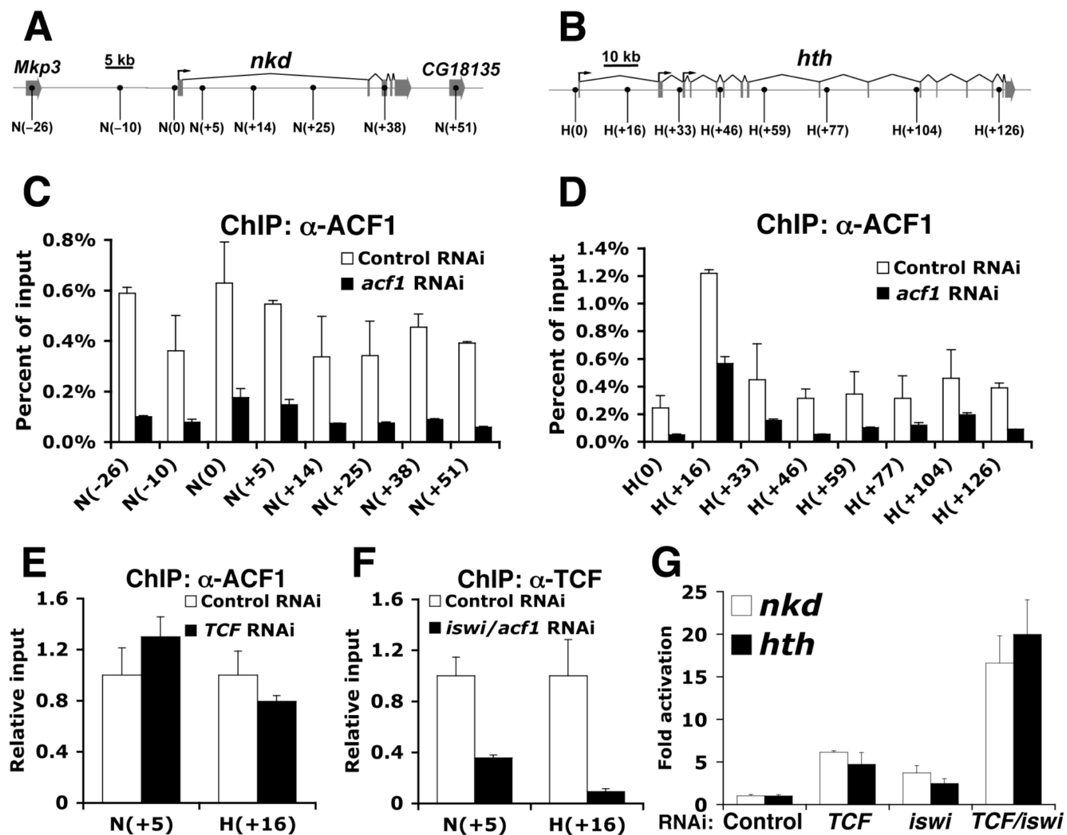
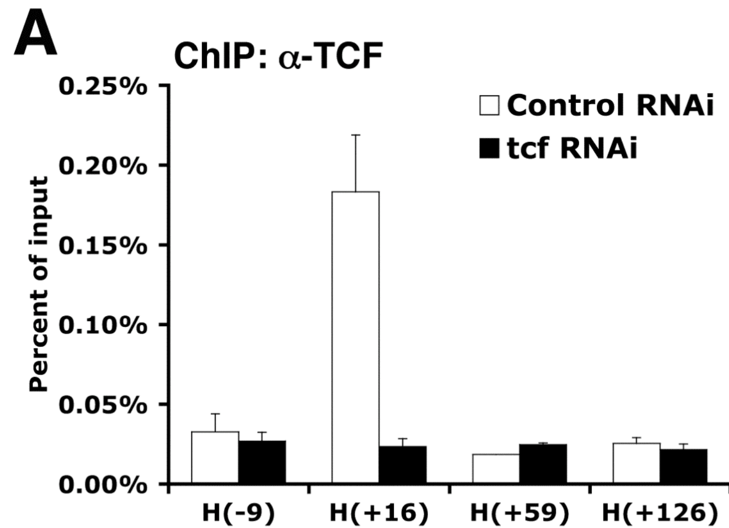


Figure 3.4. ACF1 binds to the broad regions of the *nkd* and *hth* genes. (A, B) Schematic diagrams of the *nkd* and *hth* loci with the illustrated sites used for ChIP analysis. Arrows indicate the TSSs. The numbers in parenthesis indicate the distance (in kb) from the TSS. N(–10) and N(+5) indicated the location of the two WREs of *nkd*. H(+16) is an area of *hth* bound by TCF. **(C, D)** ChIP analysis shows ACF1 binding to *nkd* and *hth*. Kc cells were treated with control dsRNA or *acf1* dsRNA for 6 days before they were harvested for ChIP analysis. For *nkd*, ACF1 binds to a broad region as well as the genes adjacent to it (**C**). For *hth*, there is a three-fold enrichment of ACF1 binding at H(+16) that was reproducibly observed (**D**). Each bar represents the mean (± S.E.) of duplicate cultures with duplicate Q-PCR reactions. **(E)** Depletion of *TCF* has no effect on ACF1 binding to *nkd* and *hth*. ChIP signals for control RNAi treated cells were normalized to 1. The efficiency of *TCF* RNAi was confirmed by Western blot and ChIP with α-TCF (data not shown). **(F)** Depletion of *iswi/acf1* reduces TCF binding to *nkd* and *hth*. The same normalization strategy was used as in (**E**). Depletion of *acf1* alone also reduces TCF binding to *nkd* and *hth*, but to a lesser degree compared to *iswi/acf1* double knockdown cells (data not shown). **(G)** Depletion of *TCF* and *iswi* cooperately derepresses *nkd* and *hth* transcript levels in Kc cells. All experiments have been performed three separate times with similar results.

(Parker *et al*, 2007b). These results suggest that ACF1 physically associates with Wg targets in the absence of Wg signaling in a broader pattern than TCF.

The third Wg target characterized in this report, *hth,* has a transcription unit nearly 130 kb in length (Fig. 3.4B) and the cis-acting elements controlling its expression have not been characterized. Using an online tool called Target Explorer (http://trantor.bioc.columbia.edu/ Target_Explorer; Sosinsky *et al*, 2003), we identified several clusters of putative TCF binding sites in the intronic regions of *hth*. ChIP analysis revealed strong TCF binding to one of these clusters, 16kb downstream of *hth* TSS, which was reduced to background levels upon *TCF* RNAi treatment (Fig. 3.5A). As was seen in the other Wg targets, ACF1 was bound to the entire *hth* locus (Fig. 3.4D). In contrast to *nkd* and *Notum*, we did observe greater ACF1 binding at the site bound by TCF than at other areas. Although the ACF1 ChIP signal at this site was only partially abolished by *acf1* RNAi, it was more dramatically reduced when both *acf1* and *iswi* were knocked down (data not shown). We conclude from these results that ACF1 directly binds to a large portion of the *hth* locus, with enrichment at the region also bound by TCF.

Since TCF and ACF1 share overlapping binding sites for all three Wg targets, we examined whether the binding of ACF1 is influenced by TCF, or vice versa. When *TCF* was depleted by RNAi, we did not observe a significant change of ACF1 binding to the WREs of *nkd*/*Notum* or the TCF bound region of *hth* (Fig. 3.4E, 3.6D). In contrast, TCF binding to these sites was significantly reduced when *iswi* and *acf1* were knocked down (Fig. 3.4F, Fig. 3.6D). *iswi*/*acf1* depletion did not reduce TCF expression as determined by Western blot (Fig. 3.5B). These results suggest that ISWI and ACF1 facilitate TCF binding to Wg targets in the absence of signaling.



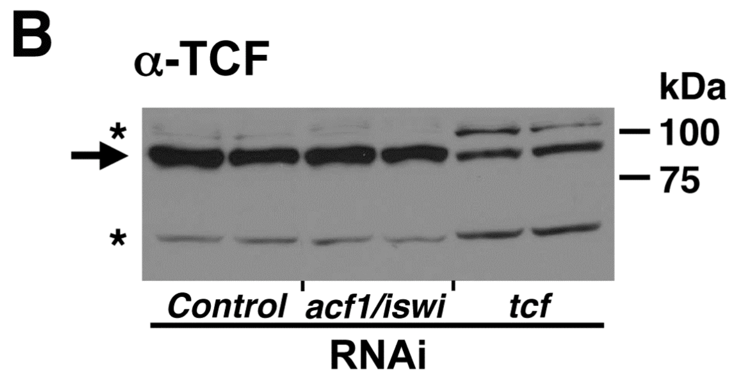


Figure 3.5 TCF preferentially binds to a specific site in the *hth* gene. (A) Kc cells were treated with control RNAi or *TCF* RNAi for six days before being harvested for TCF ChIP analysis. A 7-fold enrichment of TCF binding was seen in a region 16 kb downstream of the *hth* TSS, and the ChIP signal was reduced to background levels upon *TCF* RNAi. Data represent the means of duplicates (± S.E.), and the experiment has been performed two separate times with similar results. (B) Western blot of TCF protein was performed in control dsRNA or *iswi/acf1* dsRNA treated cells. As a control, *TCF* RNAi significantly reduced TCF protein levels. Asterisks indicate nonspecific bands.

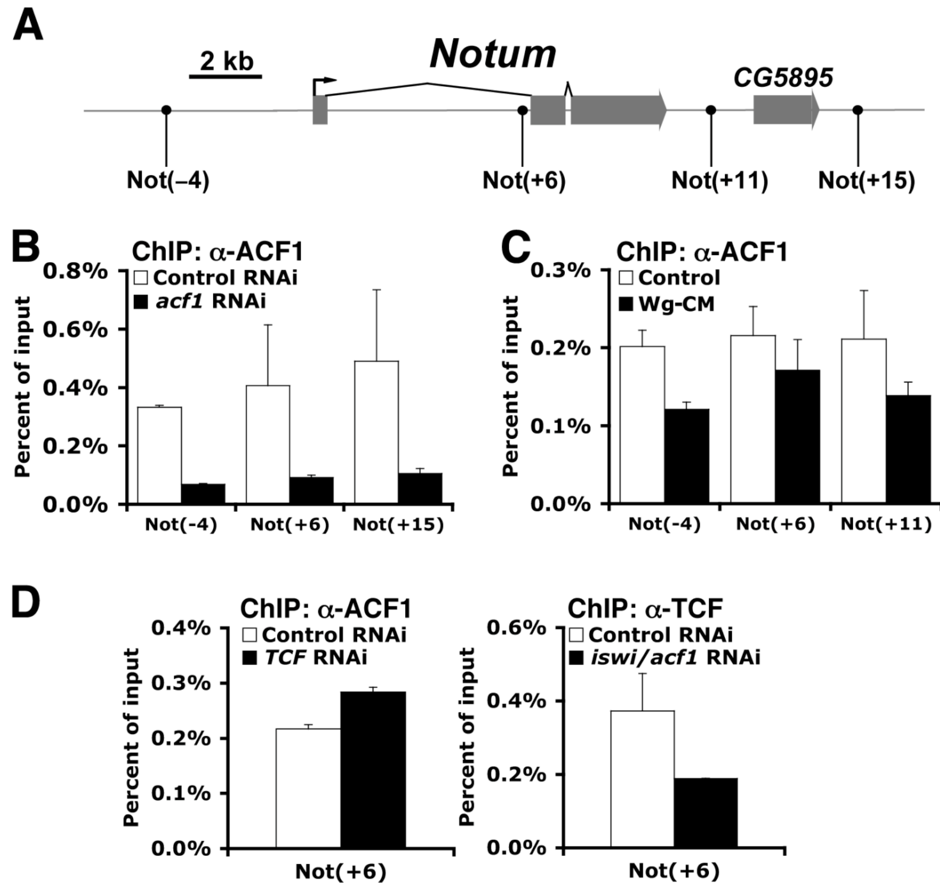


Figure 3.6. ACF1 binds to *Notum* in a similar fashion as to *nkd*. (A) Schematic diagram of the *Notum* locus with the illustrated sites used for ChIP analysis. Arrow indicates the TSS. The numbers in parentheses indicate the distance (in kb) from the TSS. Not(–4) and Not(+6) are two WREs in *Notum*. **(B)** ChIP analysis shows that ACF1 binds to a broad region on *Notum*. **(C)** Wg-CM has a modest influence on ACF1 binding to *Notum*. Same experimental conditions were used as in Fig. 3.8A, B. **(D)** Similar to what was seen for *nkd* and *hth*, ACF1 binding to *Notum* is not reduced by *TCF* RNAi, yet TCF binding to *Notum* is significantly reduced by *iswi*/*acf1* RNAi. The same experimental conditions were used as for Fig. 3.4E, F. Data represent the means of duplicates (± S.E.), and experiments have been performed two separate times with similar results.

TCF is thought to repress *nkd* expression with the transcriptional corepressor Gro (Fang et al, 2006) and the same is true for *hth* (Fig. 3.4G and data not shown). When *TCF* and *iswi* were depleted simultaneously, greater derepression of *nkd* and *hth* was observed than with either factor alone (Fig. 3.4G). This result suggests that ISWI acts in parallel with TCF to repress Wg target genes. Similar results were also observed upon depletion of *iswi* or *acf1* with *gro* (data not shown). These data suggest that ISWI/ACF1 has TCF-independent activities in repressing Wg targets.

ISWI/ACF1 represses Wg targets independent of chromatin assembly

Because of its role in chromatin assembly (Ito *et al,* 1999; Fyodorov and Kadonaga, 2002), it is possible that the derepression of Wg targets observed in cells lacking *iswi/acf1* is due to incomplete packaging of chromatin after mitosis, rather than specific transcriptional regulation. To test this possibility, the effect of *iswi/acf1* depletion on Wg targets in non-dividing Kc cells was examined. When hydroxyurea (HU), an inhibitor of DNA synthesis, was applied to Kc cells for 48 hours, cell division was effectively blocked (Fig. 3.7A). Derepression of *nkd* or *hth* by *iswi/acf1* knockdown was unaffected in the HU treated cells (Fig. 3.7B). Similar results were obtained using aphidicolin (Aph), a different DNA synthesis inhibitor (Fig. 3.7C, D). These data argue that the repression of Wg targets by ISWI/ACF1 is not due to the role of ACF/CHRAC in post-mitotic chromatin assembly.

The results described above suggest that repression of Wg targets by ISWI/ACF1 is unlikely due to their active assembly of chromatin after DNA replication. However, it is also possible that ISWI/ACF1 is required for maintaining histone/DNA integrity during

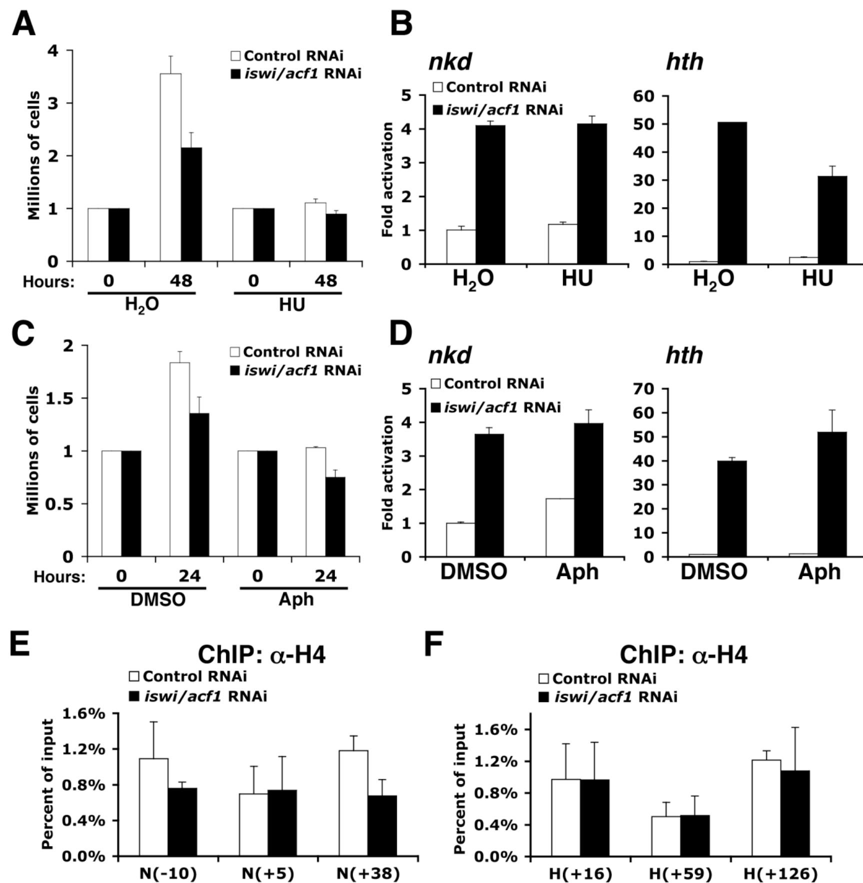


Figure 3.7. ISWI/ACF1 represses Wg targets independent of post-mitotic chromatin assembly. (A) Hydroxyurea (HU) effectively blocks cell division. Kc cells incubated with control or *iswi*/*acf1* dsRNA for four days were treated with H_2O or 5mM HU for an additional 48 hours. Cells stopped dividing, judged by cell number, upon HU treatment. In the control group, *iswi*/*acf1* RNAi decreases the cell division rate. A similar decrease in cell division was also observed with *acf1* RNAi, but not with *iswi* RNAi (data not shown). **(B)** Derepression of *nkd* and *hth* by *iswi*/*acf1* RNAi is not abolished after HU treatment. Same experimental conditions were used as in (**A**), and transcript levels of *nkd* and *hth* were measured by RT-Q-PCR. **(C)** Aphidicolin (Aph) also inhibits cell division. Kc cells incubated with control dsRNA or *iswi*/*acf1* dsRNA for four days were treated with DMSO or 25μM Aph for an additional 24 hours. Effective blockage of cell division was seen in Aph treated cells. **(D)** *iswi*/*acf1* RNAi still derepresses *nkd* and *hth* after Aph treatment. **(E, F)** *iswi*/*acf1* RNAi does not significantly affect H4 binding to the *nkd* and *hth* genes. ChIP analysis for pan-H4 was performed in control dsRNA or *iswi*/*acf1* dsRNA treated cells. Multiple sites including the *nkd* WREs and TCF binding region of *hth* were tested for H4 binding, and no obvious change was observed between control RNAi and *iswi*/*acf1* RNAi. Data shown were the means of duplicates (± S.E.), and all experiments have been performed two separate times with similar results.

interphase. To explore this possibility, ChIP analysis using histone H4 antibody was performed on the *nkd* and *hth* loci in *iswi/acf1* depleted cells. No significant change of H4 binding to various regions of *nkd* and *hth*, including the regions bound by TCF, was detected when *iswi* and *acf1* were knocked down (Fig. 3.7E, F). Taken together, these data lead us to favor a model where ISWI/ACF1 specifically repress Wg target gene transcription independently of chromatin assembly or maintenance.

Wg signaling reduces ACF1's binding to Wg targets

ACF1 is important in maintaining the silent state of Wg target genes (Fig. 3.1C) and is physically present at these loci in the absence of signaling (Fig. 3.4C, D; Fig. 3.6B). Therefore, we were curious to see whether ACF1 binding to Wg targets was regulated by Wg signaling. After a 5 hr treatment with Wg-CM, a consistent but modest reduction of ACF1 was observed at various regions for all three Wg targets (*hth*, *nkd* and *Notum*; Fig. 3.8A, B; Fig. 3.6C).

Although this result implies that ACF1's binding to Wg targets is not significantly regulated by Wg signaling, it is possible that only a portion of the cultured cells were responding to the Wg-CM stimulation. The unstimulated cells would elevate the background level of ACF1 ChIP signal and obscure a greater decrease of ACF1 binding by Wg signaling. Activation of Wg targets is correlated with the acetylation of histones H3/H4 throughout these loci (Parker *et al,* 2007b). This suggests that the Wg-stimulated chromatin could be selected by precipitation with acetylated H4 (AcH4) antibody, followed by an ACF1 re-ChIP to determine the binding of ACF1 on activated Wg targets. As a control, a pan-H4 ChIP followed by an ACF1 re-ChIP was performed. For *hth*, we

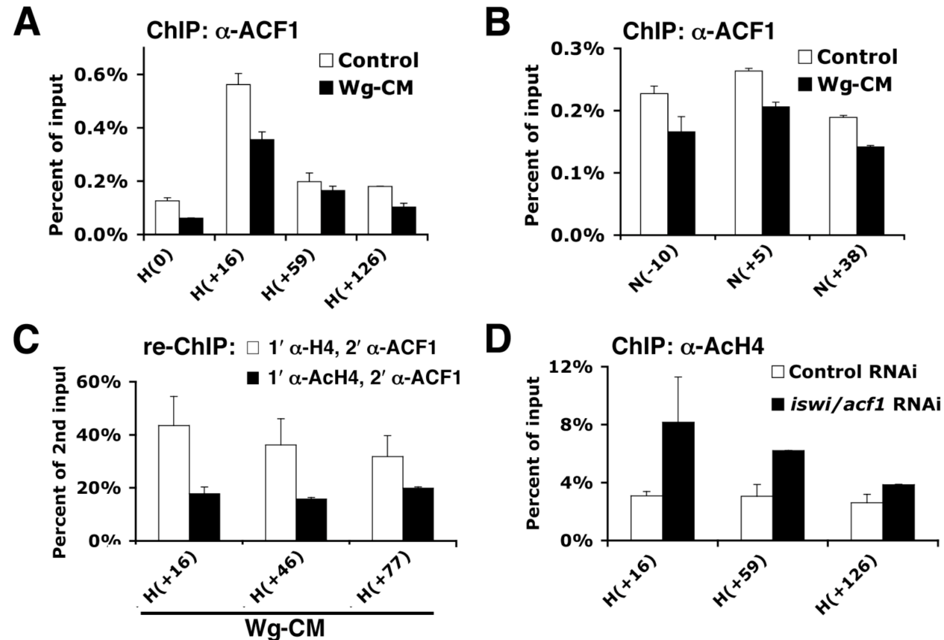


Figure 3.8. ACF1 binding to Wg targets is modestly reduced by Wg signaling. (A, B) Cells were treated with control media or Wg-CM for 5 hours before harvesting for ACF1 ChIP analysis. A modest decrease of ACF1 binding was observed across the *nkd* and *hth* loci. (C) Less ACF1 binds to AcH4 on *hth* upon Wg-CM treatment. Cells treated with Wg-CM for 5 hours underwent ChIP with either α-H4 antibody or α-AcH4 antibody, followed by a secondary ChIP with α-ACF1 antibody. The α-AcH4 antibody recognizes acetylated K5/8/12/16 on histone H4. The re-ChIP signal was normalized to the eluted solution from the first immunoprecipitate, termed the 2nd input. (D) ISWI/ACF1 antagonizes AcH4 levels on *hth* in the absence of Wg signaling. Cells were treated with control dsRNA or *iswi/acf1* dsRNA for six days before harvested for AcH4 ChIP analysis. An increase of AcH4 on *hth*, most prominently at the site bound by TCF, was observed. In general, data represent the means of duplicates (± S.E.), and all experiments have been performed at least two separate times with similar results.

observed a pronounced decrease of ACF1 binding to the AcH4 associated chromatin compared to its binding to the pan-H4 associated chromatin, with the greatest reduction observed at the location of TCF binding (H(+16); Fig. 3.8C). These results suggest that Wg signaling reduces ACF1 binding to the activated Wg target genes.

Our results are in agreement with previous studies that have shown that acetylation of H4 N-terminal tails may interfere with ISWI/ACF1 function (Corona *et al*. 2002; Shogren-Knaak *et al*. 2006). To further explore the relationship between ISWI/ACF1 activity and histone H4 acetylation at Wg targets, we performed AcH4 ChIP on unstimulated cells depleted for *iswi*/*acf1*. An increase of AcH4 binding to *hth,* especially at the TCF binding site, was observed when *iswi*/*acf1* was inhibited (Fig. 3.8D). This result suggests that ISWI/ACF1 antagonizes the acetylation of histone H4 on Wg targets in the absence of Wg signaling.

Discussion

ISWI/ACF1 transcriptionally represses Wg target genes

In the present study, loss-of-function analyses for *iswi* and *acf1* revealed that they play a negative role in regulating Wg targets in cell culture (Fig. 3.1C) and the wing imaginal disc (Fig. 3.2). The elevation of *nkd* transcripts observed in *iswi*/*acf1* depleted Kc cells was not due to a decrease in transcript turnover (Fig. 3.1F). Furthermore, reporter constructs for several Wg targets (i.e., Notum-lacZ, Nkd-lacZ and Dfz3-lacZ) were expressed ectopically in *iswi* mutant cells in the developing wing (Fig. 3.2B, F, N and data not shown). These data strongly argue that ISWI and ACF1 act as transcriptional repressors of Wg target genes.

Examination of genes not regulated by Wg signaling suggests that ISWI/ACF1 is not a general repressor of gene expression. In Kc cells, several genes were not affected by *iswi/acf1* depletion, including those adjacent to the *nkd* locus (Fig. 3.1D). In the wing imaginal disc, ISWI did not regulate Wg, Dpp-lacZ or Spalt expression (Fig. 3.3D-I), targets of Notch, Hh and Dpp signaling, respectively. These results suggest some degree of specificity for ISWI/ACF1 towards Wg targets.

En/Invected (Inv) expression was also largely unaffected in *iswi* mutant clones (Fig. 3.3A-C). This is seemingly in conflict with a previous report showing that En expression was abolished in *iswi* mutant wing discs (Deuring *et al*, 2000). Since the antibody used in our study recognized both En and Inv proteins (Patel *et al*, 1989), it is possible that the remaining Inv expression masked a reduction of En in the *iswi* clones.

Previous studies have shown that ISWI associates with ACF1 in two distinct chromatin remodeling complexes (Langst and Becker, 2001). Therefore, it is attractive to propose that ISWI and ACF1 act as a complex to repress Wg targets. However, simultaneous knockdown of *iswi* and *acf1* always resulted in higher derepression of targets than single RNAi treatments in Kc cells (Fig. 3.1C). Because RNAi does not completely abolish gene expression, these results are equivocal but could indicate that ACF1 and ISWI can function independently of each other. Interestingly, a human homologue of ACF1, WSTF, associates with a complex devoid of ISWI and regulates transcription (Kitagawa *et al*, 2003).

While ISWI and ACF1 both contribute to Wg target gene repression in Kc cells, the situation is different in the wing imaginal disc. In this tissue, complete removal of *acf1* had no effect on Wg targets, while cells lacking *iswi* showed ectopic expression of

several Wg targets (Fig. 3.2; data not shown). If ISWI and ACF1 act together to repress Wg targets in the developing wing, it is possible that ACF1 has a redundant partner. The closest relative to *acf1* in the fly genome is *toutatis (tou)*, which has been implicated in *Drosophila* neural development (Vanolst *et al.*, 2005). While *tou* mutant flies were semi-viable, flies homozygous for both *acf1* and *tou* died during late pupal stages. However, these double mutants did not display obvious aberrations in the expression of Wg targets (Chapter II). Deciphering the relationship between ISWI, ACF1 and Tou requires additional genetic analysis in flies and molecular analysis in cell culture.

A recent study revealed that ISWI was co-purified with β-catenin, implying that it plays a positive role in Wnt signaling (Sierra *et al.*, 2006). Further functional analyses are needed to corroborate such a role. Since ISWI containing complexes have been implicated in both transcriptional repression and activation (Corona and Tamkun, 2004), it is possible that ISWI plays multiple roles in regulating Wnt targets in different biological contexts.

Mechanism of ISWI/ACF1 regulation of Wg signaling

The repression of Wg target gene expression by ISWI/ACF1 was independent of Arm (Fig. 3.1E). This distinguishes ISWI/ACF1 from several other factors that antagonize Wnt/Wg by interfering with β-catenin/Arm binding to TCF, such as ICAT (Tago *et al*, 2000), Chibby (Takemaru *et al*, 2003), CtBP/APC (Hamada and Bienz, 2004), and SOX9 (Akiyama *et al*, 2004). Rather, ISWI/ACF1 is required for silencing of Wg targets in the absence of pathway activation, similar to CtBP and Gro in Kc cells (Fang *et al,* 2006) and Kaiso in *Xenopus* embryos (Park *et al,* 2005). Consistent with a

role as a direct transcriptional repressor, ACF1 was found to be associated with Wg target gene chromatin (Fig. 3.4C, D; Fig. 3.6B).

ISWI and ACF1 are known to form a complex that can efficiently package DNA and nucleosomes into chromatin *in vitro* (Ito *et al,* 1999; Fyodorov and Kadonaga 2002) and there is evidence that they are required for some aspects of chromatin organization *in vivo* (Deuring *et al*, 2000; Corona *et al*, 2002; Fyodorov *et al*, 2004). However, we found that *iswi*/*acf1* were still required for silencing Wg targets in non-dividing cells (Fig. 3.7B, D). This suggests that incomplete chromatin assembly after mitosis is not a major contributor to the derepression seen in *iswi*/*acf1* depleted cells. In addition, inhibition of *iswi*/*acf1* did not alter the deposition of histone H4 on Wg targets (Fig. 3.7E-F). These data lead us to favor a model where ISWI and ACF1 act as specific transcriptional repressors of Wg target genes.

In the absence of Wg signaling, TCF contributes to target gene silencing (Cavallo *et al*, 1998; Fang *et al*, 2006). We found that TCF's binding to specific sites in Wg targets was significantly reduced upon the depletion of *iswi*/*acf1* (Fig. 3.4F, Fig. 3.6D). While this suggests that ISWI and ACF1 act to repress these genes by promoting TCF binding, it is unlikely to be the whole story. Depletion of *iswi* or *acf1* with *TCF* or *gro* led to a non-additive derepression of Wg targets (Fig. 3.4G; data not shown), suggesting that ISWI/ACF1 acts independently of TCF in gene silencing. It appears that ISWI and ACF1 repress Wg targets through multiple mechanisms, only some of which involve TCF.

In general, ISWI/ACF1 is not found uniformly on chromatin in flies (Deuring *et al*, 2000), in murine thymocytes (Yasui *et al*, 2002), and in yeast (Gelbart *et al*, 2005).

Consistent with these findings, there are several examples in yeast and mammals where specific DNA-binding proteins recruit ISWI/ACF1 to chromatin (Goldmark *et al*, 2000; Yasui *et al*, 2002; Moreau *et al*, 2003; Bachman *et al*, 2005; Alenghat *et al*, 2006). In contrast, we found that ACF1 binds across large regions (19-126 kb) of several Wg targets (Fig. 3.4C, D; Fig. 3.6B). A previous study demonstrated widespread binding of yeast Isw2 to the DNA damage-inducible gene *RNR3*, though the region bound was less than 4 kb (Zhang and Reese, 2004). For both *RNR3* and the Wg targets identified in this report, it is not clear whether the widespread binding of ACF1 or Isw2 is required for efficient repression.

Consistent with their ability to slide nucleosomes *in vitro*, Isw1 and Isw2 are required for nucleosome positioning at the promoters of several genes they repress (Goldmark *et al*, 2000; Moreau *et al*, 2003; Zhang and Reese, 2004; Sherriff *et al*, 2007). In the case of the *PHO8* promoter, this activity is required to displace TBP under repressive conditions (Moreau *et al*, 2003). While we have not yet examined the role of ISWI/ACF1 in regulating nucleosome distribution at Wg targets (in part because of the large regions bound by ACF1), it is likely that they also act at this level in our system.

Regulation of ISWI/ACF1 by Wg signaling

Does activation of Wg signaling effect ISWI/ACF1 binding to the target genes? A modest reduction of ACF1 binding to Wg target loci was observed after Kc cells were stimulated with Wg-CM (Fig. 3.8A, B). A marked decrease of ACF1 association with AcH4 was observed compared to generic H4 (Fig. 3.8C) and depletion of *iswi/acf1* resulted in increased AcH4 at a Wg target (Fig. 3.8D). These results suggest that

ISWI/ACF1 acts antagonistically with AcH4 to regulate the transcriptional response to Wg signaling. Interestingly, a similar relationship has been suggested in dosage compensation (Corona *et al*, 2002; Shogren-Knaak *et al*, 2006).

Our laboratory has recently shown that Wg signaling induces a widespread increase in histone acetylation throughout Wg targets (Parker *et al,* 2007b). This chromatin modification requires CBP, a histone acetyltransferase that is recruited to WREs in a Wg and Arm-dependent manner and is required for target gene activation (Li *et al,* 2007b). Therefore, it is tempting to propose that Wg signaling promotes displacement/inactivation of ISWI/ACF1, by activating histone acetylation, which is necessary for transcriptional activation of Wg targets.

Finally, it is possible that the residual binding of ACF1 to target genes after Wg stimulation has functional relevance. In the presence of Wg signaling, Wg target genes were further activated upon depletion of *iswi*/*acf1* in Kc cells and in the wing imaginal disc (Fig. 3.2 and data not shown). Therefore, ISWI/ACF1 could have a dual function in regulating Wg targets. In the absence of Wg signaling, they help maintain the silent state of Wg targets. When Wg signaling is activated, the negative influence of ISWI/ACF1 may help to set the threshold for the precise activation of Wg targets.

Materials and methods

***Drosophila* genetics**

The *iswi* mutant strain $iswi^1$ was kindly provided by J. Tamkun (Deuring *et al*, 2000). For clonal analysis, $iswi^1$ was recombined onto a FRT^{42D} chromosome using standard methods (Xu and Rubin, 1993). Somatic clones of $iswi^1$ in wing imaginal discs were generated by crossing $FRT^{42D}iswi^1$ males to *yw* P[*HS-Flp*]; FRT^{42D} P[*Ubi-GFP*]

females. Clones were induced by one-hour 37°C heat shock at 48-72 hours after egg laying.

A 2.2kb fragment from approximately –4.1 to –1.9 kb upstream of the *Notum* TSS (Städeli *et al,* 2005) was cloned into pH-Pelican vector (Barolo *et al*, 2000), and the corresponding Notum-lacZ transgenic flies were generated by BestGene Inc (Chino Hills, CA). In a similar fashion, an 864bp fragment upstream of the *nkd* TSS (–10.5 to –9.7 kb) was used to generate the Nkd-lacZ reporter. Both reporters were positively regulated by Wg signaling in all tissues examined, including the wing imaginal disc (data not shown). The Dpp-lacZ line (Blackman *et al*, 1991) was obtained from the Bloomington Stock Center.

Antibodies, immunoblot and immunostaining

Rabbit and guinea pig α-ACF1 antisera were generously provided by D. Fyodorov. Rabbit polyclonal α-ISWI was from J. Kadonaga (Ito *et al*, 1999). Rabbit polyclonal α-TCF antisera and guinea pig α-Sens have been described previously (Fang *et al*, 2006). Rabbit polyclonal α-acetyl-histone H4 (#06-866) and rabbit monoclonal α-histone H4 (#05-858) were from Upstate. Mouse α-β-galactosidase was from Sigma-Aldrich. Rabbit α-β-galactosidase was from Abcam. Mouse α-Wg (4D4) and mouse α-En/Inv (4D9) were from the Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit polyclonal α-Spalt was from R. Schuh and B. Mollereau (Kuhnlein *et al*, 1994).

For immunobloting, α-rabbit ACF1 (1:5000), α-ISWI (1:2000) and α-TCF (1:2000) were followed by HRP-conjugated α-rabbit IgG (1:2000). Signal was detected with the ECL kit (Amersham Bioscience). Immunostaining of wing imaginal discs was

as described previously (Parker *et al*, 2002). The dilution factors for the primary antibodies used were: α-Sens (1:1000), α-Wg (1:100), α-En/Inv (1:20), α-Spalt (1:100), rabbit α-β-galactosidase (1:200), mouse α-β-galactosidase (1:500). Cy3- and Cy5-conjugated secondary antibodies were from Jackson Immunochemicals, and Alexa 488-conjugated secondary antibody was from Molecular Probes. All fluorescent images were obtained with Olympus FV-500 Confocal microscope, and processed in Adobe Photoshop 8.0.

***Drosophila* cell culture**

Drosophila embryonic Kc167 (Kc) cells were cultured at room temperature in the Schneider's *Drosophila* media (Invitrogen) containing 5% FBS and Penicillin/Streptomycin antibiotics. RNAi mediated knockdown of gene expressed was performed as described elsewhere (Clemens *et al*, 2000) with modifications. Briefly, when Kc cells approached confluent status after 4-5 days of culture (~8×10^6/ml), they were resuspended at 1×10^6/ml in standard media and seeded onto 12-well plates (1ml/well) or T-25 flask (6ml/well). RNA duplex was then added at a final concentration of 9μg/ml. After 4 days, cells were resuspended, diluted into 1×10^6/ml with fresh media and reseeded onto 12-well plates or T-25 flask. Cells were harvested after 2 additional days incubation. dsRNAs with a typical length of 500-700 bp were synthesized using the MEGAscript T7 *in vitro* transcription kit (Ambion). The sequences of the PCR primers for the dsRNA synthesis are: *iswi* (1st duplex: 5'-CCATCAGTTGCGGCTGCAATATGGT AA-3' & 5'-GCGGCACGCAATAGTAATGTAGTCGGAT-3'; 2nd duplex: 5'-CCACTTCATG ACTAACAGCGCTAAGAGT-3' & 5'-GCAGAATCTCCGACAGCTTCGACTTCT-3'), *acf1* (1st duplex: 5'-CGACCACGTAACTCTTTGCGCCTATCTA-3' & 5'-GCGTGTGCTGAACTT

AGAACTGACAT-3'; 2nd duplex: 5'-CGATGAATGCAACGCTGGCACTCACAT-3' & 5'-GGTCGCTTGAGGTGAACACATTCCA-3'). The sequences of primers for control, *arm* and *TCF* dsRNAs have been published previously (Fang *et al*, 2006).

Wg-CM was collected from stable *pTubwg* S2 cells provided by R Nusse, and stored at -80°C. For 1×10^6 cells, 5 hours treatment of 200μl-500μl Wg CM was typically performed prior to harvesting. Media collected from *Drosophila* S2 cells was used as control. The pharmacological reagents Hydroxyurea (Sigma-Aldrich H8627) and Aphidicolin (Sigma-Aldrich A0781) were added to the cells (final concentrations of 5mM and 25μM, respectively) after they were reseeded at 1×10^6/ml on the 4th day of RNAi treatment. For the α-amanitin experiment, cells were treated with α-amanitin (Sigma-Aldrich A2263) at a final concentration of 10μg/ml for the indicated times on the 6th day of RNAi treatment.

Real-time quantitative PCR (Q-PCR)

Samples were analyzed using an iCycler iQ real-time PCR detection system. For RT-PCR, Trizol (Invitrogen) was used to extract total RNA from 1-5×10^6 cells. Reverse transcription was performed using Stratascript reverse transcriptase (Stratagene) followed by Q-PCR analysis. *β-tubulin56D* or *arm* or *TCF* or the combination of all three were used to normalize the transcripts. Q-PCR primers were designed by using the online program Primer3 (http://frodo.wi.mit.edu/), and their sequences are available upon request.

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed according to the protocol of Upstate with minor modifications. An initial protein-crosslinking step was included by incubating cells in

10mM DTBP solution (Pierce #20665) for 30 minutes on ice. For each immunoprecipitation, 3×10^6 cells were used and the amounts of antibodies used are as following: 5μl guinea pig α-ACF1 (provided by D. Fyodorov), 10μl rabbit α-TCF (Fang *et al*, 2006), 3μl rabbit α-H4 (Upstate), 1μl rabbit α-AcH4 (Upstate). All ChIP samples were quantified with Q-PCR. The inputs refer to the samples that were not subject to immunoprecipitation. The primer sequences for ChIP sites on *nkd*, *hth* and *Notum* loci are available upon request. For the re-ChIP assay, DNA-protein complexes were eluted by incubation in 50μl 10mM DTT for 30min at 37°C. After centrifugation, the supernatant was diluted into 1 ml (20 times) with ChIP dilution buffer. Half of the eluted sample (500μl) was saved as the secondary input, and the other half was subject to immunoprecipitation by the second antibody.

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References

Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD, de Crombrugghe B (2004) Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev* **18**:1072-87

Alenghat T, Yu J, Lazar, MA (2006) The N-CoR complex enables chromatin remodeler SNF2H to enhance repression by thyroid hormone receptor. *EMBO J* **25**:3966-74

Azpiazu N, Morata G (2000) Function and regulation of homothorax in the wing imaginal disc of *Drosophila*. *Development* **27**:2685-93

Bachman N, Gelbart ME, Tsukiyama T, Boeke JD (2005) TFIIIB subunit Bdp1p is required for periodic integration of the Ty1 retrotransposon and targeting of Isw2p to S. cerevisiae tDNAs. *Genes Dev* **19**:955-64

Barolo S, Carver LA, Posakony JW (2000) GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *Biotechniques* **29**:726-32

Blackman RK, Sanicola M, Raftery LA, Gillevet T, Gelbart WM (1991) An extensive 3' cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila. *Development* **111**:657-66

Cadigan KM, Liu YI (2006) Wnt signaling: complexity at the surface. *J Cell Sci* **119**:395-402

Casares F, Mann RS (2000) A dual role for homothorax in inhibiting wing blade development and specifying proximal wing identities in Drosophila. *Development* **127**:1499-508

Cavallo RA, Cox RT, Moline MM, Roose J, Polevoy GA, Clevers H, Peifer M, Bejsovec A (1998) *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**:604-8

Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, Dixon JE (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci USA* **97**:6499-503

Clevers H (2006) Wnt/beta-catenin signaling in development and disease. *Cell* **127**:469-80

Collins RT, Treisman JE (2000) Osa-containing Brahma chromatin remodeling complexes are required for the repression of wingless target genes. *Genes Dev* **14**:3140-52

Corona DF, Clapier CR, Becker PB, Tamkun JW (2002) Modulation of ISWI function by site-specific histone acetylation. *EMBO Rep* **3**:242-7

Corona DF, Tamkun JW (2004) Multiple roles for ISWI in transcription, chromosome

organization and DNA replication. *Biochim Biophys Acta* 1677:113-9

Daniels DL, Eklof Spink K, Weis WI (2001) beta-catenin: molecular plasticity and drug design. *Trends Biochem Sci* **26**:672-8

Daniels DL, Weis WI (2005) Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* **12**:364-71

Deuring R, Fanti L, Armstrong JA, Sarte M, Papoulas O, Prestel M, Daubresse G, Verardo M, Moseley SL, Berloco M, Tsukiyama T, Wu C, Pimpinelli S, Tamkun JW (2000) The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. *Mol Cell* **5**:355-65

Diaz-Benjumea FJ, Cohen SM. (1995) Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**:4215-25

Ding Y, Dale T (2002) Wnt signal transduction: kinase cogs in a nano-machine? *Trends Biochem Sci* **27**:327-9

Fang M, Li J, Blauwkamp T, Bhambhani C, Campbell N, Cadigan KM (2006) C-terminal-binding protein directly activates and represses Wnt transcriptional targets in *Drosophila*. *EMBO J* **25**:2735-45

Fyodorov DV, Kadonaga JT (2002) Dynamics of ATP-dependent chromatin assembly by ACF. *Nature* **418**:897-900

Fyodorov DV, Blower MD, Karpen GH, Kadonaga JT (2004) Acf1 confers unique activities to ACF/CHRAC and promotes the formation rather than disruption of chromatin in vivo. *Genes Dev* **18**:170-83

Gelbart ME, Bachman N, Delrow J, Boeke JD, Tsukiyama T (2005) Genome-wide identification of Isw2 chromatin-remodeling targets by localization of a catalytically inactive mutant. *Genes Dev* **19**:942-54.

Gerlitz O, Basler K (2002) Wingful, an extracellular feedback inhibitor of Wingless. *Genes Dev* **16**:1055-9

Giraldez AJ, Copley RR, Cohen SM (2002) HSPG modification by the secreted enzyme Notum shapes the Wingless morphogen gradient. *Dev Cell* **2**:667-76

Goldmark JP, Fazzio TG, Estep PW, Church GM, Tsukiyama T (2000) The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell* **103**:423-33

Hamada F, Bienz M (2004) The APC tumor suppressor binds to C-terminal protein to divert nuclear beta-catenin from TCF. *Dev Cell* **7**:677-85

Houston DW, Kofron M, Resnik E, Langland R, Destree O, Wylie C, Heasman J (2002) Repression of organizer genes in dorsal and ventral Xenopus cells mediated by maternal XTcf3. *Development* **129**:4015-25

Ito T, Levenstein ME, Fyodorov DV, Kutach AK, Kobayashi R, Kadonaga JT (1999) ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. *Genes Dev* **13**:1529-39

Kim CH, Oda T, Itoh M, Jiang D, Artinger KB, Chandrasekharappa SC, Driever W, Chitnis AB (2000) Repressor activity of Headless/Tcf3 is essential for vertebrate head formation. *Nature* **407**:913-6

Kitagawa H, Fujiki R, Yoshimura K, Mezaki Y, Uematsu Y, Matsui D, Ogawa S, Unno K, Okubo M, Tokita A, Nakagawa T, Ito T, Ishimi Y, Nagasawa H, Matsumoto T, Yanagisawa J, Kato S (2003) The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* **113**:905-17

Kuhnlein RP, Frommer G, Friedrich M, Gonzalez-Gaitan M, Weber A, Wagner-Bernholz JF, Gehring WJ, Jackle H, Schuh R (1994) spalt encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the Drosophila embryo. *EMBO J* **13**:168-79

Langst G, Becker PB (2001) Nucleosome mobilization and positioning by ISWI-containing chromatin-remodeling factors. *J Cell Sci* **114**:2561-8

Li B, Carey M, Workman JL (2007a) The role of chromatin during transcription. *Cell* **128**:707-19

Li J, Sutter C, Parker DS, Blauwkamp T, Fang M, Cadigan KM (2007b) CBP/p300 are bimodal regulators of Wnt signaling. *EMBO J* **26**: 2284-2294

Liu F, van den Broek O, Destree O, Hoppler S (2005) Distinct roles for Xenopus Tcf/Lef genes in mediating specific responses to Wnt/{beta}-catenin signalling in mesoderm development. *Development* **132**:5375-85

Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* **20**:781-810

Merrill BJ, Pasolli HA, Polak L, Rendl M, Garcia-Garcia MJ, Anderson KV, Fuchs E (2004) Tcf3: a transcriptional regulator of axis induction in the early embryo. *Development* **131**:263-74

Moreau JL, Lee M, Mahachi N, Vary J, Mellor J, Tsukiyama T, Goding CR (2003) Regulated displacement of TBP from the PHO8 promoter in vivo requires Cbf1 and the Isw1 chromatin remodeling complex. *Mol Cell* **11**:1609-20

Park JI, Kim SW, Lyons JP, Ji H, Nguyen TT, Cho K, Barton MC, Deroo T, Vleminckx K, Moon RT, McCrea PD (2005) Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell* **8**:843-54

Parker DS, Jemison J, Cadigan KM (2002) Pygopus, a nuclear PHD-finger protein required for Wingless signaling in *Drosophila*. *Development* **129**: 2565-76

Parker DS, Blauwkamp T, Cadigan KM (2007a) Wnt/β-catenin-mediated transcriptional regulation. In *Wnt Signaling in Embryonic Development*, Sokol S., (ed), Advances in Developmental Biology, Wassarman PM (ed), Vol. 17, pp. 1-61. San Diego: Elsevier.

Parker DS, Ni Y, Li, J, Cadigna KM (2007b) Wingless signaling induces widespread chromatin remodeling of target loci. Manuscript submitted.

Patel NH, Martin-Blanco E, Coleman KG, Poole SJ, Ellis MC, Kornberg TB, Goodman CS (1989) Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**:955-68

Phillips RG, Whittle JR (1993) wingless expression mediates determination of peripheral nervous system elements in late stages of Drosophila wing disc development. *Development* **118**:427-38

Rocheleau CE, Downs WD, Lin R, Wittmann C, Bei Y, Cha YH, Ali M, Priess JR, Mello CC (1997) Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. *Cell* **90**:707-16

Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H (1998) The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**:608-12

Roose J, Huls G, van Beest M, Moerer P, van der Horn K, Goldschmeding R, Logtenberg T, Clevers H (1999) Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* **285**:1923-6

Schweizer L, Nellen D, Basler K (2003) Requirement for Pangolin/dTCF in *Drosophila* Wingless signaling. *Proc Natl Acad Sci USA* **100**:5846-51

Sherriff JA, Kent NA, Mellor, J (2007) The Isw2 chromatin-remodeling ATPase cooperates with the Fkh2 transcription factor to repress transcription of the B-type cyclin gene CLB2. *Mol Cell Biol* **27**:2848-60

Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, Peterson CL (2006) Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science* **311**:844-7

Sierra J, Yoshida T, Joazeiro CA, Jones KA (2006) The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes. *Genes Dev* **20**:586-600

Sivasankaran R, Calleja M, Morata G, Basler K (2000) The Wingless target gene Dfz3 encodes a new member of the *Drosophila* Frizzled family. *Mech Dev* **91**:427-31

Sosinsky A, Bonin CP, Mann RS, Honig B (2003) Target Explorer: An automated tool for the identification of new target genes for a specified set of transcription factors. *Nucleic Acids Res* **31**:3589-92

Städeli R, Basler K (2005) Dissecting nuclear Wingless signalling: recruitment of the transcriptional co-activator Pygopus by a chain of adaptor proteins. *Mech Dev* **122**:1171-82

Städeli R, Hoffmans R, Basler K (2006) Transcription under the control of nuclear Arm/beta-catenin. *Curr Biol* **16**:R378-85

Standley HJ, Destree O, Kofron M, Wylie C, Heasman J (2006) Maternal XTcf1 and XTcf4 have distinct roles in regulating Wnt target genes. *Dev Biol* **289**:318-28

Tabata T (2001) Genetics of morphogen gradients. *Nat Rev Genet* **2**:620-30

Tago K, Nakamura T, Nishita M, Hyodo J, Nagai S, Murata Y, Adachi S, Ohwada S, Morishita Y, Shibuya H, Akiyama T (2000) Inhibition of Wnt signaling by ICAT, a novel beta-catenin-interacting protein. *Genes Dev* **14**:1741-9

Takemaru K, Yamaguchi S, Lee YS, Zhang Y, Carthew RW, Moon RT (2003) Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. *Nature* **422**:905-9

Thorpe CJ, Schlesinger A, Carter JC, Bowerman B (1997) Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. *Cell* **90**:695-705

Vanolst L, Fromental-Ramain C, Ramain P (2005) Toutatis, a TIP5-related protein, positively regulates Pannier function during *Drosophila* neural development. *Development* **132**:4327-38

Wang S, Jones KA (2006) CK2 controls the recruitment of Wnt regulators to target genes in vivo. *Curr Biol* **16**:2239-44

Wernet MF, Labhart T, Baumann F, Mazzoni EO, Pichaud F, Desplan C (2003) Homothorax switches function of *Drosophila* photoreceptors from color to polarized light sensors. *Cell* **115**:267-79

Xu T, Rubin GM (1993) Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**:1223-37

Yasui D, Miyano M, Cai S, Varga-Weisz P, Kohwi-Shigematsu T (2002) SATB1 targets chromatin remodeling to regulate genes over long distances. *Nature* **419**:641-5

Zhang Z, Reese JC (2004) Ssn6-Tup1 requires the ISW2 complex to position nucleosomes in Saccharomyces cerevisiae. *EMBO J* **23**:2246-57

Zeng W, Wharton KA Jr, Mack JA, Wang K, Gadbaw M, Suyama K, Klein PS, Scott MP

(2000) naked cuticle encodes an inducible antagonist of Wnt signalling. *Nature* **403**:789-95

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Wnt/β-cat signaling controls a large array of events in both developing and adult tissues, which is thought to be achieved through regulation of specific target gene expression. Insight into how Wnt transcriptional targets are regulated will not only improve our knowledge of the pathway, but also provide an important context for understanding eukaryotic transcriptional control in general.

As a sizable body of work has been focusing on Wnt target activation, our knowledge of how these genes are silenced in the absence of signal is still limited. By characterizing the role of several ISWI family chromatin remodelers in Wingless (Wg) signaling, I believe my dissertation makes significant contributions to understanding the repression mechanism for Wnt targets in the context of chromatin.

ISWI/ACF1 specifically represses Wg targets

My studies showed that ISWI represses 4 Wg targets (Notum-lacZ, Sens, Nkd-lacZ and Dfz3-lacZ) in the developing wing, and ISWI/ACF1 represses 3 Wg targets (*nkd*, *Notum* and *hth*) in cultured cells. In contrast, ISWI does not affect 3 non-Wg

targets (En/Inv, Dpp-lacZ and Spalt) in the wing, nor does ISWI/ACF1 repress 3 non-Wg targets in cultured cells including two genes adjacent to *nkd* (Fig. 3.1-3.3). These results suggest that ISWI/ACF1 has considerable specificity for repressing Wg targets, rather than acting as universal repressors.

On the other hand, ISWI and ACF1 are not required for repression of all Wg targets. Loss of *iswi* does not affect the expression of Dll, a long-range Wg target in the wing (Zecca et al., 1996, data not shown). Depletion of *iswi/acf1* does not derepress *CG6234*, a direct target of Wg signaling in Kc cells (Fang et al., 2006, data not shown). These data are hardly surprising, as none of the characterized repressors in the pathway, including TCFs, represses all Wg target genes (Parker et al., 2007a). The default repression of Wnt targets could be gene-specific possibly due to the different thresholds of basal expression levels for different genes.

Although ISWI and ACF1 have certain specificity for repressing Wg targets, it is unlikely that they are dedicated repressors in the pathway. The kinked pattern of Wg expression seen in one third of *iswi* clones is probably not a defect of Wg signaling, as perturbation of Wg signaling does not lead to a distortion of Wg expression. Also, *patched*, a direct target of Hth signaling, is significantly derepressed upon inhibition of *iswi/acf1* in Kc cells (data not shown). Therefore, ISWI/ACF1 could be involved in repressing target genes of other signaling pathways. In fact, a recent report demonstrated that SNF2H, a human ISWI homologue, acts with histone deacetylase (HDAC) to repress thyroid hormone receptor (TR) regulated genes in cultured cells (Alenghat et al., 2006).

ISWI/ACF1 has been shown to participate in post-mitotic chromatin assembly and maintenance of higher-order chromosome structure (Corona and Tamkun, 2004).

Nevertheless, I observed that ISWI/ACF1 still represses Wg targets in non-dividing cells, and the integrity of histone H4 deposition is maintained upon depletion of *iswi/acf1* (Fig. 3.7). These results suggest that repression of Wg targets by ISWI/ACF1 is independent of their activity in chromatin assembly and maintenance.

A more comprehensive understanding of ISWI/ACF1's role in transcription can be achieved by a whole genome expression analysis using microarray. Previously, two microarray studies have been done for ISWI-type factors. The first was performed in yeast *isw2* mutant. For the *isw2* single mutant, only 3 genes in the whole genome are significantly derepressed (>3 fold). In contrast, a much larger portion of genes (114) are derepressed in the absence of *isw2* and *rpd3*, the latter encoding a histone deacetylase, suggesting the cooperation between these two proteins in repression (Fazzio et al., 2001). The second microarray was performed in fly *Nurf301* (encoding a cofactor of an ISWI complex) mutant, where the expression of 477 genes (both activation and repression) is affected (Badenhorst et al., 2005). These results indicate that ISWI-type factors are not global regulators of gene expression.

To examine how specifically ISWI/ACF1 represses Wg targets, microarray analysis can be performed in Kc cells, and the expression profiles between control RNAi and *iswi/acf1* RNAi can be compared. Comparing the list of genes affected by *iswi/acf1* RNAi to the list of Wg-induced genes in a separate microarray (T. Blauwkamp, unpublished) will be informative on how prevalent ISWI/ACF1 mediated repression of Wg targets.

More experimental conditions can be added to the microarray analysis. It would be interesting to examine the expression profiles of *iswi* RNAi alone and *acf1* RNAi

alone, especially for the genes that are affected by *acf1* RNAi but not by *iswi* RNAi. Since I have observed an ISWI-independent ACF1 activity for some Wg targets in Kc cells (see a separate section), it would be interesting to know to what extent this activity can be observed at the genome level.

A cooperation between ISWI and TCF in repressing two Wg targets (*nkd* and *hth*) was observed in fly cells (Fig. 3.4G). In contrast, another Wg target, *Notum*, appears predominantly repressed by TCF itself, since *TCF* RNAi causes >30 fold derepression of *Notum* in the absence of signal and no further derepression is seen upon *iswi/TCF* RNAi (data not shown). Comparing the derepression profiles at the genome level between *iswi* RNAi, *TCF* RNAi and *iswi/TCF* RNAi conditions will be useful to determine how general a mechanism of parallel repression is adopted for Wg targets.

Widespread binding of ACF1 to Wg targets

ACF1 was found bound to broad regions of several Wg targets (*nkd*, *Notum* and *hth*), spanning up to 130 kb. In addition, the binding of ACF1 extends to two genes adjacent to *nkd*, even though they are not regulated by ISWI/ACF1 (Fig. 3.4). This is reminiscent to a previous report showing that yeast Isw2 binds to a broad region of the DNA damage-inducible gene *RNR3* (spanning 3.8kb). The binding of Isw2 also extends to the regions where the nucleosome positioning is not regulated by Isw2 (Zhang and Reese, 2004).

Upon Wg signaling stimulation, a modest reduction of ACF1 binding to Wg targets was observed (Fig. 3.8). Moreover, less ACF1 binds to acetylated histones in the presence of signal, suggesting that histone acetylation antagonizes ACF1 binding

to Wg targets. Combined with the previous observation in the lab that Wg signaling induces widespread histone acetylation on its targets (Parker et al., 2007b), we propose a model where this broad acetylation displaces or inactivates ACF1 binding, resulting in an alteration of chromatin conformation favorable for Wg target activation (Figure 4.1).

How broadly does ACF1 bind to Wg targets, or does ACF1 bind everywhere in the genome reflecting its chromatin maintenance activity? I consider the latter possibility less likely since there are quite a few good examples both in yeast and mammals showing that ISWI/ACF1 preferentially binds to the enhancers of its target genes, often recruited by specific DNA-binding proteins, to silence their expression (Goldmark et al., 2000; Yasui et al., 2002; Moreau et al., 2003; Alenghat et al., 2006).

To explore how widespread the ACF1 binding is, loci further upstream and downstream of Wg targets can be tested by ACF1 ChIP, and it will be comforting to see that the binding of ACF1 on chromatin eventually drops off at some points away from Wg targets. In addition, it will be interesting to explore how broadly the ACF1 binding is reduced in the presence of signal, since this has not been tested especially for the areas outside of Wg targets. If a more localized reduction of ACF1 binding to Wg targets is observed, it will argue that the reduction is due to a specific Wg signaling influence rather than a non-specific effect of Wg-CM.

Is ACF1 bound broadly to Wg targets in flies as well? From my studies, the best derepressable Wg target in *iswi* mutant clones is Notum-lacZ in the developing wing (Fig. 3.2E-H). As my colleagues in the Cadigan lab are establishing the ChIP

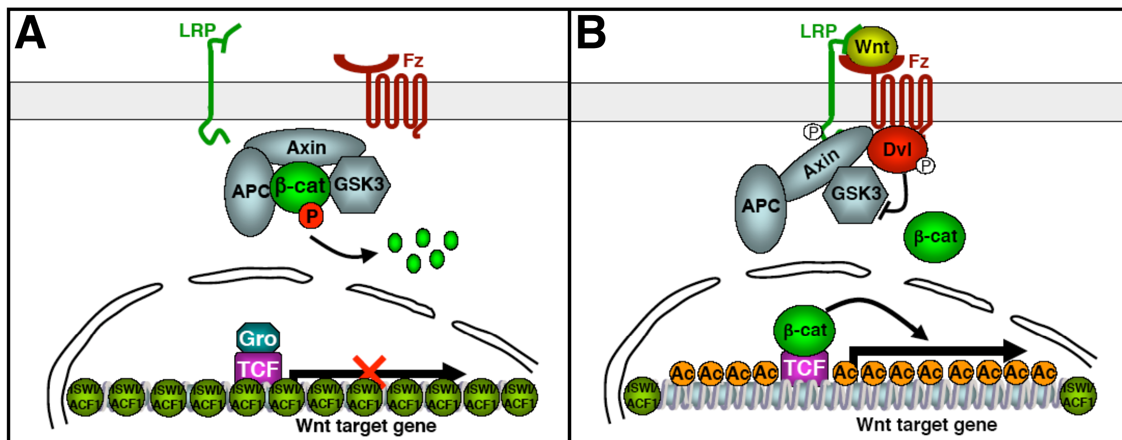

Figure 4.1 Model of ISWI/ACF1's action in Wnt signaling. (A) In the absence of Wnt signaling, ACF1 binds to broad regions of Wnt targets (much broader than TCF binding) to repress Wnt target expression. (B) Upon Wnt stimulation, binding of β-cat to TCF induces widespread acetylation of histones across Wnt targets (possibly by histone acetyltransferase CBP), which displaces/inactivates ACF1 to activate Wnt target expression.

assay in wing imaginal discs, the binding of ACF1 to this target and its regulation by Wg signaling can be examined in the foreseeable future. To turn on or shut off the pathway in the wing, drivers expressed in the major domains of the wing (Vg-Gal4 or C96-Gal4) can be used to misexpress UAS lines of constitutively active form of Arm or dominant-negative form of TCF, respectively. The validity of ACF1 ChIP signal can be confirmed by using *acf1* mutant wing discs as a control. One caveat for this experiment is the artificial nature of the reporter gene, which may bear different chromosome organization from the endogenous gene. Therefore, it is worthwhile testing the ACF1 binding profile at the endogenous *Notum* locus as well, especially regarding how widely ACF1 binds to this locus.

Is widespread binding of ACF1 important for Wg target repression? One approach to address this question is to force ACF1 to bind locally to Wg targets, and test whether under these conditions it can still repress Wg targets. For this purpose, ACF1 can be fused to TCF, and presumably this fusion protein recruits ACF1 to WREs due to the strong DNA binding ability of TCF. As a control, a mutant form of TCF that does not bind DNA (HMG deletion) can be fused to ACF1, and this presumably abolishes the localized binding of the fusion protein to WREs. After the establishment of stable cell lines for these two constructs, ACF1 ChIP can be done in these cells to see whether the localized vs. widespread binding of ACF1 to Wg targets is achieved. This can be followed by a functional analysis to see whether the fusion proteins rescue the derepression of Wg targets caused by endogenous *acf1* knockdown. If the mutant TCF fusion can rescue but the wild-type TCF fusion can

not, this will argue that widespread binding of ACF1 is functional important for repression. Similar experiments can also be carried out in flies for Notum-lacZ, given the advantage that *acf1* mutant is viable.

Different activities between ISWI and ACF1

Thus far, all the genetic analysis in the wing centers on ISWI, while all the ChIP studies in Kc cells are oriented toward ACF1. The dichotomous focus is due to a lack of Wg phenotypes in *acf1* mutant flies for the former, and limited amount of ISWI antibodies available for the latter. Even if people in the ISWI community consider that ACF1 usually acts with ISWI (not vise versa), we should be careful not to take it for granted since we did observe ISWI-independent activity for ACF1 in both flies and cultured cells.

In the developing wing, neither *acf1* nor *tou/acf1* displays obvious defects in Wg signaling. In contrast, loss of *iswi* causes derepression of several Wg targets. Considering ISWI is found in multiple complexes (Fig. 1.2), it is curious to know which ISWI complex is responsible for the Wg phenotype. NURF complex might be involved, as *nurf301* (the unique factor in NURF complex) mutant reveals phenotypes similar to those of *iswi* mutant (Badenhorst et al., 2002). Strong evidence supports the hypothesis that Nurf301 directly regulates gene activation, though a repression activity is also suggested in JAK/STAT signaling (Badenhorst et al., 2002; Badenhorst et al., 2005; Wysocka et al., 2006). Interestingly, Nurf301 and ACF1 cooperately repress *nkd* in Kc cells (data not shown). To further test the role of Nurf301 in Wg signaling in flies, *nurf301* mutant clones can be induced in the wing and the effect on Wg targets can be

examined. If no obvious defect is observed, *nurf301* clones can also be generated in *tou*/*acf1* mutant background to test whether these three related factors act redundantly repressing Wg targets, although it would be technically challenging to perform this experiment.

It is worth noting that even though *iswi* mutants display derepression of Wg targets, the penetrance is never 100% (varies from 43% to 95%). Interestingly, *iswi/acf1* double mutant flies die earlier than *iswi* mutant flies, suggesting an ISWI-independent function of ACF1 during animal development (data not shown). Generating *iswi* clones in *acf1* mutant background will be informative to see whether they both contribute to repressing Wg targets, as we saw in cell culture.

In contrast to the broad binding of ACF1 to Wg target genes, preliminary results for ISWI ChIP reveal that ISWI preferentially binds to the area where TCF is bound at *hth* locus (Fig. 4.2). Consistently, the ACF1 binding to this site (H16), and this site only, is partially reduced upon *acf1* knockdown, but further reduced to background levels upon *acf1/iswi* knockdown (Fig. 3.4D). These results suggest that ACF1 works with ISWI at localized site of Wg targets but binds to other regions independent of ISWI. Undoubtedly, more sites need to be examined for *hth* as well as for other Wg targets to confirm that ISWI has a distinct binding profile from ACF1. In addition, other ChIP experiments we have done for ACF1 (dependency on TCF, Wg signaling influence, etc.) can also be done for ISWI (if more ISWI antibody is available) to see how similarly to or differently from ACF1 it behaves in Wg target repression.

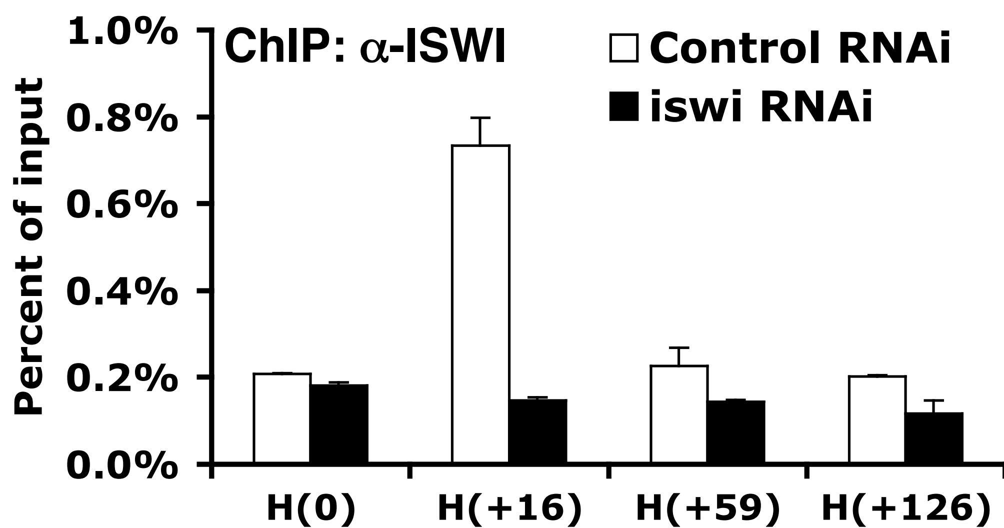

Figure 4.2 ISWI preferentially binds to the site where TCF binds in the *hth* locus. Kc cells were treated with control RNAi or *iswi* RNAi for six days before subjecting to ISWI ChIP analysis. A 3-4 fold enrichment of ISWI binding was seen in a site (H(+16)) where TCF preferentially binds, and the ChIP signal was reduced to background levels upon *iswi* RNAi. Rabbit polyclonal α-ISWI was from J. Kadonaga (Ito et al., 1999).

Action of ISWI/ACF1 with other repressors

Given that ISWI/ACF1 does not have a specific DNA sequence recognition motif, it is unclear how these factors are recruited to Wg targets loci. Since ACF1 and TCF synergistically repress Wg targets and the ACF1 binding to Wg targets is not affected upon depletion of *TCF*, it is unlikely that TCF recruits ACF1 (Figure 3.4E,G). More experiments need to be done to see whether this is true for ISWI as well.

Previous studies in the lab showed that C-terminal binding protein (CtBP) represses Wg targets independently of TCF (Fang et al., 2006). Interestingly, ISWI and CtBP synergistically repress *nkd* in Kc cells (data not shown). As both ISWI and CtBP bind to the similar region of a Wg target *hth*, ChIP experiment can be done to determine whether one influences the binding of the other.

What factor(s) could recruit ISWI/ACF1 to Wg targets? One candidate is Tramtrak (Ttk), a protein containing BTB/POZ and zinc finger domains (Kelly and Daniel, 2006). It has been shown that a Ttk-like protein in *Xenopus*, xKaiso, can repress Wnt targets independently of TCF (Park et al., 2005). Moreover, fly Ttk binds to CtBP *in vitro* (Wen et al., 2000). Interestingly, Ttk works together with TCF to repress *nkd* in Kc cells (Fig. 4.3A). In addition, depletion of *ttk* leads to a reduction of TCF or CtBP binding to a WRE of *nkd* (Fig. 4.3B). These results suggest that like its vertebrate counterpart, Ttk plays a role in silencing Wg targets.

Several more experiments can be done to further dissect the function of Ttk in Wg signaling. To see whether Ttk recruits ISWI/ACF1 to Wg targets, ISWI or ACF1 ChIP can be done in cells depleted of *ttk*. Since we have obtained some Ttk antibody (Badenhorst, 2001), Ttk ChIP can be carried out to see whether it directly binds to Wg

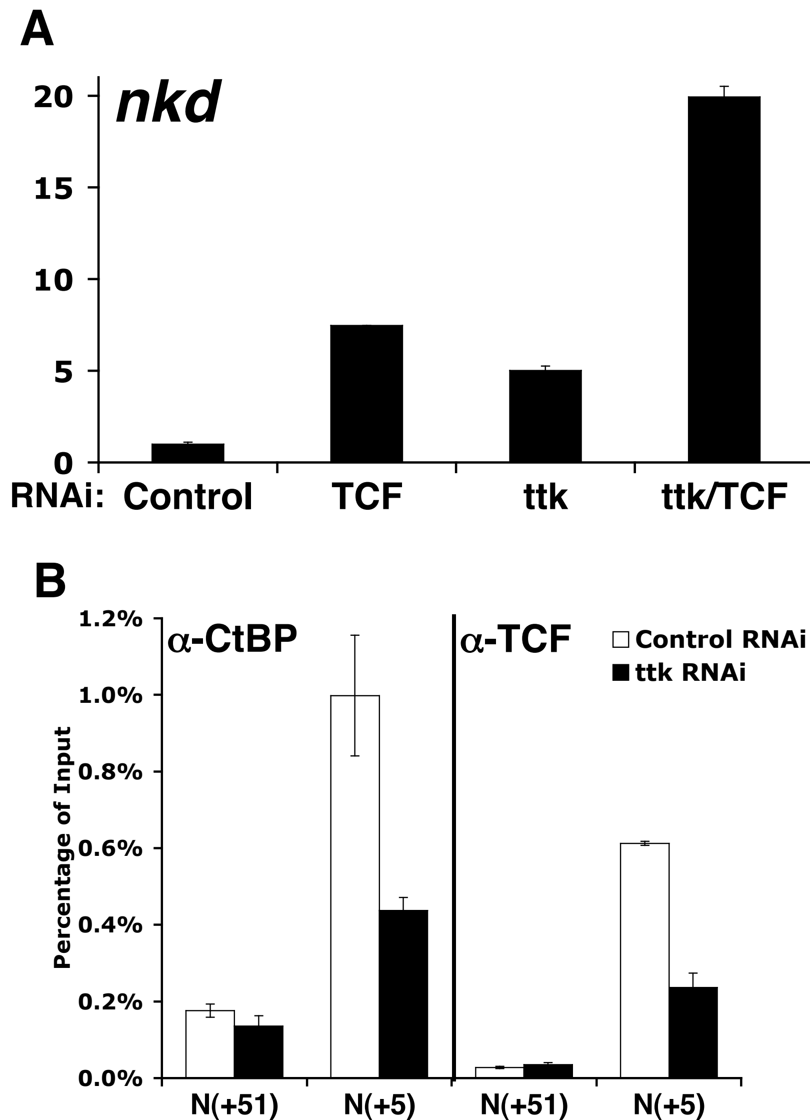

Figure 4.3 Ttk represses *nkd* expression and facilitates CtBP/TCF binding to *nkd*. (A) Kc cells were treated with indicated dsRNAs for six days, and transcripts of *nkd* were measured by Q-RT-PCR. *TCF* RNAi or *ttk* RNAi results in 5-7 fold derepression of *nkd*, while *TCF/ttk* RNAi leads to 20 fold derepression of *nkd*. (B) ChIP analysis for CtBP or TCF was performed in cells treated with *ttk* RNAi. Compared to the control, both CtBP and TCF's binding to the intronic WRE of *nkd* (N(+5)) is reduced upon *ttk* depletion.

targets. The study of Ttk can also be extended to flies, where Wg targets in the developing wing can be examined in *ttk* mutant clones (*ttk* mutant acquired from Lai and Li, 1999). These genetic and molecular analyses will give us a better idea of how Ttk, TCF, ISWI/ACF1 work together to repress Wg target genes.

Future perspectives

Overall speaking, my study of several ISWI-type chromatin remodelers stays at genetic and molecular level. A further understanding of ISWI/ACF1's repression mechanism on Wg targets will be at the level of nucleosomes. How do they position nucleosomes around Wg target loci *in vivo*? How is nucleosome positioning regulated by Wg signaling? These are intriguing questions, yet they can not be answered until the *in vivo* nucleosome positions around WREs have been mapped by micrococcal nuclease digestion assay. There are good examples in both yeast and mammals demonstrating that establishing such a system is very helpful to dissect the nucleosome remodeling mechanism in the transcriptional context (Fazzio and Tsukiyama, 2003). Yet in the Wnt field, this aspect of research has not been extensively explored especially for endogenous genes. Therefore, I envision that my PhD work is not the end, but rather the prelude of a new chapter of studying transcriptional regulation of Wnt target genes in the chromatin context, where the combined insights from genetic, molecular and nucleosomal analyses will surely warrant plenty of fruitful discoveries.

References

Alenghat, T., Yu, J. and Lazar, M.A. (2006) The N-CoR complex enables chromatin remodeler SNF2H to enhance repression by thyroid hormone receptor. *Embo J*, **25**, 3966-3974.

Badenhorst, P. (2001) Tramtrack controls glial number and identity in the Drosophila embryonic CNS. *Development*, **128**, 4093-4101.

Badenhorst, P., Voas, M., Rebay, I. and Wu, C. (2002) Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev*, **16**, 3186-3198.

Badenhorst, P., Xiao, H., Cherbas, L., Kwon, S.Y., Voas, M., Rebay, I., Cherbas, P. and Wu, C. (2005) The Drosophila nucleosome remodeling factor NURF is required for Ecdysteroid signaling and metamorphosis. *Genes Dev*, **19**, 2540-2545.

Corona DF, Tamkun JW (2004) Multiple roles for ISWI in transcription, chromosome organization and DNA replication. *Biochim Biophys Acta* 1677:113-9

Fang, M., Li, J., Blauwkamp, T., Bhambhani, C., Campbell, N. and Cadigan, K.M. (2006) C-terminal-binding protein directly activates and represses Wnt transcriptional targets in Drosophila. *Embo J*, **25**, 2735-2745.

Fazzio, T.G., Kooperberg, C., Goldmark, J.P., Neal, C., Basom, R., Delrow, J. and Tsukiyama, T. (2001) Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol*, **21**, 6450-6460.

Fazzio, T.G. and Tsukiyama, T. (2003) Chromatin remodeling in vivo: evidence for a nucleosome sliding mechanism. *Mol Cell*, **12**, 1333-1340.

Goldmark, J.P., Fazzio, T.G., Estep, P.W., Church, G.M. and Tsukiyama, T. (2000) The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell*, **103**, 423-433.

Ito, T., Levenstein, M.E., Fyodorov, D.V., Kutach, A.K., Kobayashi, R. and Kadonaga, J.T. (1999) ACF consists of two subunits, Acf1 and ISWI, that function cooperatively in the ATP-dependent catalysis of chromatin assembly. *Genes Dev*, **13**, 1529-1539.

Kelly, K.F. and Daniel, J.M. (2006) POZ for effect--POZ-ZF transcription factors in cancer and development. *Trends Cell Biol*, **16**, 578-587.

Lai, Z.C. and Li, Y. (1999) Tramtrack69 is positively and autonomously required for Drosophila photoreceptor development. *Genetics*, **152**, 299-305.

Moreau, J.L., Lee, M., Mahachi, N., Vary, J., Mellor, J., Tsukiyama, T. and Goding, C.R. (2003) Regulated displacement of TBP from the PHO8 promoter in vivo requires Cbf1 and the Isw1 chromatin remodeling complex. *Mol Cell*, **11**, 1609-1620.

Park, J.I., Kim, S.W., Lyons, J.P., Ji, H., Nguyen, T.T., Cho, K., Barton, M.C., Deroo, T., Vleminckx, K., Moon, R.T. and McCrea, P.D. (2005) Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell*, **8**, 843-854.

Parker, D.S., Blauwkamp, T. and Cadigan, K.M. (2007a) Wnt/β-catenin-mediated transcriptional regulation. In *Wnt Signaling in Embryonic Development*, Sokol S., (ed), Advances in Developmental Biology, Wassarman PM (ed), Vol. 17, pp. 1-61. San Diego: Elsevier.

Parker DS, Ni Y, Li, J, Cadigna KM (2007b) Wingless signaling induces widespread chromatin remodeling of target loci. Manuscript submitted.

Wen, Y., Nguyen, D., Li, Y. and Lai, Z.C. (2000) The N-terminal BTB/POZ domain and C-terminal sequences are essential for Tramtrack69 to specify cell fate in the developing Drosophila eye. *Genetics*, 156, 195-203.

Wysocka, J., Swigut, T., Xiao, H., Milne, T.A., Kwon, S.Y., Landry, J., Kauer, M., Tackett, A.J., Chait, B.T., Badenhorst, P., Wu, C. and Allis, C.D. (2006) A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature*, **442**, 86-90.

Yasui, D., Miyano, M., Cai, S., Varga-Weisz, P. and Kohwi-Shigematsu, T. (2002) SATB1 targets chromatin remodeling to regulate genes over long distances. *Nature,* **419**, 641-5.

Zecca, M., Basler, K. and Struhl, G. (1996) Direct and long-range action of a wingless morphogen gradient. *Cell*, **87**, 833-844.

Zhang, Z. and Reese, J.C. (2004) Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in Saccharomyces cerevisiae. *J Biol Chem*, **279**, 39240-39250.