Analysis of chromatin modification and remodeling in the transcriptional regulation of the Wnt/Wg pathway

by

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To my family

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Chapter I

General Introduction

The Wnt signaling pathway plays an essential role throughout animal development

Wnts are a family of secreted glycolipoproteins found throughout the animal kindom. The name Wnt comes from the contraction of *Drosophila* Wingless (Wg) (Baker 1987; Rijsewijk et al. 1987) and mammalian oncogene Int1 (Nusse and Varmus 1982). The 'canonical' Wnt pathway utilizes the cell junction protein β-catenin as its transcription activator and therefore is often referred to as Wnt/β-catenin signaling (for recent reviews, see (Cadigan and Peifer 2009; MacDonald et al. 2009). There are multiple Wnt induced signaling pathways and the Wnt/β-catenin pathway is the best understood and will be the main focus throughout this thesis.

The Wnt signaling pathway is one of a handful of cell-cell communication pathways that control many aspects of development (Cadigan and Nusse 1997; Logan and Nusse 2004; Clevers 2006). For example, loss of Wnt1 in mice, formerly Int1, causes a loss of midbrain in embryogenesis (McMahon and Bradley 1990) whereas Wnt3a mutation leads to defects in somites and tailbud formation (Takada et al. 1994) .

In *Drosophila*, epidermis segmentation is severely affected in *wg* mutant. The alternating denticles and naked belts in wild type embryos are replaced by continuous denticles in the mutant (Siegfried et al. 1992). In *Xenopus*, the injection of mouse Wnt1 mRNA into ventral blastomeres of a 4-cell embryo results in body axis duplication (McMahon and Moon 1989; Itoh et al. 2005). Fig1. 1 illustrates some of the described phenotypes.

The Wnt signaling pathway is required throughout animal development. For example, in fly embryos *wg* mutant causes epidermis segmentation defects (Siegfried et al. 1992) as well as defects in heart formation (Wu et al. 1995). In addition, adult flies carrying a hypomorphic allele of *wg* lack normal wings (Sharma and Chopra 1976). How does a single signaling pathway control so many aspects of development? One of the answers lies in the pathway's ability to regulate different targets in a tissue and developmental time specific manner. Therefore, understanding the transcriptional regulation of the Wnt pathway is an essential part of Wnt pathway research and this understanding may shed light on the mechanism of transcription of other developmental pathways besides the Wg pathway as well.

Disruption of Wnt signaling is linked with various diseases

As stated above, the Wnt signaling pathway plays various important roles throughout development of metazoan animals. So it is intuitive that the alteration of this pathway would have pathological consequences. Indeed, many diseases have been found to be associated with either gain of function or loss of function of the Wnt pathway. For a recent review, please refer to Macdonald et al. (MacDonald et al. 2009) and also the Wnt home page http://www. stanford. edu/~rnusse/wntwindow. html.

The Wnt signaling pathway has been connected to many types of cancers, including colon, ovarian, brain, liver and oesophageal cancer. (Salahshor and Woodgett 2005; Polakis 2007). Of these, colorectal cancer is the most tightly associated with aberrant Wnt pathway. Mutation in the APC gene, which encodes a negative regulator of the Wnt pathway, is found in almost all cases of familial adenomatous polyposis coli, a heritable predisposition to colorectal cancer. In addition, biallelic muatations in APC are found in a majority of sporadic colorectal tumors (Groden et al. 1991; Kinzler et al. 1991).

Recent studies also identified an additional mutation as a colon cancer susceptibility marker (Gudmundsson et al. 2007; Tomlinson et al. 2007; Yeager et al. 2007; Zanke et al. 2007) and the mutation is localized to a potential enhancer of the Wnt target gene *myc* (Tuupanen et al. 2009). Besides *myc*, a small number of Wnt direct targets have also been linked with Wnt related cancers (See the Wnt homepage for a list: http://www.stanford.edu/~rnusse/pathways/targets.html). The knowledge of Wnt transcription regulation will help us to understand the mis-regulation of these targets in cancers and also aid in the identification of more direct targets.

Another area that has attracted a lot of recent interest is the role of the Wnt pathway in the bone mass regulation. Loss of function mutation of LRP5, a Wnt coreceptor, is found in osteoporosis pseudoglioma syndrome, a genetic disorder with low bone mass as one of the feature symptoms (Gong et al. 2001). In contrast, some patients with high bone density diseases have a gain of function mutation in LRP5 (Boyden et al. 2002; Little et al. 2002a; Little et al. 2002b). Due to these associations, the Wnt pathway has become a popular therapeutic target for treating osteoporosis.

Molecular mechanism of the β–catenin dependant pathway

Wnt/β–catenin signaling revolves around the key activator β–catenin. When cells are not stimulated by Wnt, cytoplasmic β–catenin levels are kept low by a destruction complex, comprised of two scaffolding proteins, APC and Axin1, as well as glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). β-catenin is sequentially phosphorated by GSK3 and CK1 and the phosphorylated β–catenin is then ubiquitinated and degraded by the proteosome (For a recent review, see (MacDonald et al. 2009). In the nucleus, the transcription factor TCF binds to the Wnt target genes and represses their transcription in the absence of β–catenin.

When the ligand Wnt is present, it binds to the receptor Frizzled (Fz) and co-receptors (LRP5/6), LRP6 is phosphorylated by GSK3 and CK1 which recruits Axin1. This recruitment depends on Disheveled (Dvl) and the recruitment of Axin1 further enhances the phosphorylation of LRP6. The result of this possible positive feedback loop is the disruption of the destruction complex, which releases β–catenin from being degraded (MacDonald et al. 2009). Stabilized β–catenin can then enter the nucleus, and the binding of β–catenin to TCF transforms TCF from a repressor to an activator and the target gene transcription is turned on. Please see figure 1-2 for a diagram of the process.

Transcription Switches in Signaling pathways

As stated above, the Wnt signaling pathway uses a switch mechanism in its transcription regulation, where the transcription factor TCF represses transcription in the absence of the ligand and repression is relieved and switched to activation when the signaling is turned on (reviewed by Parker et al. 2007, (Stadeli et al. 2006; Willert and Jones 2006). Interestingly, a similar mechanism is used in other signaling pathways

(Barolo and Posakony 2002). For example, in the canonical Notch signaling pathway, the transcription factor CSL binds to its targets and functions as a repressor until the switch is flipped upon ligand binding to the receptor. The conformational change of the Notch receptor makes a cleavage site on the receptor accessible and the cleaved Notch intracellular domain then translocates to the nucleus, switching CSL from a repressor to an activator (Kopan and Ilagan 2009). A similar mechanism is used by the nuclear receptors. Nuclear receptors undergo conformational changes after ligand binding, and together with the recruited co-activators, the transcription repression by the nuclear receptors is relieved and transcription machinery is then recruited (Lonard and O'Malley B 2007). A common theme here is that the same *cis* and *trans* factors are used for repression and activation. The different conformations of the transcription factors and difference in co-factors result in the opposite transcriptional outcomes.

Targets of Wnt signaling are bound by the transcription factor TCF

TCF is a member of a family of high mobility group (HMG) DNA-binding proteins. There is only one TCF protein in flies and worms, while mammals have four TCFs: TCF1, Lef-1, TCF-3 and TCF4. Lef-1 and a Xenopus TCF XTCF-3 were the first TCFs found to interact with β–catenin (Behrens et al. 1996; Huber et al. 1996; Molenaar et al. 1996). The involvement of TCF in the Wnt pathway was further confirmed by the functional data showing that loss of function mutations of TCF phenocopy the Wnt mutation (Molenaar et al. 1996; Brunner et al. 1997; van de Wetering et al. 1997; Schweizer et al. 2003). TCF family members bind to a conserved sequence CCTTTGAT through the HMG domain, and the reporter constructs consisting of concatemerized HMG binding sites are able to drive Wnt dependant expression both in cultured cells and

in animals (Korinek et al. 1997; DasGupta and Fuchs 1999; Dorsky et al. 2002; Maretto et al. 2003; Barolo 2006). Interestingly, some in vivo HMG binding sites differ significantly from the conserved site (Chang et al. 2008a). This high degeneracy makes the *in silico* target identification a great challenge, and the in vivo target recognition mechanism by TCF remains a mystery. Some recent work by the Cadigan lab identified another consensus transcription factor binding site in Wg targets in flies. This site, named the helper site (GCCGCCR; R=A or G) is bound by the C-clamp domain of TCF (Atcha et al. 2007; Chang et al. 2008b). Although some mammalian TCF isoforms also contain a C-clamp motif that allows it to bind to a similar sequence (RCCG; R=A or G) in vitro, the functional importance of this domain in vivo remains unknown (Atcha et al. 2007).

TCF represses Wnt targets in the absence of signaling

In the absence of the Wnt signaling, TCF represses Wnt targets (Cavallo et al. 1998). Reduced levels of TCF in fly embryos suppress the segment polarity phenotype caused by the Wg null mutation (Cavallo et al. 1998). Consistent with this, mutation of TCF binding sites in the WRE of a Wnt target *dpp* results in ectopic expression of the reporter in fly visceral mesoderm (Yang et al. 2000). In contrast to flies which have only one TCF, vertebrates seem to have evolved a dedicated TCF for repression, TCF-3. TCF-3 knockout mouse embryos often have duplicated nodes and notochords (Merrill et al. 2004), a phenotype similar to overexpression of Wnt (Popperl et al. 1997). Importantly, the expression of a Wnt reporter is still maintained in its normal expression domain, suggesting that the Wnt activation is not affected (Merrill et al. 2004). TCF

achieves this repression at least partly by recruiting corepressors. The molecular mechanism of two of such corepressors will be reviewed here.

Groucho (Gro) is one of the first corepressors identified. Gro represses Wnt targets by competing with Armadillo (Arm, the fly β-catenin) for TCF binding (Cavallo et al. 1998; Daniels and Weis 2005; Fang et al. 2006). The repression by Gro could be achieved by either directly condensing chromatin structure (Sekiya and Zaret 2007) or recruiting additional chromatin remodelers such as histone deacetylases HDACs (Chen et al. 1999; Billin et al. 2000).

CtBP is another corepressor that is required to keep repress Wnt targets in the absence of signaling. Whether CtBP interacts with TCF is a controversial topic. Mouse CtBP has been shown to interact with the C-terminus of TCF in an in vitro interaction assay (Valenta et al. 2003). Similar interaction has also been reported between Xenopus TCF-3 C-terminal domain and fly CtBP (Brannon et al. 1999). However the evidence for TCF/CtBP interaction in vivo is mostly lacking except for a weak partial co-localization between human TCF4 and CtBP which depends on the C-terminal CtBP binding domain of TCF4 (Cuilliere-Dartigues et al. 2006). In fact, mammalian CtBP and TCF-4 have been specifically shown to not interact either functionally or physically (Hamada and Bienz 2004). Then what is the repression mechanism by CtBP? Two models have been proposed to address this question. In the first model, CtBP binds to the destruction complex member APC and the CtBP-APC complex prevents β-catenin from binding to TCF (Hamada and Bienz 2004; Sierra et al. 2006), thus serving as a β-catenin buffer. In the second model, CtBP binds to Wnt targets as a direct corepressor. Interestingly, this binding does not depend on TCF, so CtBP may repress the transcription in parallel with

TCF (Fang et al. 2006), consistent with the lack of physical interaction between CtBP and TCF in vivo (Hamada and Bienz 2004) .

TCF becomes a transcription activator upon Wnt pathway activation

β-catenin accumulates in the cytoplasm and then enters the nucleus when its constitutive degradation is relieved by Wnt signaling. The binding of β-catenin with TCF switches TCF from a repressor to an activator. To ensure that the Wnt targets are not turned on by small amount of β-catenin leaking into the nucleus in the absence of the signaling, TCF-β-catenin interaction is regulated by several factors.

Chibby(Cby), a coiled-coil domain containing protein conserved from flies to humans is one such factor (Takemaru et al. 2003). Cby binds to β-catenin (Fig1-3) and the overexpression of Cby in mammalian cells inhibits Wnt reporter activation. Knockdown of Cby with RNAi in fly embryos also partially recues the Wg null allele phenotype (Takemaru et al. 2003). A more recent study showed that the Cby's repression function in the Wnt signaling pathway is a result of shuttling β-catenin outside of the nucleus. A β-catenin mutant unable to bind to Cby preferentially localizes to the nucleus (Li et al. 2008). By shuttling β-catenin outside of the nucleus, Cby ensures that enough β-catenin accumulates in the nucleus before the signaling is turned on.

ICAT is another β-catenin-TCF interaction buffer. ICAT can interact with β-catenin and this interaction inhibits the β-catenin-TCF interaction (Tago et al. 2000; Tutter et al. 2001). Structural studies have revealed that ICAT binds to the same portion of β-catenin as TCF, suggesting direct competition between ICAT and TCF for β-catenin binding (Daniels and Weis 2002; Graham et al. 2002).

When enough β-catenin finally accumulates in the nucleus, its binding with TCF turns transcription on with the help of many coactivators (Fig1-3). Some coactivators are almost dedicated coactivators in the Wnt signaling, whereas a majority are general transcription coactivators that also play positive roles in other signaling pathways. Many of these general coactivators are proteins regulating chromatin structure and will be discussed in a later section. Here I will focus on the Wnt specific coactivators.

Legless-Pygopus complex is primarily a dedicated Wnt coactivator (Mosimann et al. 2009). Legless (Lgs) is recruited to the amino-terminal portion of β-catenin (Fig1-3,(Kramps et al. 2002; Hoffmans and Basler 2004) which then recruits Pygopus (Pygo). The Lgs-Pygo complex is required for all the Wg-dependant phenotypes tested in flies (Belenkaya et al. 2002; Kramps et al. 2002; Parker et al. 2002; Thompson et al. 2002). Two domains on Lgs protein are sufficient for the Lgs function in flies: the HD1 domain interacts with Pygo and the HD2 domain interacts with Arm (Kramps et al. 2002). Besides serving as a linker between Pygo and Arm, overexpression of BCL-9 (mammalian Lgs) activates a β-catenin dependant reporter and this activation function seems to reside in a domain outside of HD1 and HD2 (Sustmann et al. 2008). Lgs binds to Pygo through Pygo's PHD domain and the transactivation function of Pygo is believed to be provided by the NHD domain. So what is the biochemical function of the Lgs/Pygo complex? Besides interacting with Lgs, the Pygo PHD domain also can bind to methylated H3K4, a hallmark for active transcription. In flies the simultaneous binding of Lgs HD1 domain to the PHD domain enhances its affinity for the methylated H3K4 (Fiedler et al. 2008) although the functional importance of this enhancement has been challenged (Kessler et al. 2009). Other general transcription related proteins such as

mediator complex (Carrera et al. 2008) and core promoter recognition complex TFIID (Wright and Tjian 2009) have also been reported to be recruited by Pygo.

Chromatin regulation in Wnt signaling

Besides the various coactivators and corepressors reviewed above, the many transcriptional regulators in the Wnt pathway transcription also directly regulate chromatin structure (Mosimann et al. 2009). In eukaryotes, about 147bp of double stranded DNA is wrapped around a histone octamer consisting of H2A, H2B, H3 and H4. This assembly, referred to as a nucleosome is the basic building block of the genetic material. Higher order chromatin compaction then further condenses the string of nucleosomes in order to pack the huge amount of the genetic material in a relatively small nucleus. While the higher chromatin structure can also play a role in transcription regulation (Lieberman-Aiden et al. 2009), this thesis will focus on the role of histone-DNA interaction in the transcription regulation. The tight interaction between histone and DNA prevents the transcription machinery from accessing DNA and chromatin regulates transcription by assuming either 'loose' or 'tight' histone-DNA interaction. Chromatin modification happens in two ways: histone-DNA interaction can be directly remodeled by ATP-dependant chromatin remodelers (Ho and Crabtree); also post-translational modifications of the N-terminal of histones can either directly affect the chromatin compaction or recruit other factors that regulate transcription (Campos and Reinberg 2009).

ATP-dependent chromatin remodeling and Wnt/Wg signaling

Several ATP-dependant chromatin remodelers have been connected with the Wnt signaling pathway and I will review two of them below.

Brg-1 is the ATPase of the Swi/Snf type ATP-dependent chromatin remodeling complex. A yeast two-hybrid screen for β-catenin binding proteins identified Brg-1 as a direct binding partner (Barker et al. 2001). Please refer to figure 1-3 for a summary of proteins binding to β-catenin/Arm in the nucleus. In mammalian cell lines, overexpression of Brg-1 enhances the activation of a Wnt reporter by β-catenin and a mutant form of Brg-1 that lacks the ATPase activity functions as a dominant negative in the activation of the endogenous Wnt targets (Barker et al. 2001). In flies, the small eye phenotype caused by the overexpression of a constitutively active form of Arm is partially rescued by the heterozygous Brm (the fly Brg-1) null allele. The above data suggests that Brg-1 in mammals and Brm in flies are positive regulators for the Wnt/Wg signaling (Barker et al. 2001).

Interestingly, another fly Swi/Snf complex member Osa has been implicated in the repression of Wg targets in flies. Osa mutants display ectoptic expression of *nub* in fly wing imaginal discs, a putative Wg target (Collins and Treisman 2000). In fly embryos, mutation of Osa also derepresses a reporter construct UbxB-lacZ whose expression depends on both Wg and Dpp signaling. Interestingly, this derepression is abolished when the Dpp response element is mutated, but not by mutation of the Wg response element. This data suggests that the repression of the UbxB reporter by Osa is not entirely dependent on the Wg response element, thus putting the directness of this repression in question. Alternatively, one could argue that Osa directly represses UbxB, but the repression is in parallel with Wg, similar as the case in CtBP. The same study also showed the repression function of Osa on several other Wg targets in different tissues (Collins and Treisman 2000).

The two studies introduced above both show convincing data that the Swi/Snf complex can play both a positive and a negative role in the Wnt pathway regulation. However, neither of the two show evidence that the regulation is direct. While it is entirely possible that Swi/Snf regulates Wnt/Wg targets transcription in a context dependant manner, directness of the regulation must first be established before further investigation. Without the evidence of direct regulation, the repression function of the Swi/Snf complex could be explained by the activation of a Wnt repressor which in turn represses Wnt targets.

Imitation SWI (ISWI), another ATP-dependent chromatin remodeler, also has important roles in the Wnt/Wg signaling pathway. ISWI binds to the C-terminus of β-catenin which inhibits the in vitro transcription activated by Lef-1 (mammalian TCF)/β-catenin complex (Tutter et al. 2001; Sierra et al. 2006). There are multiple ISWI-containing complexes; NURF and ACF are two of them. ISWI and ACF1, the two members in the ACF complex have been shown to negatively regulates Wg signaling transcription both in cultured fly cells and in wing imaginal discs (Liu et al. 2008). In wing imaginal discs, two direct Wg targets reporters, notum-lacZ and nkd-lacZ, are derepressed when ISWI is knocked out. Interestingly, in another study the same mutant allele of ISWI caused a loss of activation of the Wg target Sens in the wing discs (Song et al. 2009). NURF301, the largest subunit of the NURF complex is also required for the activation of Sens in this tissue. Both groups have shown convincing data that the regulation is likely direct as ISWI physically associates with the regulated genes. The dual function of ISWI could be attributed to different ISWI containing complexes or it is possible that the same complex could have opposite functions in different context.

Histone modifications and Wnt/Wg signaling

The N-termini of histone subunits protruding from the nucleosome are subject to intensive posttranslational modifications (Allis et al. 2007; Campos and Reinberg 2009). Acetylation, methylation, ubquitination and phosphorylation can happen on almost every exposed polar residue. The shear number of the possible combinations from these modifications suggested the idea of a 'histone code' which is a direct correlation between the histone modification status and transcription outcome. While such a code may exist, it is a very complicated one. It has become apparent that no single histone modification can direct the transcriptional outcome although some general rules apply (Wang et al. 2008). Histone hyperacetylations are generally associated with transcription activation and hypoacetylated histones are normally found on repressed genes. H3K4 and H3K36 methylations are generally connected with activation while H3K9 and H3K27 methylation usually mark the repressed chromatin (Allis et al. 2007). While the genome-wide mapping of those histone modifications provides huge amount of information (Wang et al. 2008), more mechanistic insights of how the histone modifications function come from studies of specific regulated pathways and their targets. Several histone modifications and their modifying enzymes have indeed been implicated in Wnt/Wg pathway regulation and I will review a couple of them below.

Histone acetylations are generally connected with transcription activation. In vertebrate systems, histone acetyltransferases CBP and p300 interact with β-catenin/Arm (Hecht et al. 2000; Takemaru and Moon 2000). The activation of both endogenous Wnt/Wg targets and reporter constructs is sensitive to the inhibition of CBP/p300. Curiously, the activation function of p300 does not seem to require its acetyltransferase

activity (Hecht et al. 2000) as overexpression of CBP lacking the enzymatic activity activates Wnt reporters just as well as the wild type. In contrast to this, p300 was shown to acetylate β-catenin and the acetylated β-catenin has increased affinity to TCF (Levy et al. 2004). In this study, both the acetyltransferase activity of p300 and the acetylated residue of β-catenin are important for Wnt activation (Levy et al. 2004). Adding more complexity to the CBP/p300 role in the Wnt signaling in vertebrates is a later study from the Cadigan lab demonstrated that p300 can also bind to TCF and functions as a repressor in human cells (Li et al. 2007). This dual function of CBP/p300 was also observed in flies (Waltzer and Bienz 1998; Li et al. 2007). For repression, CBP is thought to acetylate TCF in its Arm-binding domain to prevent TCF from binding to Arm (Waltzer and Bienz 1998). However, whether this acetylation is functionally important is still not clear as a mutation in the HAT (histone acetyltransferase) domain of CBP does not affect its repression function (Li et al. 2007). CBP also binds to Arm in flies and this binding likely helps to recruit CBP to Wg targets (Li et al. 2007). The activation function of CBP is abolished when its HAT domain is mutated. In conclusion, it is clear that CBP/p300 play both positive and negative roles in the Wnt/Wg signaling in both vertebrates and invertebrates. But whether its HAT activity is required for either function is still controversial. The most convincing experiment would be a rescue assay with either wildtype or HAT mutant CBP in both activation and repression assays. But since CBP knockdown severely affects the general health of cells/tissues, such an experiment has not been done yet. What is also missing in this field is the investigation of the histone acetylation status of the Wnt/Wg targets and whether CBP/p300 plays a role in acetylating the chromatin of those targets.

H3K4 methylation is another active histone mark that has been connected with the Wnt/Wg signaling pathway. The major enzymes responsible for this modification are SET domain containing MLL (mixed lineage leukaemia complexes) proteins which have been found to bind to the C-terminus part of β-catenin (Sierra et al. 2006). MLL is recruited to a Wnt enhancer in a signal dependant manner. This recruitment is accompanied by an increase of H3K4me3 at the enhancer (Sierra et al. 2006).

Recently, H3K79 methylation has also been connected to the Wnt/Wg signaling (Mohan et al.). H3K79 is methylated by Dot1 (Feng et al. 2002; Lacoste et al. 2002; Ng et al. 2002a; van Leeuwen et al. 2002). Although the best known function for H3K79 methylation in yeast is telomeric silencing, H3K79 methylation was found to colocalize with active histone marks, such as H3Ac, H4Ac and H3K4me3 in flies (Schubeler et al. 2004). Biochemical purification of the Dot1 containing complex revealed β-catenin as a binding partner. Knocking down of dDot1/Grappa in flies with RNAi causes a loss of activation of Wg target Sens in fly wing discs. The same phenotype was also observed with the knockdown of two other subunits of the complex, dAF10/Alh and dSkp1/SkpB. The requirement of the dDot1 complex in Wg signaling is not universal as two other Wg targets in the Wing disc, Dll and Vg, do not require dDot1 for activation (Mohan et al.). In addition to dDot1, the E3 ubiquitin ligase Bre1 is also required for the activation of Sens in wing discs. This is consistent with the previous study showing that H2B monoubiquitination is a prerequisite for H3K79 methylation (Ng et al. 2002b).

Polycomb Group Proteins

Polycomb group (PcG) proteins were originally identified in flies as repressors of homeotic (Hox) genes (Lewis 1978; Struhl 1981). Besides this famous function of PcG

proteins, they also perform many other important tasks which will be reviewed later. Here I will first review the PcG complex members and their biochemical properties and molecular functions. PcG complexes consist of at least three subcomplexes: PhoRC, PRC1 and PRC2 (Fig1-4(Muller and Verrijzer 2009).

PhoRC complex and Polycomb recruitment

In flies, the PhoRC complex contains two members, Pho and dSfmbt. Pho and its relative Pho-like are the only sequence specific DNA binding proteins in PcG complexes. Binding of Pho is required for the recruitment of other PcG complex members. When Pho is depleted with RNAi, the binding of both PRC1 and PRC2 members to the polycomb response element (PRE) of Ubx (a Hox gene) is abolished (Wang et al. 2004). Unsurprisingly, the binding of Pho to the PRE is not affected when members of PRC1 or PRC2 are knocked down. The core consensus sequence recognized by Pho has been defined biochemically as GCCAT (Mohd-Sarip et al. 2005). Point mutations in this sequence abolished the repression mediated by PcG proteins (Fritsch et al. 1999; Busturia et al. 2001).

dSfmbt exists in a complex with Pho and the recruitment of dSfmbt depends on Pho (Klymenko et al. 2006). There is no detectable interaction between the PhoRC complex and other PRC members, suggesting that PhoRC is a distinct complex (Klymenko et al. 2006). Functionally, in either dSfmbt null clones or Pho/Pho-like double null clones, the expression of Ubx is elevated.

Although the sequence GCCAT is required for the Pho binding to PREs and is functionally important in mediating the PcG complexes repression, it is not the only cis-

element required. A long list of DNA binding proteins and their binding motifs have been implicated in PcG targets silencing (reviewed in (Muller and Kassis 2006). These proteins include: GAGA factor (GAF) or Trithorax-like, Pipsqueak, Zeste, Grainyhead, Dsp1 and Sp1/KLF family members. With such a long list of factors, it is very hard to identify what actually defines a PRE. The answer is likely to be different in different contexts and one study provided an example of motifs sufficient to define a PRE. In this study (Dejardin et al. 2005), a synthetic reporter containing binding motifs of Pho, GAF, Zeste and Dsp1 in their natural spacing and orientation was able to induce a PcG dependant repression although it failed to reproduce all the repression phenotypes. So are Pho, GAF, Zeste and Dsp1 four of the most important repressors linked to PcG inhibition of transcription? The answer is still not clear because the mutants of those binding factors themselves either show no obvious defects in PcG silencing or seem to play opposite roles in the PcG silencing depending on context. For example, mitotic clones lacking GAF in wing discs do not have increased expression of Ubx (Brown et al. 2003). In another study, deletion of either GAF or Zeste or both binding sites in a synthetic reporter reduced the expression pattern of this reporter (Laney and Biggin 1992). This study however is complicated by the fact that the binding motifs in this synthetic reporter are arranged in a way not found in nature. In conclusion, Polycomb mediated repression is a coordinated action by many factors. The requirement and function of each specific reporter is likely to be target and tissue specific.

In contrast to the PcG recruitment in flies described above, what recruits Polycomb in vertebrates in a DNA sequence specific manner was not known until very recently. The Jumonji C domain containing protein Jarid2 is suggested to be a recruiter

of polycomb complexes in vertebrates (Li et al. ; Pasini et al. ; Peng et al. 2009; Shen et al. 2009). Jarid2 interacts with all core subunits in the PRC2 complex, but no interaction was detected between Jarid2 and PRC1 complex subunits. Jarid2 and PRC2 complex bind to very similar genomic regions and the recruitment of PRC2 to a majority of genomic loci is significantly reduced in Jarid2 shRNA treated cells (Pasini et al. ; Peng et al. 2009).

While it is clear that Jarid2 recruits PRC2, the biochemical and biological function of Jarid2 in polycomb silencing is still controversial. The Jumonji C domain containing families are known to be histone demethylases that remove methyl groups from lysine residues (Swigut and Wysocka 2007; Agger et al. 2008). This activity is in opposition to H3K27 methylation, the main biochemical function of the polycomb complexes. However, Jarid2 may lack histone demethylase activity because it has mutations in the cofactor binding domain which is essential for its demethylase activity (Shen et al. 2009). Consistent with this, Jarid2 itself also showed no demethylase activity in vitro (Shen et al. 2009). However, addition of increasing amounts of Jarid2 to the PRC2 complex inhibits the complex's methyltransferease activity in vitro and this inhibition is specific for H3K27 as the methylation of H3K9 is not affected (Peng et al. 2009; Shen et al. 2009). Consistent with Jarid2 inhibiting PRC2's methyltransferase function, the H3K27me3 enrichment on endogenous targets is either not changed or modestly increased with Jarid2 knockdown in some studies (Li et al. ; Peng et al. 2009; Shen et al. 2009). On the other hand, when a different set of genes were examined, shRNA of Jarid2 clearly decreases the H3K27me3 levels on these genes, to the same extent when PRC2 subunit is knocked down (Li et al. ; Pasini et al.).

The biological function of Jarid2 in polycomb silencing is equally perplexing. Jarid2 has been identified as a repressor long before its association with PcG (Kim et al. 2003). In fact, overexpression of Gal4-Jarid2 can repress UAS-luciferase reporter as a result of the Gal4-UAS association. Furthermore, PRC1 and PRC2 members are recruited to the UAS-luciferase construct, showing that this repression is likely to be mediated by PcG (Pasini et al.). In contrast, Jarid2 as well as other polycomb subunits have been connected with the induction of gastrulation genes (Peng et al. 2009) in Xenopus embryos and the activation of differentiation markers in ES cells (Pasini et al. ; Shen et al. 2009). For both the gastrulation genes and differentiation markers, the core PRC subunits are important for the induction of these genes and at least a couple differentiation genes seem to be directly regulated (Pasini et al. 2007). So it is no surprise that Jarid2 also functions in activating these genes considering that it is a recruiter of PcG. Considering the controversy of Jarid2's role in PcG mediated H3K27 methylation, the more interesting question is in PcG silencing, whether Jarid2 plays a helping role or an antagonizing role. More work examing the PcG repressed targets in vertebrates may clear this issue up.

PRC2 and histone methylation

PRC2 has four core subunits, including E(z)/Ezh2, ESC/EED, Su(z)12/SUZ12 and NURF55. E(z), a SET domain containing protein can add one, two or three methyl groups to H3K27. The methylated H3K27 is generally a repressive histone mark and this modification contributes to most known polycomb repression functions. All four subunits are required for maximum methyltransferase activity in vitro (Ketel et al. 2005; Nekrasov et al. 2005). Loss of function of ESC/EED or Su(z)12/SUZ12 also results in a

global decrease of H3K27 methylation as well as Hox gene derepression (Cao and Zhang 2004; Pasini et al. 2004; Ketel et al. 2005). Although ESC knock out flies are viable and only have subtle PcG phenotypes (Struhl 1981), double knockout of ESC and ESCL, a close relative of ESC, in flies causes death by the end of larval stage and a loss of H3K27 methylation (Kurzhals et al. 2008; Ohno et al. 2008).

Although E(z)/Ezh2 is able to catalyze mono, di and tri methylations on H3K27, only H3K27me3 is traditionally connected with transcription repression. Polytene chromosome staining revealed that H3K27me and H3K27me2 is more widely spreaded on chromosomes than H3K27me3 (Ebert et al. 2004). This pattern difference may suggest that the three modified states of H3K27 do not play equal roles in transcription regulation. This differential function is further supported by the identification of a PRC2 variant containing Pcl/PHF1 which specifically catalyze the H3K27me3, but not the other two states (O'Connell et al. 2001; Tie et al. 2003; Nekrasov et al. 2007). In fact, the removal of Pcl in flies results in an increase of H3K27me and H3K27me2 on select PcG targets (Nekrasov et al. 2007). This increase is quite mysterious because in flies with Pcl RNAi, the binding of E(z) to chromosomes is largely gone (Savla et al. 2008). A double knock down of Pcl and E(z) would clarify the dependence of this increase on E(z). If this increase turns out to be E(z) independent, it may suggest the presence of another H3K27 methyltransferase in flies.

Due to the difference of binding patterns between H3K27me3 and H3K27me2 (Ebert et al. 2004), it is suspected that H3K27me2 may not regulate transcription. However, a recent study suggested that H3K27me2 may repress RB and E2F target genes (Lee et al.). The RB/E2F pathway regulates processes like cell division, cell death and

development. Lee and coworkers examined differentiation specific target genes repressed by RB/E2F. The authors found that in contrast with the HOX gene PRE which is enriched with H3K27me3, the RB/E2F targets are enriched with H3K27me2. Knock down of either RB/E2F or E(z) decreases the binding of H3K27me2 on their targets. The authors however have not examined whether H3K27me3 was decreased in these treated cells. So it can not be ruled out that the decrease of H3K27me2 is just a by-product of H3K27me3 demethylation which actually represses target genes. This study also found that E(z) knockdown causes an increase of acetylation on H3K27. This finding agrees with another study claiming an antagonizing relationship between H3K27me3 and H3K27Ac (Tie et al. 2009).

PRC1 complexes: H2A ubiquitination and chromatin compaction

In flies, the PRC1 complex contains four core subunits: dRing, Pc, Psc and Ph. Mammalian PRC1 complexes are more complicated because each core subunit has multiple paralogues (Simon and Kingston 2009). Instead of reviewing all possible combinations between these paralogues in mammals, I will focus on the case in flies in this thesis.

PRC1 was originally purified from fly embryos (Shao et al. 1999). The incubation of this complex with chromatin prevents the chromatin remodeling by Swi/Snf suggesting a repressive role of PRC1 in transcription by compacting chromatin (Shao et al. 1999; Francis et al. 2004). Electron microscopy images show that the compaction of chromatin by PRC1 does not require the histone tails, thus decoupling the covalent modification of histones and chromatin compaction (Francis et al. 2004). The PRC1 subunit Psc is essential for this compaction as a mutant version of Psc severely affected

the chromatin compaction but the PRC1 complex assembly is not affected (Francis et al. 2004).

The chromatin compaction by PRC1 provides an appealing mechanism for PcG silencing. However, a couple of studies have shown that PREs are almost nucleosome-free (Mishra et al. 2001; Mohd-Sarip et al. 2006). Then where does the PRC1 compact chromatin? A study by Lavigne and coworkers showed that the recruited PRC1 can recruit another chromatin templates and chromatin remodeling by Swi/Snf on this secondary recruited chromatin is inhibited by PRC1 (Lavigne et al. 2004). This study provides a possible mechanism for PcG complexes to induce a chromosome looping which may facilitate the chromatin compaction by PRC1 on targets other than PREs. Whether such a chromosome loop exists in vivo needs further investigation and new technologies such as chromosome confirmation capture (3C) can be helpful in this aspect (Vassetzky et al. 2009).

Another biochemical function of PRC1 is H2A-K119 ubiquitylation, executed by dRing, an E3 ubiquitin ligase. Interestingly, the H2A ubiquitylation does not depend on Pc or Ph, two other core PRC1 subunits. Instead, this modification requires dKDM2, a H3K36 demethylase, which was copurified with dRing in Pc depleted cells. Strong interaction data showed that there is a novel complex called dRAF complex containing dRing, Psc and dKDM2. This complex ubiquitylates H2A in a Pc and Ph independent way. dKDM2 mutant genetically enhances the Pc mutant phenotype in flies, suggesting its participation in PcG silencing (Lagarou et al. 2008). dKDM2 itself is a histone demethylase, specifically removing H3K36me2 (Lagarou et al. 2008). The finding of this new complex provides another possible mechanism for PcG silencing.

Besides the four core subunits of PRC1 described above, Scm is another potential PRC1 subunit. Transheterozygous Scm/Pc adults show more severe homeotic phenotype than Pc/+, suggesting a role of Scm in PcG silencing (Bornemann et al. 1998). In fact, the original purification of PRC1 found Scm as an associated subunit (Shao et al. 1999), although later studies failed to detect in vivo interaction between Scm and Ph (Peterson et al. 2004). Consistent with its function in PcG silencing, Scm was found to bind to the same region as Pho (Wang et al.), although the binding does not depend on Pho. Furthermore, different from a PRC1 knockdown, where the binding of Pho and PRC2 is not significantly affected (Wang et al. 2004), the knockdown of Scm almost abolished the binding of PRC1 and PRC2 (Wang et al.). This phenotype is very similar to the knockdown of Pho, so it was proposed that Scm may bind to PREs independent of other Polycomb complexes and this binding somehow stabilizes the binding of Polycomb complexes.

Genome wide mapping of polycomb binding

In order to identify additonal target genes and processes regulated by PcG, genome wide mapping of Polycomb subunits has been performed by multiple groups (reviewed in (Ringrose 2007). Indeed, this effort has revealed the binding of PcG to a number of genes outside of the Hox clusters, and many of which are important development regulators. The function of PcG in some of these processes will be discussed in the next section. I will focus on the mechanistic implications of the mapping in this section. The mapping of PRC1, PRC2 and GAF in flies confirmed that PRC1 and PRC2 colocalize at the same genomic region with H3K27me3. H3K27me3 occupies a broader region than E(z) and Psc, whereas Pc relatively has a more widespreaded binding

profile than E(z) and Psc (Schwartz et al. 2006; Tolhuis et al. 2006). How the localized E(z) catalyze methylation in a remote region may be explained by chromosome looping as discussed before. Because Pc binds to a broader region, more like H3K27me3 than other PRC1 subunits, this may reflect the ability of Pc's chromo domain to bind to methylated H3K27 (Eissenberg 2001). Whether this PRC1 independent binding of Pc plays a role in Polycomb silencing needs further investigation. Consistent with the lack of Polycomb phenotype in GAF mutant (Brown et al. 2003), there is limited co-localization between GAF and PRC1 (Negre et al. 2006), suggesting that GAF is not required in all polycomb silencing. It was also shown that PcG complexes bind to many genes that are regulated dynamically and there are major differences in the PcG binding patterns between fly embryos and larval tissues (Kwong et al. 2008; Oktaba et al. 2008). Although increasing evidences point to a dynamic regulatory mechanism by PcG, direct support for such a regulation is still lacking.

The studies in mammalian cells generally agree with the findings in flies. Many developmental regulators are found to be PcG targets (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). Furthermore, genes activated during differentiation in ES cells tend to be PcG targets. The role of PcG in stem cell proliferation and differentiation will be discussed later.

Biological function of PcG

As described previously, PcG complexes have been connected with multiple cellular and developmental events beyond Hox gene repression, including cell cycle regulation (Martinez and Cavalli 2006), X-chromosome inactivation (Heard 2005) and genomic imprinting (Sha 2008). An area that has attracted a lot of attention recently is

the role of PcG complexes in the stem cell establishment, maintenance and differentiation which I will discuss in more detail here.

Initial genome wide mapping of PcG members show that PcG binds to a subset of genes that are upregulated in differention, suggesting that PcG is required for ES cell maintenance by repressing the differentiation associated genes (Boyer et al. 2006; Lee et al. 2006). Further studies of the role of PcG in stem cells, however, is complicated by the requirement of PcG in stem cell maintenance. For example, ES cells can be derived from EZH2 (mammalian E(z)) mutant as well as Suz12 (mammalian Su(z)12) mutant embryos, indicating PcG silencing is probably not required for the establishment of stem cells (Pasini et al. 2007; Shen et al. 2008). In fact, Chamberlain and colleagues showed that pluripotency markers are still maintained in Eed^{null} ES cells (Eed is a mammalian PRC2 subunit) (Chamberlain et al. 2008), and a similar phenotype was also observed in $Suz12^{-/-}$ ES cells (Pasini et al. 2007). In addition, Eed^{null} cells can also be found in all tissues when Eed^{null} ES cells are injected in blastocyst embryos, showing that Eed^{null} ES cells indeed are still pluripotent (Chamberlain et al. 2008). On the other hand, PcG proteins may be required for ES cell differentiation. For example, $Suz12^{-/-}$ cells fail to differentiate properly (Pasini et al. 2007), a phenotype that might be regulated by PRC1 and PRC2 redundantly (Leeb et al.). Mutation of PcG members also causes a defect in the activation of differentiation related genes (Shen et al. 2008). The differentiation defects of PcG mutants are somewhat difficult to explain considering that PcG proteins clearly repress differentiation associated genes in ES cells to prevent differentiation (Boyer et al. 2006; Lee et al. 2006).

The discovery of the mammalian PRC recruiter Jarid2 adds further complexity to the story. Jarid2 plays dual roles in PcG silencing. On one hand, it is essential for the PRC recruitment. On the other hand, Jarid2 may inhibit the methyltransferase activity of PRC2, thus negatively regulating the repression (Peng et al. 2009; Shen et al. 2009). In Jarid2 mutant cells, the differentiation markers failed to be turned on which indicates an active repression is in place by PcG (Shen et al. 2009). This phenotype is probably due to the inhibitory role of Jarid2 on the methylatransferase activity. But how the repression by PcG is maintained in cells lacking Jarid2 is not clear. A recent study by Leeb et al. provided another important clue as to how PcG complexes regulate ES cell differentiation. In this study, they showed that while single knockdown of either PRC1 or PRC2 does not cause a detectable defect in differentiation, double knockdown of both complexes abrogates differentiation (Leeb et al.). It is worth noting that the differentiation markers are still turned on in double knockdown cells at least to the same level (higher for some markers), but the differentiated cell counts decrease. This suggests that in those cells, differentiation can still initiate, but the differentiated cells do not survive (Pasini et al. 2007).

Summary of results

Although the Wnt/Wg signaling has been intensively studied, the knowledge of how the chromatin alteration is regulated on Wnt target genes is largely lacking. This thesis will examine the role of chromatin remodeling in the Wg pathway regulation in flies.

Chapter II: Wg induces widespread histone acetylation. In contrast to many previous studies which identify histone acetylation as an active histone mark normally

enriched at distinct loci, widespread histone acetylation across the whole target region is found. The widespread histone acetylation is not a byproduct of transcription activation and is not found on all active genes. Although the histone acetylatransferase CBP is required for the widespread histone acetylation, CBP itself is only localized to the identified enhancer.

Chapter III: Polycomb group proteins and H3K27 methylation are required to repress Wg target transcription in the absence of signaling. Depletion of Polycomb group proteins causes an upregulation of Wg target genes as well as a decrease of the H3K27 methylation levels. Wg signaling activation, however, does not always displace this repressive mark.

Chapter IV: Possible dual roles of Brm complexes in the Wg transcription regulation. Depletion of the ATP-dependant chromatin remodeler Brm complexes causes either loss of activation or derepression in a target gene specific manner. This chapter will also examine the directness of the regulation.

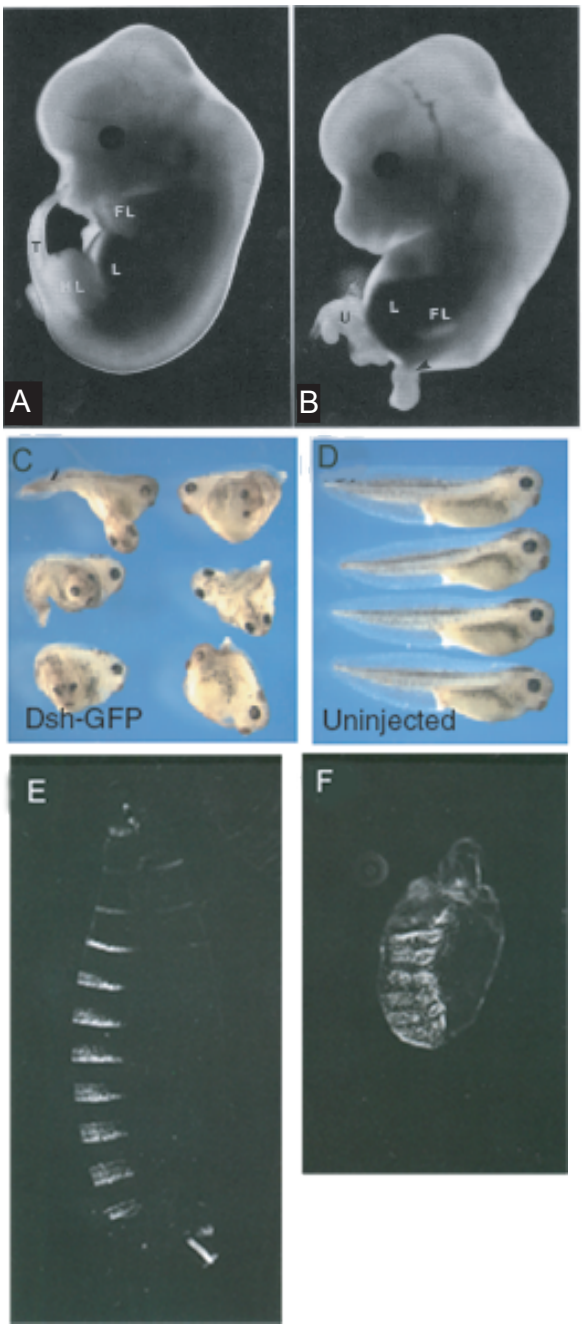

Fig1-1 Representative phenotypes of Wnt mutants (A and B) Dark field view of a (A) wt 12.5-dpc mouse embryo and (B) *wnt3A* mutant (Takada et al.,1994). (C and D) Photographs of Xenopus embryos (C) One ventral vegetal blastomere was injected with 1ng Dsh-GFP. (D) unjected control. (Itoh et al. 2005) (E and F) Cuticles of wt(E) and *wg* mutant(F) flies. (Siegfried et al. 1992)

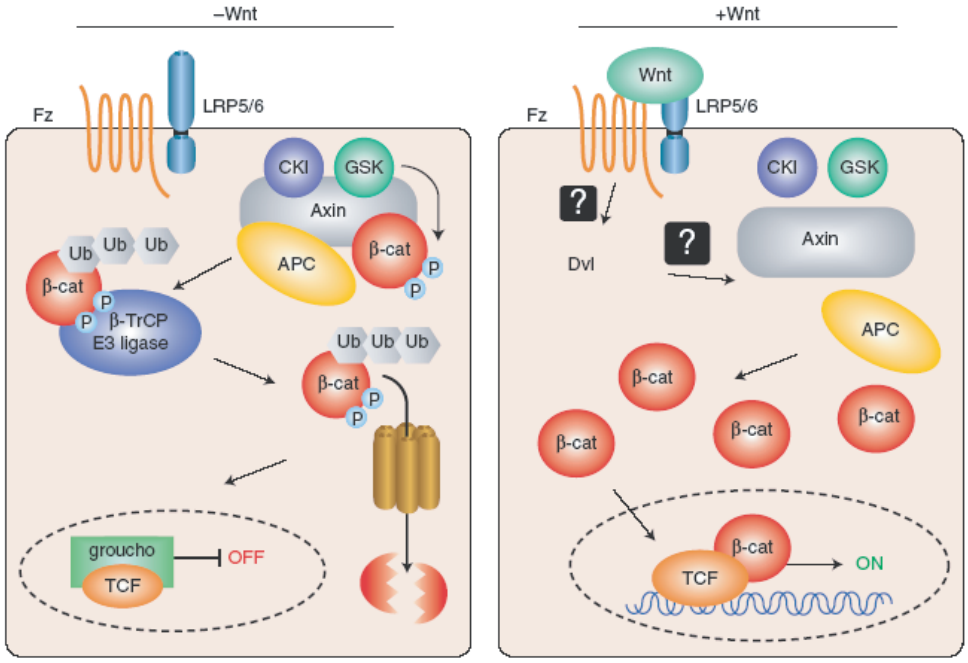

Fig1-2. Simplified diagram of the Wnt signaling pathway. (A) In the absence of signaling, β–catenin is phosphorylated by GSK3 and CKI, which is facilitated by Axin and APC. The phosphorylated β–catenin is then degraded by the proteosome. In the nucleus, TCF binds to the Wnt responsive genes and represses them. (B) When Wnt binds to its receptor Frizzled (Fz), the Wnt co-receptors LRP5/6 are phosphorylated by GSK3 and CKI which is required to recruit Axin to the membrane. β–catenin is then released from phosphorylation and degradation. Nuclear binding of β–catenin to TCF turns on the transcription of the Wnt target genes. (Figure taken from Cadigan and Peifer 2009)

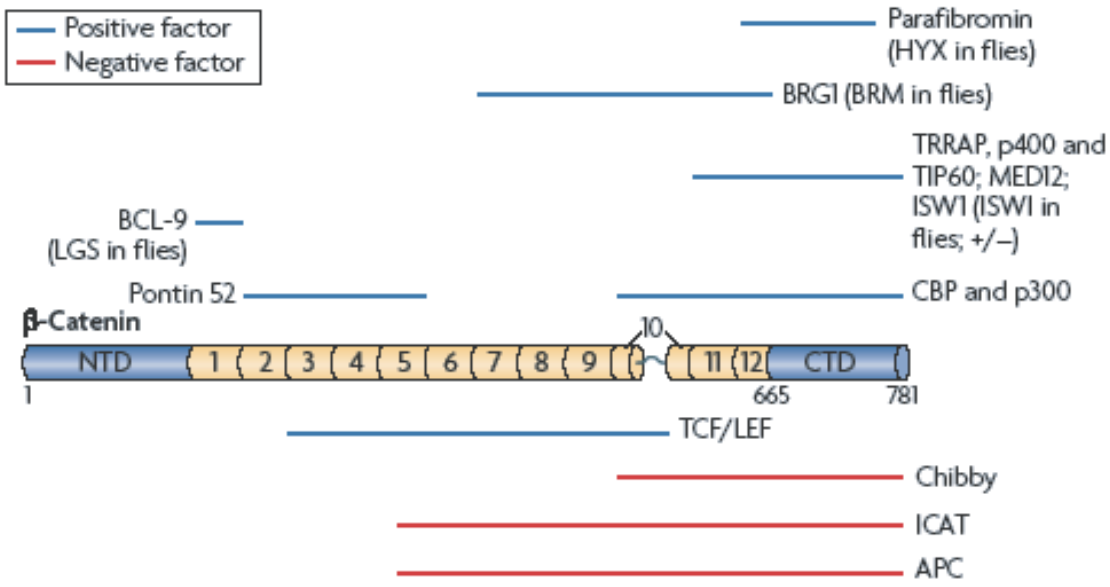

Figure1-3. Summary of proteins interacting with beta-catenin/Arm in the nucleus. beta-catenin/Armadillo (Arm) has 12 Arm repeats in the middle flanked by an N-terminus domain(NTD) and a C-terminus domain(CTD). Figure taken from Mosimann et al., 2009. Brahma related gene 1 (BRG1), Adenomatous polyposis coli (APC), CREB binding protein (CBP), B-cell CLL/lymphoma 9 (BCL-9), transformation/transcription domain-associated protein (TRRAP), mediator complex subunit 12 (MED12), Imitation SWI (ISWI).

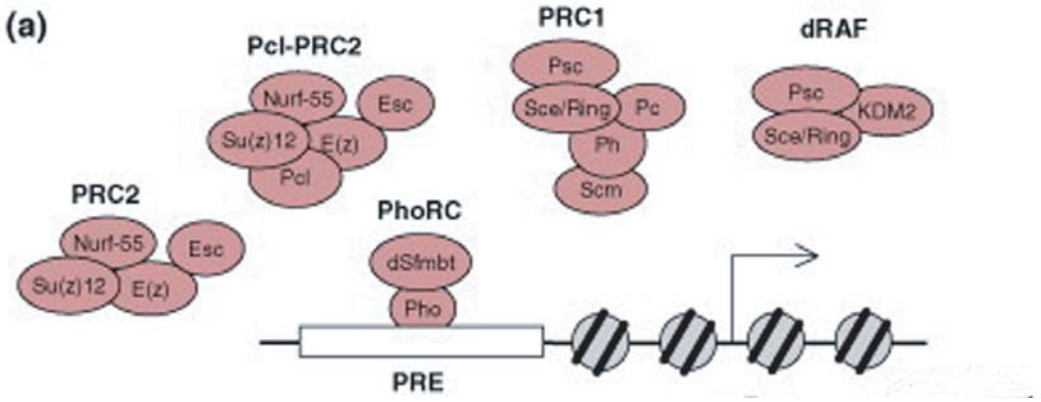

Fig1-4. Cartoon of the PcG complexes in flies. PhoRC, PRC1 and PRC2 are the three sub-complexes. Pcl-PRC2 and dRAF are the variants to the originally idenfied PRC2 and PRC1 respectively. (Figure adapted from Muller et al., 2009) Abbreviations: Pleiohomeotic (Pho), Scm-related gene containing four mbt domains (Sfmbt), Enhancer of zeste (E(z)), Supressor of zest 12 (Su(z)12), Extra sexcombs (Esc), Nucleosome remodeling factor of 55kD (Nurf-55), Polycomb like (Pcl), Posterior sex combs (Psc), Sex combs extra (Sce), Polycomb (Pc), Polyhomeotic (Ph), Sex comb on midleg (Scm), Lysine demethylase 2 (KDM2).

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Chapter II

Wingless signaling induces widespread chromatin remodeling of target loci

Abstract

Wnt/Wingless (Wg) signaling plays important developmental roles in animal development. Without Wnt/Wg stimulation, the key activator β-catenin/Armadillo (Arm) gets constitutively degraded by the proteosome. When the signal is turned on, β-catenin/Arm is released from this degradation and the accumulation of β-catenin/Arm in the nucleus causes the transcription factor TCF to switch from a repressor to an activator. How the Wnt/Wg signaling pathway regulates chromatin structure is not well understood. In this report, we examined the post-translational histone modifications involved in the Wnt/Wg signaling activation process. Surprisingly, H3Ac and H4Ac are increased over the entire target gene locus, covering over 30kb of chromatin. This widespread chromatin remodeling is specific for the Wg regulated targets and we only observed peaks of acetylated histones around the transcription start site on the constitutively active genes. The widespread chromatin modification is not a result of transcription elongation, but does depend on the histone acetyltransferase CBP.

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I generated the following data:

Figure 2-3 C-F

Figure 2-4

Figure 2-5

Figure 2-7

Figure 2-10

Figure 2-12

Figure 2-14

Introduction

Eukaryotic cells have developed an intricate mechanism to package their large amount of genetic material into relatively small nuclei. Double-stranded DNA is wrapped around the histone octameric core, forming a 'beads-on-the-string' structure; each bead is called a nucleosome. Nucleosomes are then further compacted to form chromatin. The tightly packed chromatin not only helps to store the genetic material but also serves as a barrier for transcription and the remodeling of chromatin is an essential mechanism in transcription regulation (Campos and Reinberg 2009)

Chromatin structure can be changed in two ways. The tightness of nucleosome packaging as well as the position of nucleosomes can be directly altered by proteins called ATP-dependant chromatin remodelers (Ho and Crabtree). In addition, the N-terminus of several histone subunits is subject to posttranslational modifications. The histone modifications can exert either a positive or a negative effect on transcription, but the connection is not always straightforward and likely to be context dependant (Berger 2007; Campos and Reinberg 2009).

Histone acetylation on histone subunits H3 and H4 is primarily found to be enriched at activated genes (Wang et al. 2008) and the enrichment is normally restricted to the transcription start site. Histone acetylation controls chromatin structure and transcription in two manners: direct alteration of the histone-DNA contact or recruitment of other chromatin remodelers (Choi and Howe 2009). Histone modifications are deposited by a group of enzymes called histone acetyltransferases (HAT or KAT), (Allis et al. 2007). Despite the importance of histone acetylation in transcription regulation, the

role of histone acetylation and HATs in Wnt/Wg signaling pathways is not well understood.

The correct expression of Wnt/Wg targets requires the coordinated action of many proteins in the nucleus (Cadigan and Peifer 2009). When Wnt/Wg signaling is off, phosphorylated and ubiquitinated β-catenin/Arm is degraded by the proteosome (Cadigan and Liu 2006). To prevent the transcription activation by low levels of nuclear β-catenin/Arm that may have escaped degradation, β-catenin/Arm buffers such as Chibby (Takemaru et al. 2003; Li et al. 2008) and ICAT (Tago et al. 2000; Tutter et al. 2001) bind with β-catenin/Arm to sequester it from chromatin. On the chromatin, the transcription factor TCF functions as a repressor (Cavallo et al. 1998), together with other corepressors like Gro (Cavallo et al. 1998) and CtBP (Fang et al. 2006), keeping Wnt/Wg targets off. When Wnt/Wg binds to its receptor, the degradation of β-catenin/Arm is inhibited. The recruitment of β-catenin/Arm by TCF converts TCF from a repressor to an activator. Together with coactivators, the TCF/Arm complex turns on the transcription of Wg targets.

Many coactivators were discovered through their binding to β-catenin/Arm and CBP/p300 is one of them (Hecht et al. 2000; Takemaru and Moon 2000). CBP and its relative p300 are histone acetylatransferases (HATs) with broad substrate specificity (Marmorstein and Roth 2001). It has been suggested to play both positive and negative roles in the Wnt/Wg pathway regulation (Waltzer and Bienz 1998; Hecht et al. 2000; Takemaru and Moon 2000; Li et al. 2007). It was suggested that CBP represses Wg targets by interacting with and acetylating TCF which prevents the Arm recruitment (Waltzer and Bienz 1998). At the same time, mutation in CBP's HAT domain does not

abolish the repression activity of CBP, putting the CBP repression mechanism in question (Li et al. 2007). CBP is also required for Wnt/Wg activation (Hecht et al. 2000; Labalette et al. 2004; Levy et al. 2004; Li et al. 2008). CBP was shown to acetylate β-catenin which increases its affinity for TCF (Labalette et al. 2004; Levy et al. 2004). However the HAT domain was shown to be dispensable for Wnt activation in other studies (Hecht et al. 2000; Li et al. 2008).

In this chapter, I will discuss our findings regarding the role of histone acetylation and CBP in the Wg pathway regulation. Wg activation induces widespread histone acetylation, a phenomenon not commonly observed. We hypothesize that the widespread histone acetylation is Wg specific and does not depend on transcription elongation. Furthermore, the widespread acetylation requires the histone acetyltransferase CBP, but surprisingly CBP is only localized to the identified enhancer. How the widespread histone acetylation is induced by localized CBP will be discussed.

Material and methods

This chapter contains data generated by Dave Parker, Zhenglong Li and Yunyun Ni. I will only include the material and methods for the experiments I performed. The remaining methods can be found in Parker et al. 2008.

Cell culture

KC cells were grown in the Drosophila Schneider media with 10%FBS. S2 cells stably transformed with a tubulin-Wg construct were used to produce Wg-conditioned medium (WCM). S2-Wg cells were grown in the presence of hygromycin until a concentration of at least 6 million/ml was reached. Cells were then precipitated and

medium was removed. Cells were then resuspended in fresh medium with no hygromycin without dilution and allowed to grow for another 4-6 days. After palleting and discarding the cells, the conditioned medium was filtered and concentrated (optional) before storage in the -80^{o}C freezer.

dsRNA was added directly to the culture medium at the concentration of 10ug/10^{6} cells. 4 days of addition of the dsRNA, cells were diluted 1:4 and allowed to grow for another 2-3 days before harvesting for assay.

WCM media was added to cells 5 hours before assay unless otherwise noted.

α-amanitin was added at the final concentration of 10ug/ml cells 2 hours before the WCM treatment. Cells were then incubated in the presence of WCM for another 5 hours before harvesting for assay.

Fly Genetics

Daughterless-Gal4 females were crossed with UAS-GPI-dFz2 or UAS-Wg males respectively. Embryos were kept at 29^{o}C and 4-10 hour old embryos were collected for ChIP assay.

Chromatin Immunoprecipitation

About 3×10^{7} Kc cells were cross linked with 1% final concentration of formaldehyde for 20 minutes at room temperature. After washing in PBS, cells were lysed and sonicated for 3 pulses of 10 seconds at the power output of 4 on a sonic dismembratro (Fisher Scientific Model 100). 3×10^{6} cells were used for each pulldown and ChIP was performed according to the Millipore ChIP protocol. Antibodies used in

this study include: anti-TCF generated by the Cadigan lab (Fang et al. 2006), anti-CBP was obtained from M. Mannervik (Lilja et al. 2007), AcH3 (Millipore 07-593), AcH4 (Millipore 06-866) and H3K4me3 (Millipore 07-473). Immunoprecipitates were analyzed with quantitative PCR.

For embryos ChIP, collected embryos were dechorionated in 50% bleach for 2 minutes and washed with water and 0. 7% NaCl-0. 1% Triton X-100. Embryos were fixed in 2% formaldehyde and heptane (1:3) for 15 minutes with rigorous shaking at room temperature. After the fixation, embryos were washed with heptane and then resuspended in PBS/glycine/Triton (125 mM glycine + 0. 1% Triton X-100 in PBS) to stop the crosslinking. After another wash in cold PBT (PBS + 0. 1% Triton X-100), embryos were aliquoted into 70ul and resuspended in 400ul SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris, pH8. 1) and ground with disposable pestle. Extracts were then flash frozen in liquid nitrogen and stored at -80^{o}C freezer. 70ul of embryos provide enough extract for 10 precipitations.

RNA extraction and RT-qPCR

RNA was prepared with trizol (Invitrogen) according to the manufacturer's instructions. cDNA was made by reverse transcription using reverse transcriptase from Invitrogen (Superscript RT) and oligo(dT) as primers. Quantitative PCR was performed using iCycler IQ real-time detection system(Bio-Rad). The primers used include: *5'TAAAATTCTCG-GCGGCTACAA3' and 5'CGCACCTGGTGGTACATCAG3' for nkd, 5'AGAGCAGCAGAAGCGTTAGC3' and 5'AAAGCCGGAGAAGCTACAAA3' for Notum,*

5'AGACCT-ACTGCATCGACAAC3' and 5'GACAAGATGGTTCAGGTCAC3' for β-tubulin56D (β-tub56D).

Data analysis

All data are presented as mean of duplicates and error bars indicate standard deviation generated by computer programs.

Results

Wg signaling induces TCF recruitment and histone acetylation at the *nkd* locus

The Cadigan lab previously identified *the naked cuticle (nkd)* gene as a direct Wg target both in KC cells and in various fly tissues (Fang et al. 2006; Li et al. 2007; Chang et al. 2008a). The major Wingless-response-element (WRE) is located 5kb downstream of the transcription start site (Fig 2-1A). Figure 2-1B shows that in response to treatment with Wingless conditioned media (WCM) treatment, *nkd* transcript levels increase rapidly over the course of 5 hours. The activation of *nkd* depends on TCF, Arm and Pygo as the dsRNA knockdown of these proteins dramatically reduces the activation of *nkd* (Fig 2-1C). TCF preferentially binds to *nkd* WRE compared with *nkd* ORF before the signal is turned on and this binding increases after cells are treated with WCM. The mechanism of this increase is not known but it is Arm dependent (Fig 2-1D). When another antibody recognizing a different portion of TCF was used, similar increase of ChIP signal was seen, suggesting that the increase is unlikely due to the increased

antibody accessibility (Fig 2-1E). When antibodies specifically recognizing the acetylated H3K9/K18 or H4K8/K12/K16 were used, an increase of signal on *nkd* WRE upon signal activation was seen. This increase is reduced when TCF or Arm is knocked down. When compared with the WRE, the enrichment of AcH3 and AcH4 remains low at the ORF in all conditions tested (Fig 2-1F and G).

Wg activation stimulates the enrichment of TCF and Arm at WRE

The Cadigan lab previously found that TCF is recruited to the intronic *nkd* WRE, but is absent at some other locations (Fang et al. 2006). In this study, we designed more primer sets targeting most of the predicted TCF binding sites (open boxes in Fig 2-1a) as well as some other regions. Consistent with our previous findings, TCF is only enriched at two identified functional WREs (Chang et al. 2008a). At the WRE about 10kb upstream of the *nkd* TSS, no preferential binding of TCF is observed before the signal is turned on and a small increase of TCF binding occurs with signal stimulation. The WRE at 5kb downstream of the *nkd* TSS recruits TCF when the signal is off and the recruitment is dramatically enhanced when the signal is turned on (Fig 2-2a). The profile of Arm at the *nkd* locus is very similar to TCF and the smaller peak at 30kb downstream of the TSS was not reproducibly observed (Fig 2-2b).

Wg activation induces widespread histone acetylation on *nkd*

In contrast to the localized recruitment of TCF and Arm in the *nkd* locus, when the binding pattern of AcH3 and AcH4 was examined, an increase across the entire *nkd* locus was found (Fig 2-3a,b). In both cases, the highest enrichment roughly centers around the WRE at 5kb downstream of the TSS. The single peak at 22kb downstream of

the TSS is reproducible but no functional WREs have been identified in this region. In the absence of the signal, both AcH3 and AcH4 show a peak at 10kb downstream of the TSS and the functional importance of this peak is not determined. When we examined another histone marker H3K4me3, normally associated with activation, we found a single peak around the transcription start site when the signal is activated (Fig 2-3c). This is consistent with the general understanding of this histone marker (Wang et al. 2008) and reassures us that the widespread AcH3 and AcH4 is not due to the increased histone binding across the locus. In fact, when we examined the unmodified H3 or panH4 (all forms of H4) across the *nkd* locus, we saw no increase of H3 and H4 (Fig 2-4a,b).

Since other studies reported that histone acetylations are localized mainly around TSSs (Wang et al. 2008), we wanted to test whether this is true for non-Wg targets in our KC cells. We tested three constitutively active promoters: the promoter of *pygo* and *rough deal*, the promoter of *TCF* and the promoter of *β-tub56D*. Different from the widespread histone acetylations seen on *nkd*, those promoters showed high level of acetylations at the TSS but the signal dropped dramatically at sites 3kb upstream or downstream of the TSS (Fig 2-3d,e,f).

Wg-dependent widespread histone acetylation of *nkd* occurs independently of transcription

The increased histone acetylation was observed upstream of the TSS, so it is unlikely that the widespread histone acetylations are due to transcription elongation. To test this possibility formally, we used α-amanitin to inhibit transcription before cells were treated with WCM. Figure 2-5a shows that the transcription activation of *nkd* by WCM

was indeed severely affected by α-amanitin. The increase of the TCF enrichment at *nkd* WRE was not affected by α-amanitin, suggesting that the Wg activation cascade upstream of TCF was not affected (Fig 2-5b). The H3K4 methyltransferases SET1 and Trithorax are known to be recruited to promoters by RNA PolII (Ng et al. 2003; Smith et al. 2004). Consistent with α-amanitin being an RNA PolII inhibitor (Wieland and Faulstich 1991), the spike of H3K4me3 induced by Wg around the *nkd* TSS is abolished when cells are pretreated with α-amanitin (Fig 2-5c). However, the presence of α-amanitin has no effect on the increase of AcH3 and AcH4 (Fig 2-5d,e), arguing that this increase is not a by-product of transcription initiation/elongation.

Wg induced histone acetylations are also widespread in the *notum* locus

Like *nkd*, the Wg target *notum* (also known as wingful) is expressed in response to Wg signaling throughout fly development (Gerlitz and Basler 2002; Giraldez et al. 2002; Hoffmans et al. 2005). The activation of *notum* in KC cells depends on TCF, Arm and Pygo (Fig 2-6b). Two WREs have been identified in the *notum* region. The first WRE, located upstream of the *notum* TSS is shown to be activated by WCM in fly S2 cells (Hoffmans et al. 2005). In this study, another WRE in the first intron of *notum* was identified which is also highly responsponsive to Wg (Fig 2-11d). TCF is preferentially recruited to the two WREs upon Wg stimulation (Fig 2-6c). In the absence of signal, the intronic WRE at 5kb downsteam of the TSS shows significant TCF binding compared with non-WRE regions whereas the WRE upstream of the TSS does not detectably recruit TCF in these cells. Similar with our results in the *nkd* locus, widespread AcH3 and AcH4 in the *notum* locus is induced in response to Wg. The peaks of acetylations are also roughly centered around the two WREs. The highest peaks for both AcH3 and

AcH4 at the intronic WRE appear to have shifted upstream compared with the binding pattern of TCF and the importance of this is not clear (Fig 2-6d,e).

To test whether the widespread histone acetylation is also relevant in systems other than the KC cells, we performed ChIP on fly embryo extracts. Daughterless-Gal4 was used to drive the expression of either Wg or a dominant negative form of the Wg receptor dFz2 (Cadigan et al. 1998) to create the Wg on and Wg off environment respectively. TCF ChIP showed that in embryos without an active Wg signal, the upstream WRE is bound by TCF and the binding is further increased when Wg is turned on (Fig 2-7a). The intronic WRE however does not show significant TCF recruitment in either Wg on or Wg off embryos. This difference may reflect a tissue specific usage of enhancers, a phenomenon that has been documented before for both mammalian TCF and fly TCF (Wohrle et al. 2007; Chang et al. 2008a). When we tested the AcH3 enrichment in the *notum* locus in embryos, we saw a similar widespread increase of AcH3 (Fig 2-7b).

CBP is required for activation of *nkd* and *notum*

CBP is an attractive candidate for the histone acetyltransferase responsible for the widespread histone acetylations. CBP is a co-activator that has HAT activity with a broad substrate spectrum including H3 and H4 (Bannister and Kouzarides 1996; Ogryzko et al. 1996). The Cadigan lab identified CBP as a Wg co-activator previously in both KC cells and wing imaginal discs. The direct interaction between Arm and CBP may help recruit CBP to the *nkd* WRE (Li et al. 2007). Can CBP be the HAT responsible for the widespread AcH3/AcH4 on *notum* and *nkd*? It was first confirmed that CBP is required for the activation of *nkd* and *notum* (Fig 2-8 A and B). When CBP is depleted

with dsRNA, the transcription activation of *nkd* and *notum* is severely reduced, but the Wg dependant TCF recruitment on *nkd* WRE and the accumulation of Arm is not affected showing that the Wg pathway activation cascade upstream of TCF is not disrupted by CBP knockdown(Fig 2-8 C and G). Because CBP is a general co-activator, the depletion of CBP often has an adverse affect on cell health. The transcription of several house keeping genes: *tub*, *arm* and *TCF* were tested and their transcription is not affected by *CBP* dsRNA suggesting that the general health of the cell is good (Fig 2-8 D-F).

Localized CBP is required for the widespread histone acetylation

Is CBP required for the widespread acetylation of Wg target upon pathway stimulation? In order to answer this question, the enrichment of AcH3/AcH4 on *nkd* and *notum* was tested in cells depleted with CBP before Wg treatment. For *nkd*, the WCM caused a widespread increase of AcH3 and AcH4 in the locus in cells treated with control dsRNA. CBP depletion reduces the amount of AcH3 and AcH4 on *nkd* to the level lower than in the control (no Wg added) cells (Fig 2-9 A and B), suggesting that even before Wg activation, there is some CBP dependent histone acetylation on *nkd*. This acetylation could contribute to the basal level of *nkd* transcription (Fig 2-5A). In the case of *notum*, CBP knockdown decreased AcH3/AcH4 to a level comparable with control cells without Wg treatment (Fig 2-9 C and D).

Although depletion of CBP abolished the Wg dependent widespread histone acetylations, CBP itself is localized to the WRE only (Fig 2-10). The possibilities of how the localized CBP promotes the widespread AcH3/AcH4 will be discussed later.

Are histone acetylations spreading?

We have shown that Wg induced widespread histone acetylations in the Wg target loci, but are the WREs the origin of the widespread AcH3/AcH4? If WREs are the origin, one would expect a delay between the onset of increase at WREs and the increase far away from the WRE. Time course experiments have been performed to test this hypothesis. In these experiments, WCM was added and the levels of AcH3 in the *nkd* region were measured at 0, 0.5, 1, 1.5, 2, 3 and 4 hours after Wg activation. Unfortunately, in our experimental setting no obvious time delay between the increase of acetylations on WREs and the increase elsewhere was detected (data not shown).

It is possible that the initial spreading from the WRE is too fast to be captured with our experimental protocol. Alternatively, there may be many other functional TCF sites in addition to the ones in the WREs and the TCF binding to these sites are too weak to be detected by ChIP. HATs may also be recruited to these 'weak' TCF sites which cause widespread acetylations. If the latter hypothesis is true, one would expect to find functional DNA elements throughout the *nkd* and *notum* region. Reporter assays were performed to test whether there were other functional but weak TCF sites in the *nkd* region. A 7.1kb fragment centered around the *nkd* intronic WRE was cloned and this fragment showed high Wg responsiveness in KC cells transfected with a stable Arm (Fig 2-11B). Interestingly, the deletion of a 1kb fragment including the identified WRE completely blocked the response. This result shows that the deleted 1kb fragment is absolutely essential for the Wg responsiveness and the remaining DNA sequence in the 7. 1kb fragment can not respond to Wg on its own.

The more direct test of whether AcH3/AcH4 spreads from the WREs would be to mutate the WREs and examine whether histone acetylation is abolished across the Wg targets loci. Since techniques to mutate the genomic DNA in fly cells are not yet available, such experiment has to be performed on transfected constructs. Unfortunately, we have not been able to perform ChIP on transiently transfected constructs in KC cells. An alternative of performing ChIP on the stably integrated constructs in fly embryos will be described in the discussion.

Discussion

Wg activation induces widespread histone acetylation of target loci

Several genome wide studies agree that histone acetylation is normally restricted to a distinct region such as TSS (Bernstein et al. 2005; Heintzman et al. 2007; Wang et al. 2008). However, our study showed that in both fly cell culture and fly embryos, Wg activation causes increase of AcH3/AcH4 both upstream and downstream of the TSS, spreading up to 45kb genomic region (Fig2-3 A and B, Fig 2-6 D and E, Fig 2-7 B). The increase in widespread acetylation did not depend on active transcription since Wg signaling still caused these modifications when RNA PolII was inhibited(Fig 2-5 D and E). The widespread acetylation is also not a result of a general increase in histones. H3K4me3 is only enriched at the TSS (Fig 2-3 C) upon Wg stimulation, showing that not all histone modifications are widespread on Wg targets. Furthermore, H3 and H4 levels in chromatin are not changed by Wg activation (Fig 2-4).

Although AcH3 and AcH4 are normally found in distinct loci, some repressive histone markers such as H3K9 methylation in heterochromatin silencing and H3K27 methylation in polycomb silencing are known to span large regions of chromatin (Danzer

and Wallrath 2004; Ringrose 2007). In the case of H3K27 methylation, it is not clear whether the H3K27 methylation originates at PREs and spreads out (Muller and Verrijzer 2009). A direct way to test whether particular enhancers serve as origins of the spreading of histone markers is to mutate the enhancer element and examine the spreading.

Although in our hands, we were not able to perform ChIP on transiently transformed reporter constructs in KC cells, we did detect TCF binding as well as Wg dependant increase of AcH3/AcH4 on the *notum* reporter integrated into fly genome in embryos (Fig 2-12). This provides a system where we can directly test the origin model of widespread AcH3/AcH4. We can, for example, examine the chromatin of the integration site to see whether there is a Wg dependant increase of acetylated histones. Then we can mutate the WRE on the inserted DNA and ask whether this mutation abolishes the increase of AcH3/AcH4 on the reporter and on the surrounding genome.

Role of CBP in the widespread histone acetylations

The role of CBP in the Wnt/Wg pathway has a complicated history. In flies, CBP has been shown to acetylate TCF and this acetylation abrogates TCF's ability to bind to Arm, suggesting a negative role of CBP in the Wg pathway (Waltzer and Bienz 1998). Loss of CBP causes ectopic expression of some Wg targets, providing a functional support of the model (Waltzer and Bienz 1998). The negative role of fly CBP in Wg pathway was also observed by another study, however, the repression is not affected by a mutation in the HAT domain of CBP (Li et al. 2007). The latter result suggested that the acetylation of TCF by CBP may not be essential for repression. Alternatively, the HAT mutant CBP could function as a dominant negative because Li et al has also identified a

positive role of CBP in the Wg regulation where CBP directly interacts with Arm and the HAT domain of CBP is required for the activation. The positive role of CBP in the Wnt signaling has also been reported in vertebrates (Hecht et al. 2000; Sun et al. 2000; Takemaru and Moon 2000). CBP can acetylate lysines on β-catenin, thus increasing its affinity to TCF (Wolf et al. 2002; Labalette et al. 2004; Levy et al. 2004). Whether this mechanism is functionally important is still controversial as CBP lacking the HAT activity can still activate TCF reporters (Hecht et al. 2000).

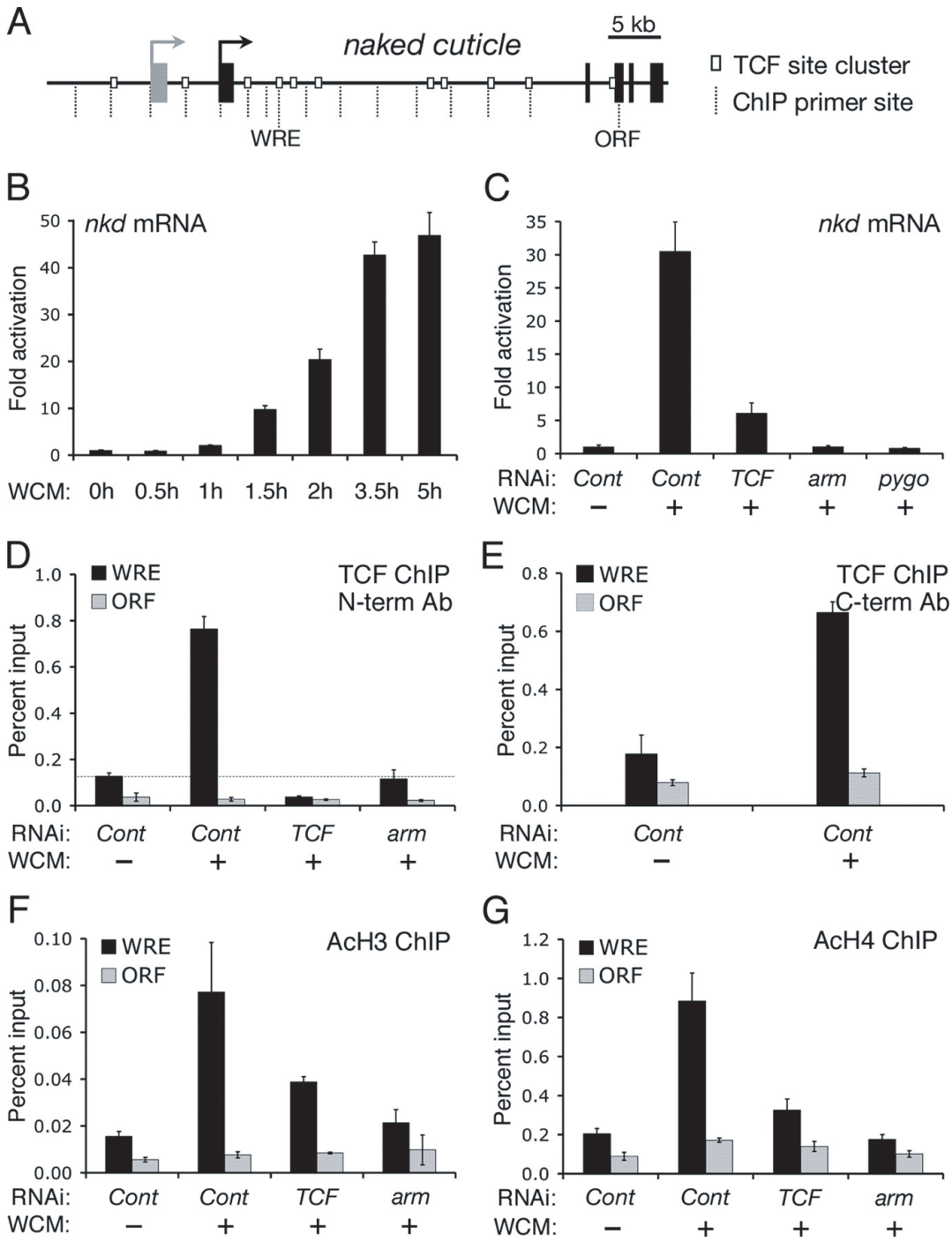
CBP is a well known histone acetyltransferase (Bannister and Kouzarides 1996) and its ability to acetylate histones are associated with gene activation in many processes such as embryonic neural differentiation in mice (Wang et al.). In Wnt/Wg signaling, much emphasis has been on the acetylation of TCF and Arm by CBP, but the role of CBP on chromatin modification has been neglected so far (Waltzer and Bienz 1998; Levy et al. 2004). In this report, we showed that depletion of CBP abolished the Wg dependant increase of AcH3/AcH4 on *nkd* and *notum* (Fig 2-9). In contrast to the widespread AcH3/AcH4, CBP is restricted to the WRE. The localized CBP could induce the widespread AcH3/AcH4 by forming chromosomal loops (Fig 2-13). Alternatively, CBP or the modified histones on WRE may recruit other HATs to catalyze the acetylations outside WRE. To test whether there are other HATs involved in Wg targets activation, I used dsRNA to deplete the known HATs in fly cells and found no obvious activation defects (Fig 2-14). Surprisingly, in this experiment none of the dsRNA causes an obvious health issue of cells, whereas CBP dsRNA normally affect the health of KC cells. So it is possible that CBP is the dominant HAT in KC cells. Alternatively, two or more

HATs may have redundant roles in the Wg activation and single depletion of just one may not be sufficient to cause a phenotype.

Why do Wg targets have widespread acetylation?

We have shown that Wg induces widespread acetylation on Wg targets both in Kc cells and in fly embryos. In contrast, several constitutively active genes in Kc cells only display a sharp peak of histone acetylation around their promoters. It is thus interesting to speculate what the widespread acetylation is good for. One possible explanation is that it is required to counteract some repressive histone marks which are also widespread in the absence of signaling. Chapter III will examine one of such repressive marks.

Figure 2-1. Wg signaling induces TCF recruitment and histone acetylation at the *nkd* locus. (A) Cartoon of the *nkd* locus. *nkd* exons are represented with black boxes. Locations of ChIP primer sets used are shown with dashed lines. Predicted TCF clusters are depicted in open boxes. (B) nkd transcription is rapidly activated by WCM. KC cells were treated with WCM for indicated length and the transcript of nkd is measured by RT-qPCR. Relative activation normalized by beta-tub56D is shown. (C) Activation of *nkd* depends on Wg components. Cells were treated with control, TCF, Arm or Pygo dsRNA for 6 days before WCM was added. Transcript of nkd was measured 5.5 hours after the addition of WCM. (D) TCF is recruited to *nkd* WRE. Cells were treated with indicated dsRNA with or without WCM. ChIP was performed with TCF antibody recognizing the N-terminus of TCF. Enrichment of TCF on either WRE or ORF was expressed as the DNA present in the pull down portion divided by the DNA present in the pre-pull down portion. (E) TCF ChIP with another TCF antibody recognizing the C-terminus of TCF. (F) Increased recruitment of AcH3 to nkd WRE with WCM. Cells were treated as before and antibody recognizing acetylated H3 was used. (G) Increased recruitment of AcH4 to nkd WRE with WCM. Cells were treated as before and antibody recognizing acetylated H4 was used. Figure and legend adapted from Parker et al. 2008.


A
naked cuticle
5 kb
TCF site cluster
ChIP primer site
WRE
ORF
B
nkd mRNA
Fold activation
WCM: 0h 0.5h 1h 1.5h 2h 3.5h 5h
C
nkd mRNA
Fold activation
RNAi: Cont Cont TCF arm pygo
WCM: – + + + +
D
WRE
ORF
TCF ChIP
N-term Ab
Percent input
RNAi: Cont Cont TCF arm
WCM: – + + +
E
WRE
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TCF ChIP
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RNAi: Cont Cont
WCM: – +
F
WRE
ORF
AcH3 ChIP
Percent input
RNAi: Cont Cont TCF arm
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G
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RNAi: Cont Cont TCF arm
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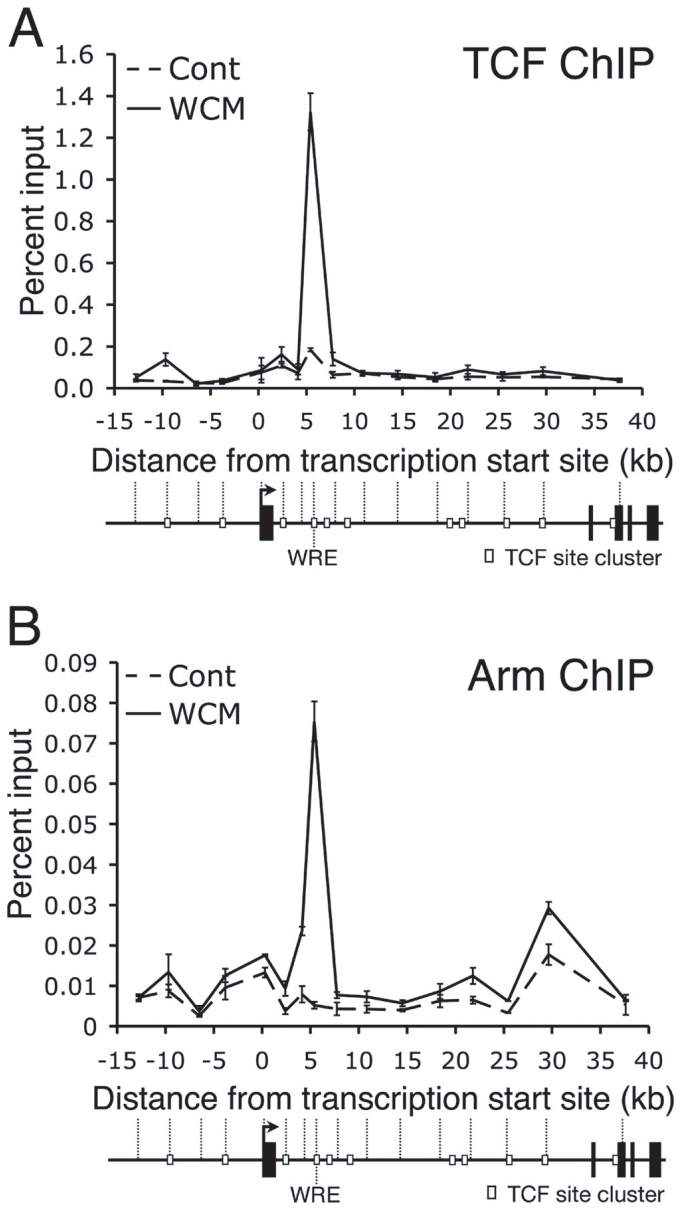
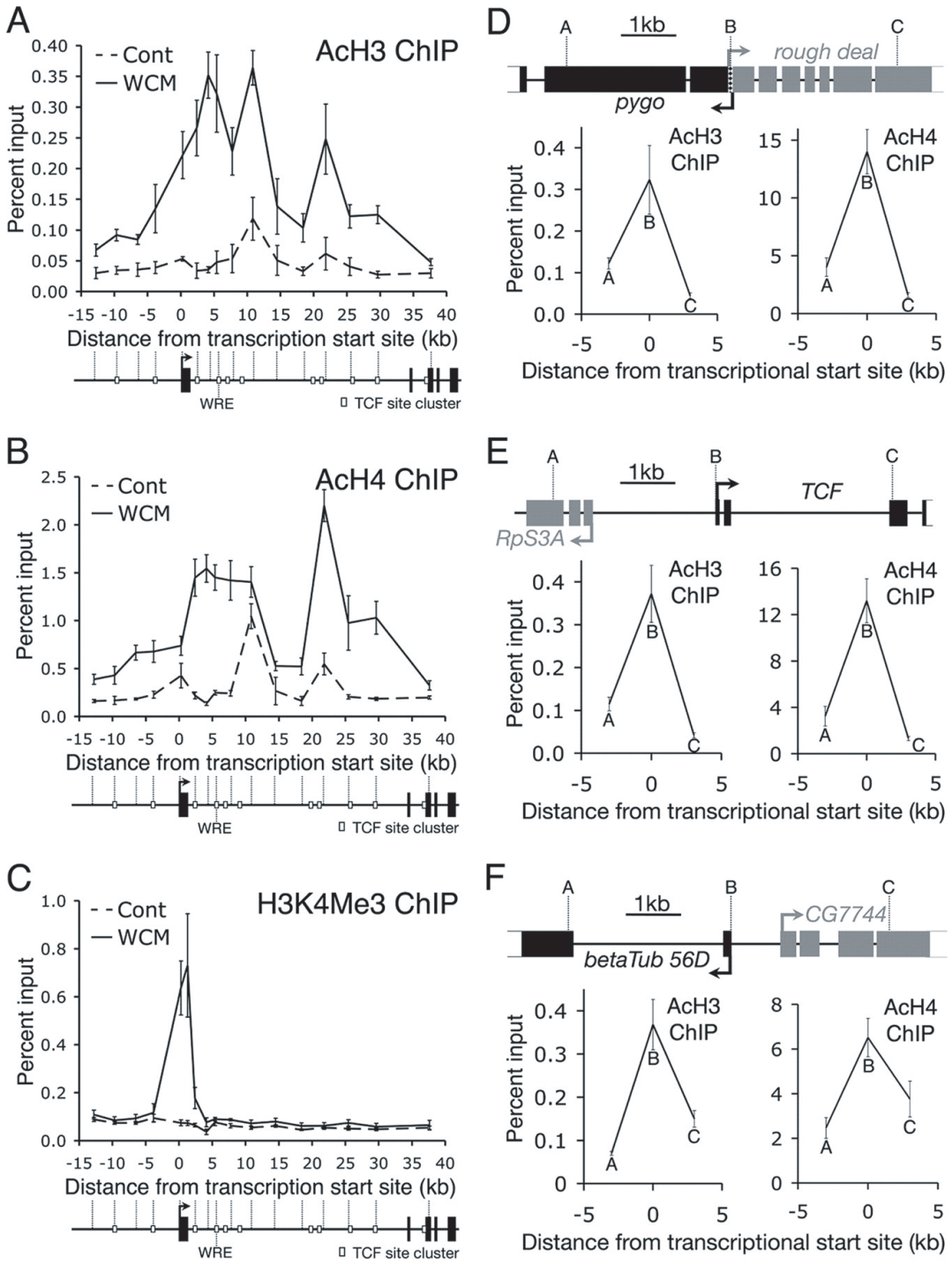


Figure 2-2 Wg induces the recruitment of TCF and Arm to nkd WRE. (A) TCF ChIP results show that TCF is recruited to the WRE in the absence of signaling (dashed line) and the recruitment is increased by Wg activation. (B) Arm ChIP results show very similar binding pattern as TCF with a single dominant peak located at the WRE. The second big peak at 30kb downstream of the TSS was not reproducibly observed. Figure and legend adapted from Parker et al. 2008

Fig 2-3. Wg specifically induces widespread histone acetylation. (A)AcH3 is increased across nkd region when Wg is activated. (B) AcH4 is increased across nkd region when Wg is activated. (C) H3K4me3 is only increased at the nkd TTS when Wg is activated. (D-F)In contrast to the Wg targets, the housekeeping genes show a sharp peak of AcH3/AcH4 around their TTSs. (D)pygo/rough deal. (E)TCF (F)beta-tub 56D Figure and Legend adapted from Parker et al. 2008


A
AcH3 ChIP
- - Cont
— WCM
Percent input
Distance from transcription start site (kb)
WRE
TCF site cluster
B
AcH4 ChIP
C
H3K4Me3 ChIP
D
1kb
rough deal
pygo
AcH3 ChIP
AcH4 ChIP
Distance from transcriptional start site (kb)
E
TCF
RpS3A
F
CG7744
betaTub 56D

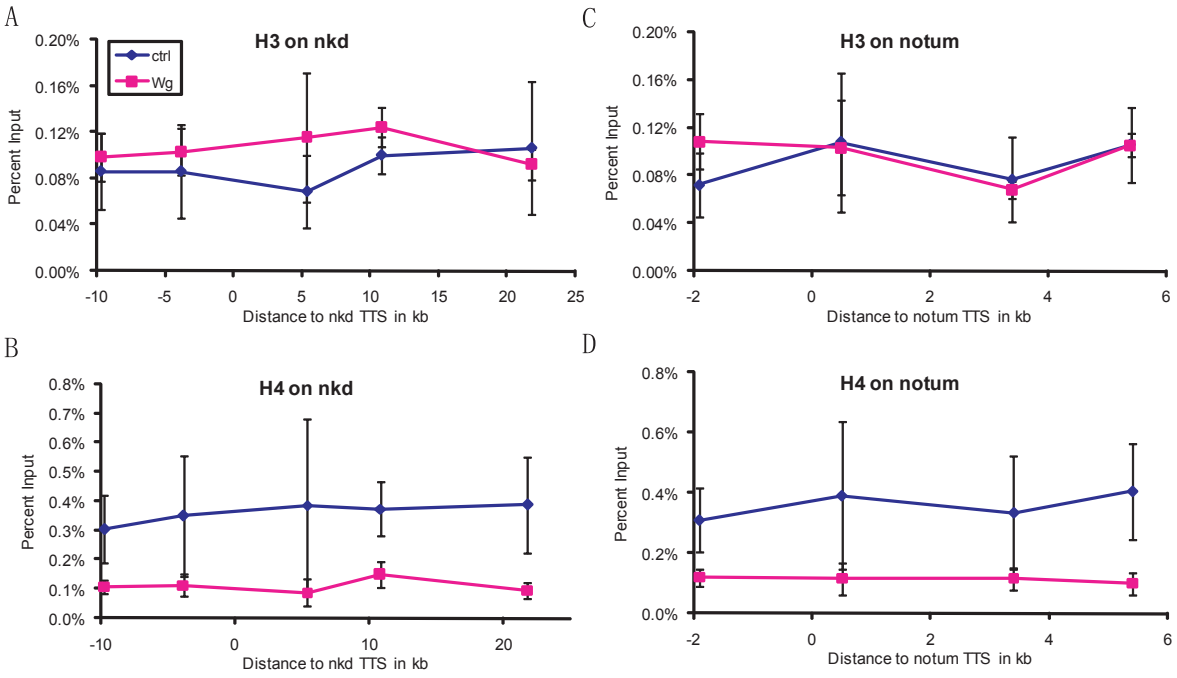


Fig2-4 Wg does not increase the level of H3 and H4 on nkd and notum. Kc cells were treated with WCM for 5 hours before harvested for ChIP assay. Immunoprecipitation with antibodies recognizing unmodified H3 and all forms of H4 was performed. H3 enrichment is not significantly changed on *nkd*(A) and *notum*(C) with Wg stimulation. The total H4 level is slightly decreased in response on *nkd*(B) and *notum*(D). Error bars represent standard deviation of ChIP duplicates.

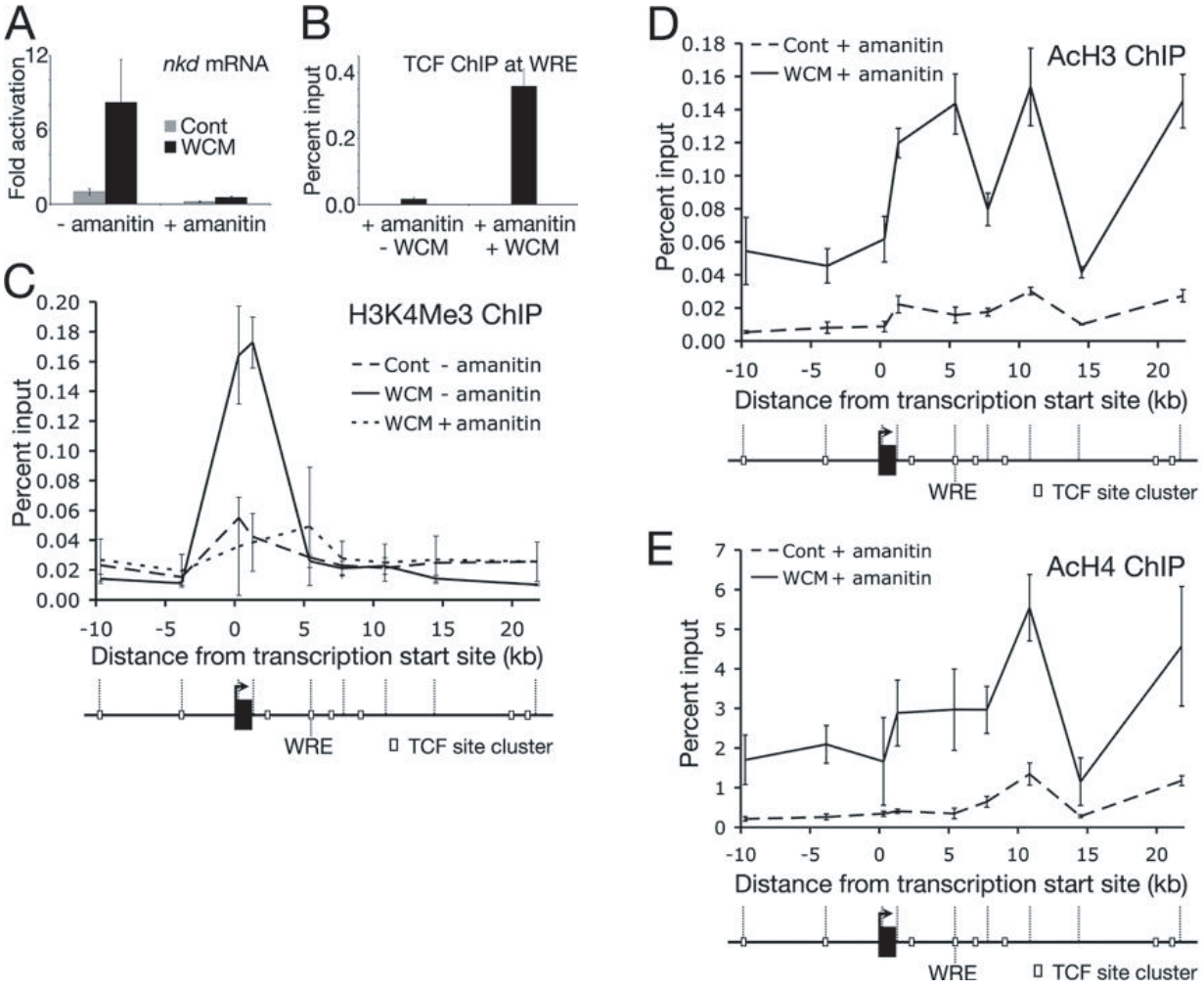


Fig 2-5 Wg dependent increase of AcH3/AcH4 is not transcription dependant. (A)Amanitin inhibits transcription of *nkd*. Cells were treated with amanitin for 2 hours before WCM addition.Relative *nkd* transcript level was normalized to that of *beta-tub* which is unchanged.(B) The increase of TCF responding to Wg is unchanged by amanitin. (C) Amanitin efficiently abolished the increase of H3K4me3 at the *nkd* TSS. (D and E) AcH3 and AcH4 are still enriched across nkd *with* Wg activation even when transcription is inhibited. Figure adapted from Parker et al 2008

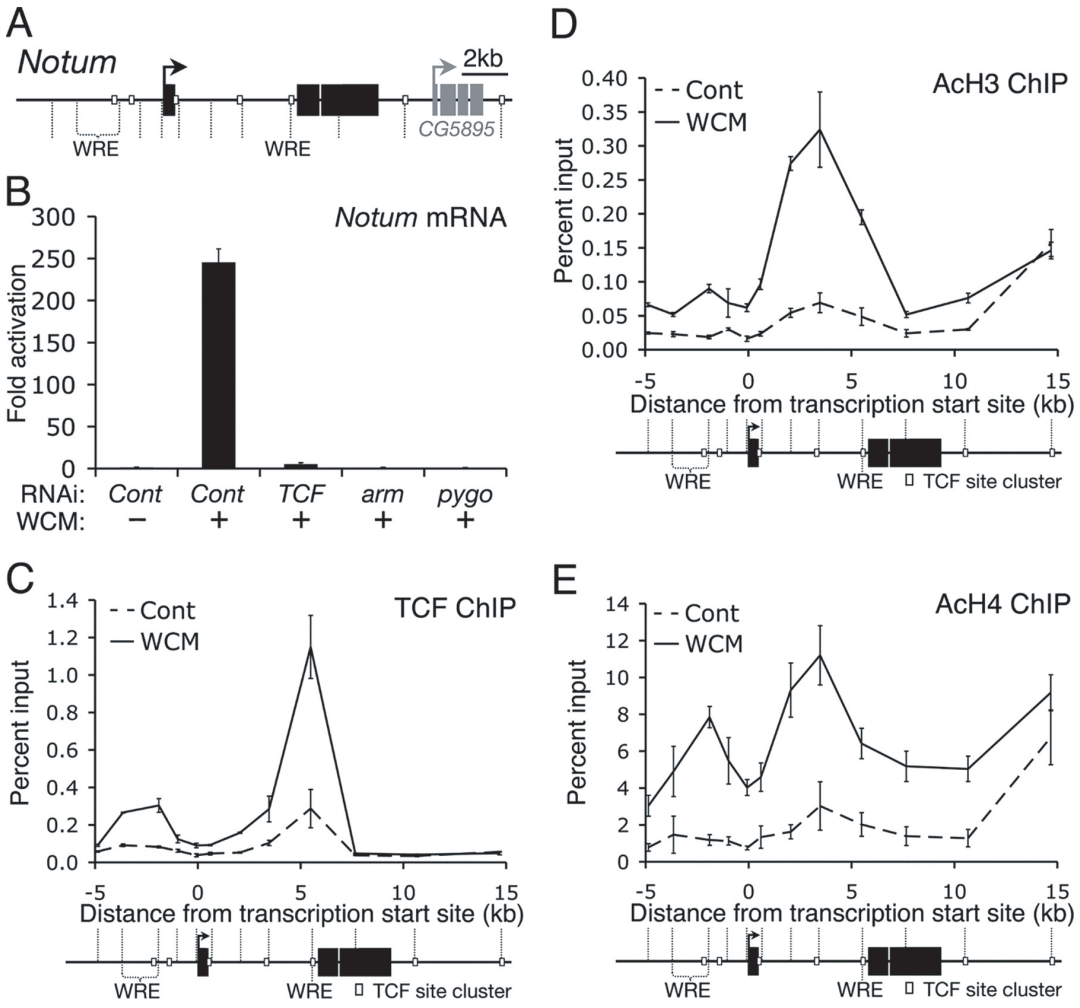


Fig 2-6 AcH3/AcH4 are increased across notum in response to Wg activation. (A) Cartoon of the *notum* locus. *notum* exons are represented by black boxes. Open boxes indicate predicted TCF sites and dashed lines mark the locations of ChIP primers.(B)Wg activates *notum* transcription in a TCF, Arm and Pygo dependant manner. (C) TCF is localized to two *notum* WREs. The intronic WRE recruits TCF in the absence of signaling (dashed line) and the recruitment is increased with Wg activation (solid line). The upstream WRE only shows TCF recruitment in the presence of Wg signaling. (D and E) AcH3 and AcH4 are increased across the notum region stimulated by Wg. Figure adapted from Parker et al. 2008

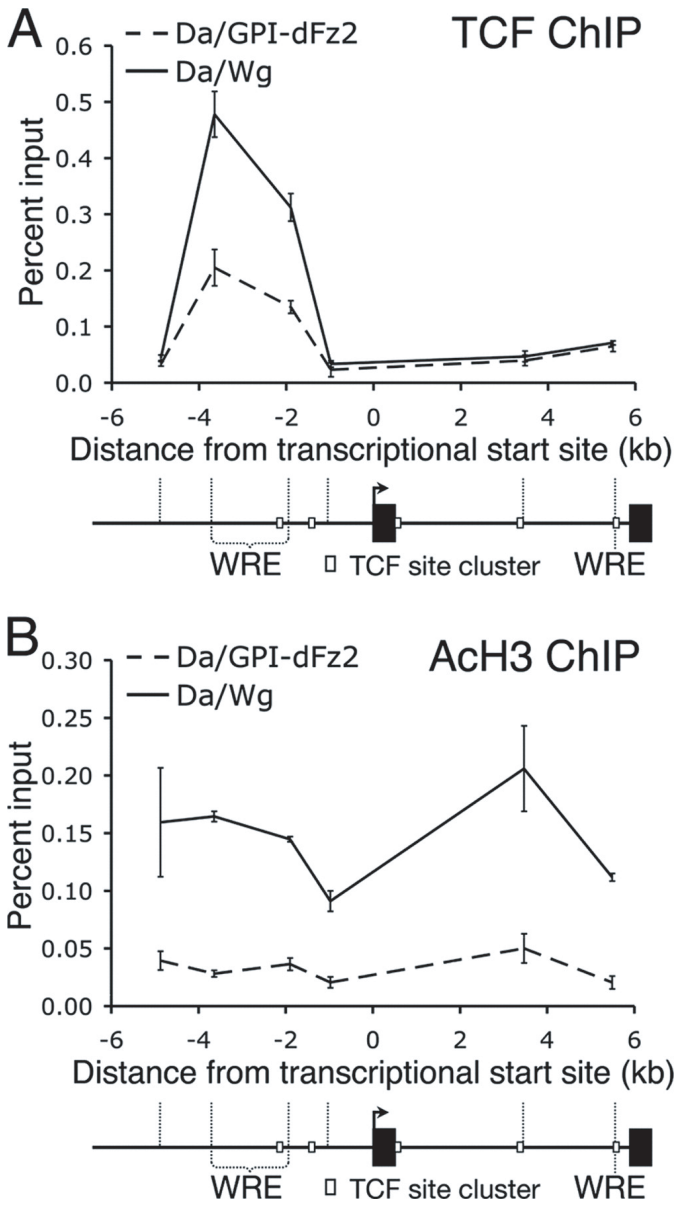


Fig2-7 Wg dependant widespread AcH3/AcH4 on *notum* in fly embryos. (A)Dominant negative dFz2(GPI-dFz2) and Wg were expressed under the control of an ubiquitously expressed promoter Daughterless in fly embryos to create Wg off and Wg on conditions respectively. TCF is recruited to the upstream *notum* WRE in the Wg off embryos (dashed line) and the recruitment is enhanced in the Wg on embryos (solid line). (B) AcH3 level is elevated across the notum region in response to Wg activation. Figure adapted from Parker et al. 2008

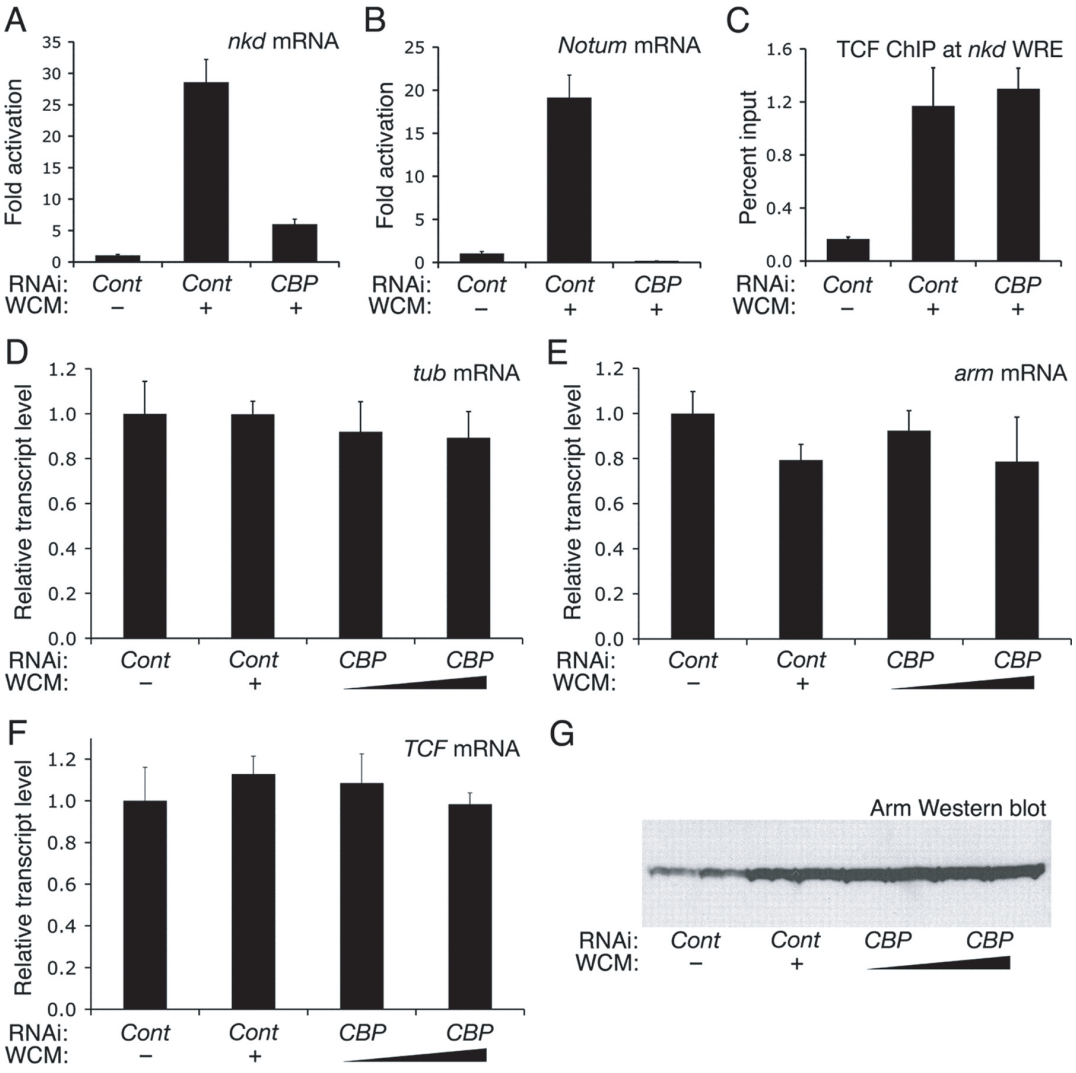


Fig 2-8 CBP is required for the Wg induced activation of *nkd* and *notum*. (A and B) The expression of *nkd* and *notum* is activated by WCM treatment and the activation is severely decreased when CBP is depleted by dsRNA. (C)The increased recruitment of TCF to *nkd* WRE is not affected by CBP depletion. (D-E) The expression of house-keeping genes is not affected by CBP depletion. (D)*tub* (E)*arm* (F)*TCF*. (G) The accumulation of Arm in response to Wg is not affected by CBP knockdown. Figure adapted from Parker et al. 2008

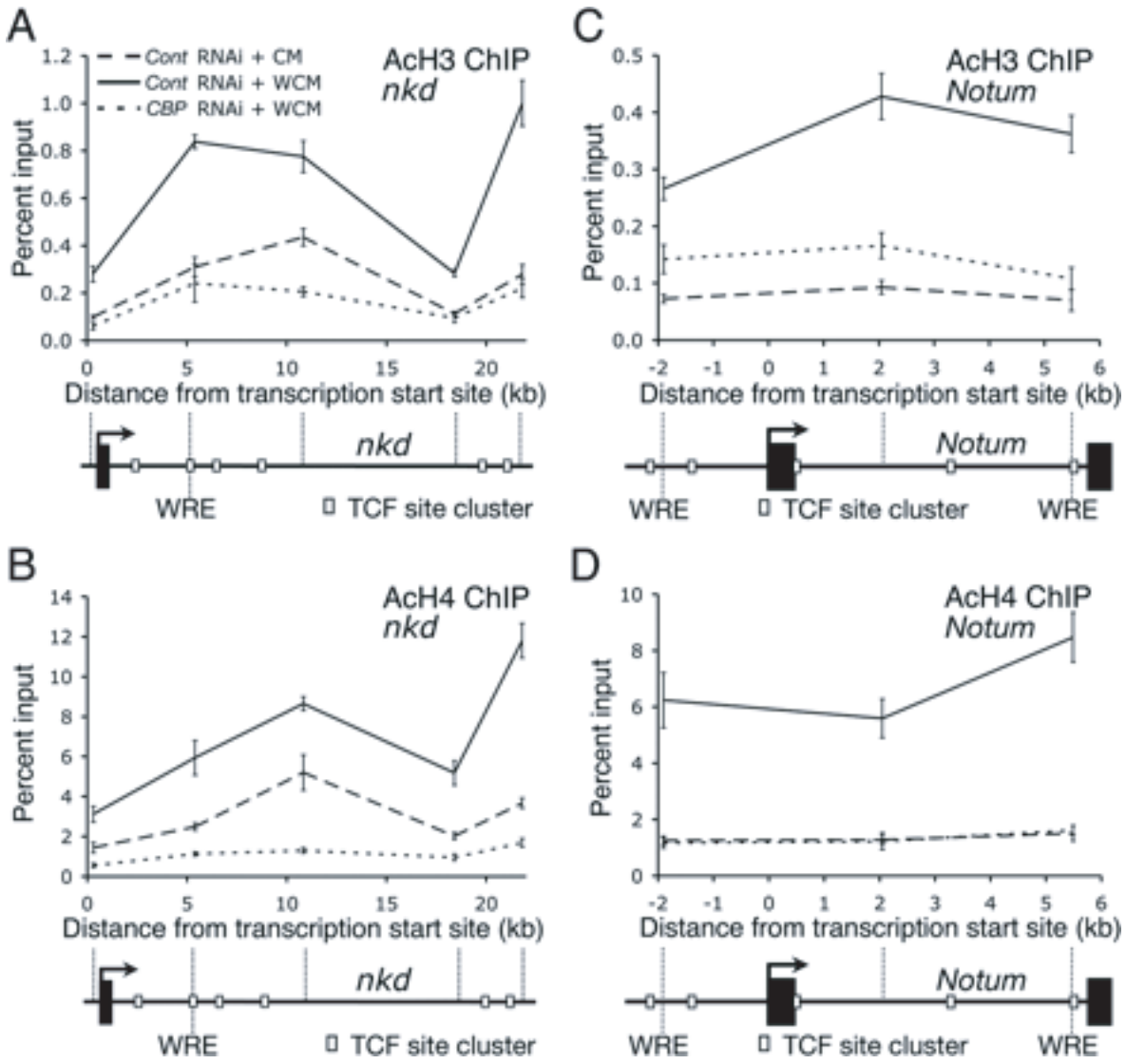


Fig 2-9 CBP is required for widespread histone acetylations across *nkd* and *notum*. (A and B) AcH3(A) and AcH4(B) level on *nkd* is decreased to a level below the unstimulated cells (dashed line) when CBP is depleted before Wg activation (dotted line). (C and D) AcH3 (C) and AcH4 (D) level on *notum* is decreased close to the level in unstimulated cells (dashed line) when CBP is depleted before Wg activation (dotted line). Figure adapted from Parker et al. 2008

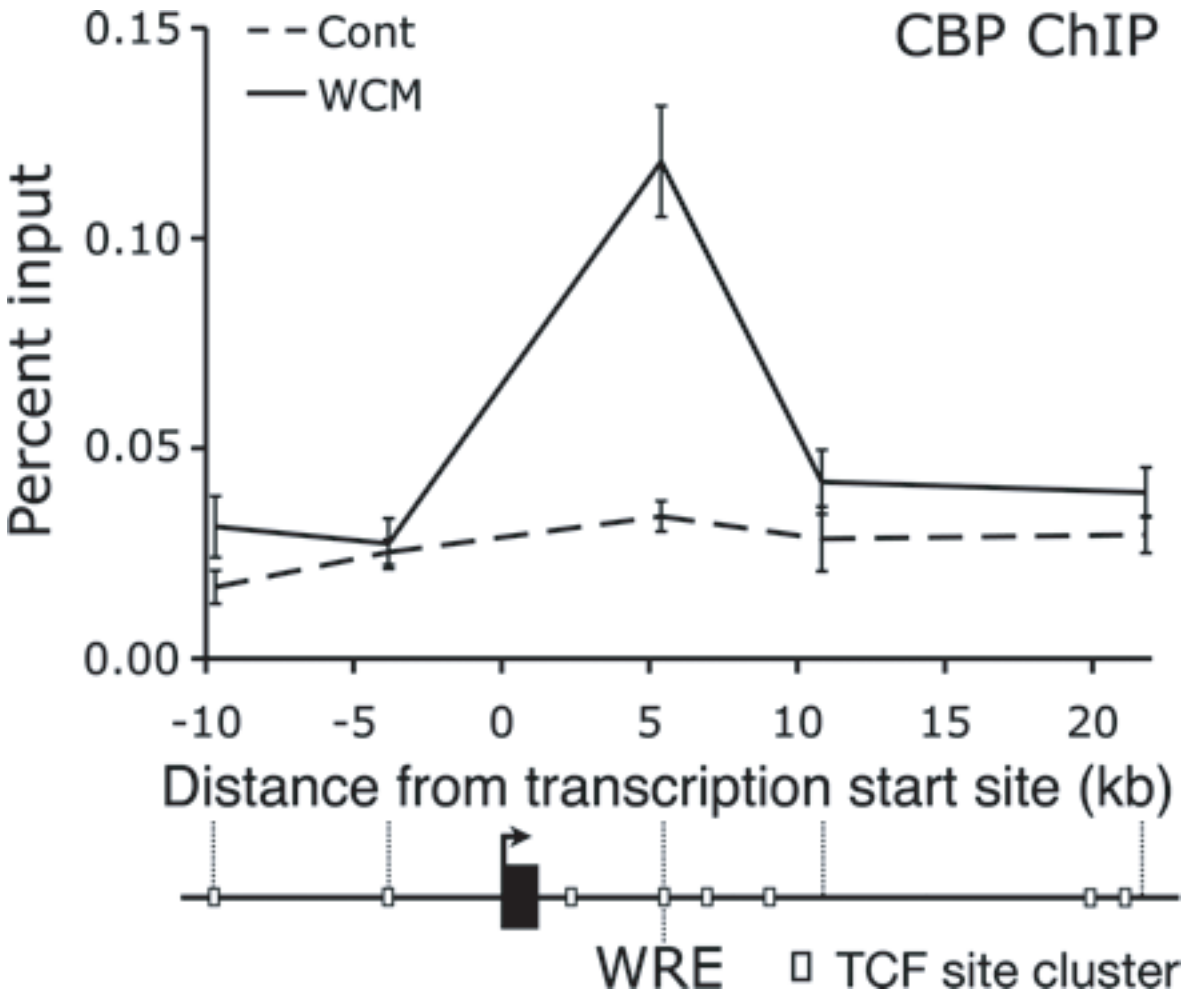


Fig 2-10 CBP is localized to *nkd* WRE. Cells were treated with WCM for 5 hours before ChIP was performed with CBP antibody. Figure adapted from Parker et al. 2008

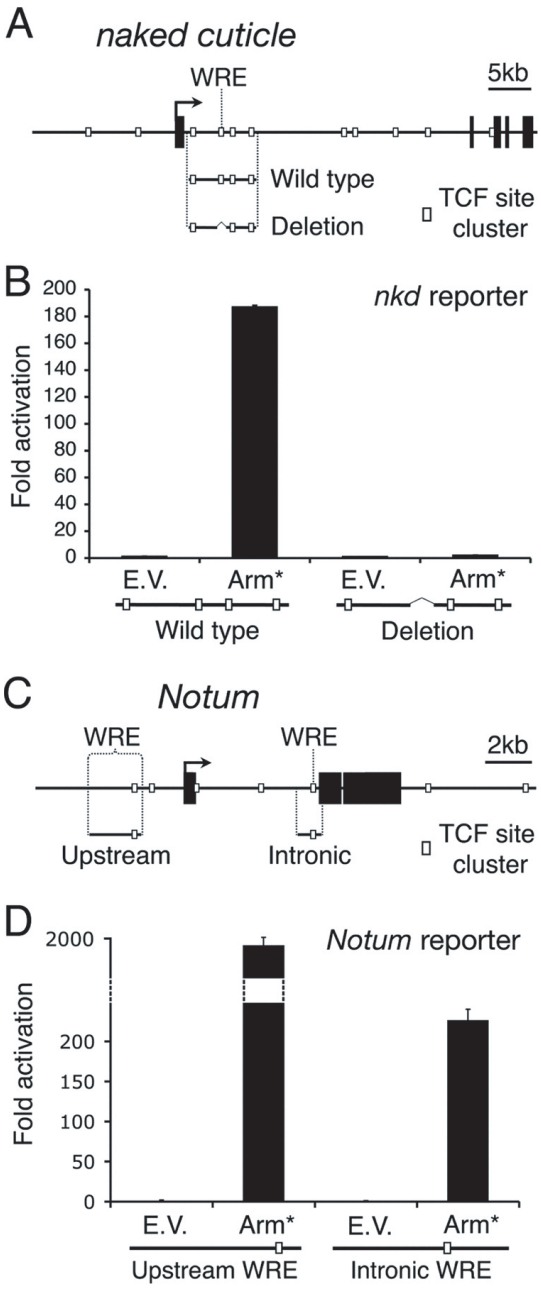


Fig 2-11 *nkd* and *notum* WREs are functional. (A) Cartoon of *nkd*. The cloned enhancer including WRE is labled as well as the deletion. (B) While wildtype reporter responds to Wg robustly, deletion of the WRE completely block the reporter's activity. (C) Cartoon of *notum*. The location of upstream WRE and intronic WRE is labled. (D) Both *notum* WREs are highly responsive to Wg activation. Figure adapted from Parker et al. 2008

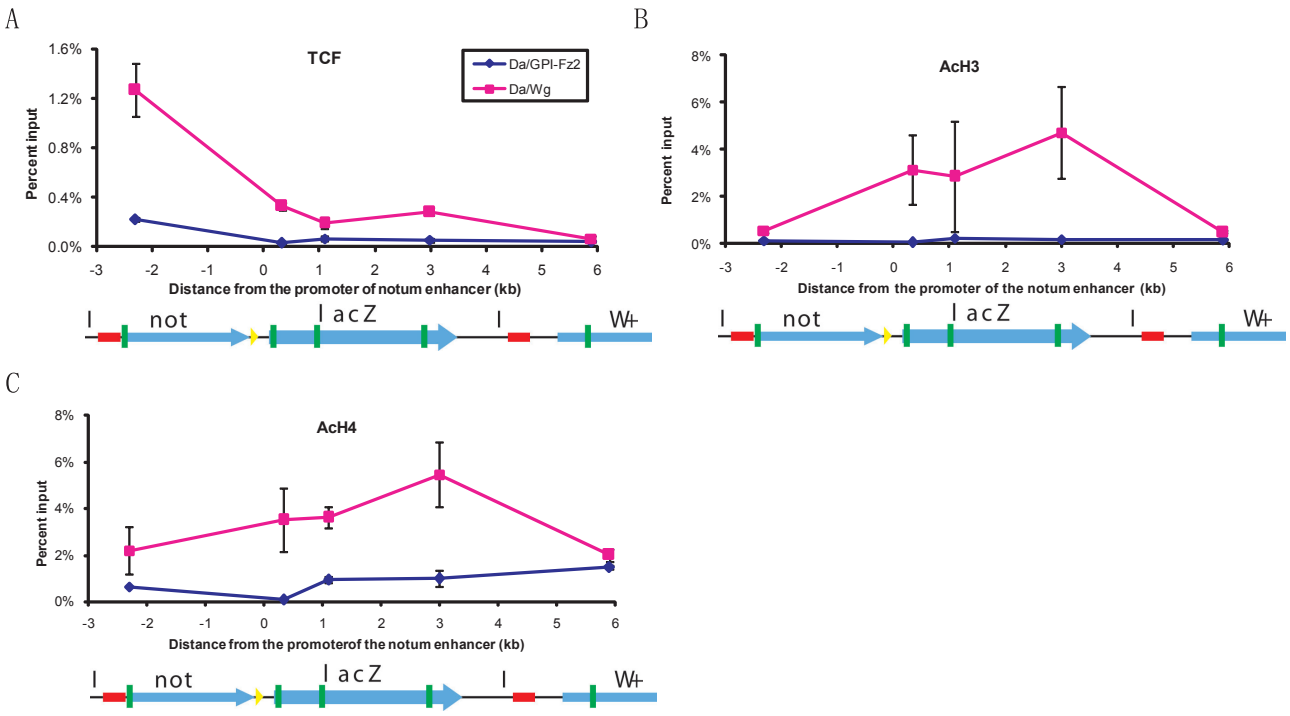


Fig 2-12. Wg induce widespread histone acetylations on stably integrated *notum* reporter in fly embryos.4-10 hr embryos were collected from Da/GPI-dFz2 or Da/Wg respectively and ChIP with the indicated antibodies was performed. The cartoon under each panel shows the construct structure. *notum* upstream enhancer (labeled not, blue box) was used to drive the expression of lacZ (blue box) and w+ (blue box) served as a selection marker for transgenic flies.Red boxes indicate the position of insulators (labeled I) and the yellow arrow represents the Hsp70 promoter. ChIP primers used are represented by green boxes.(A) Activation of Wg induces preferential recruitment of TCF to the *notum* enhancer. We also observed a small increase of TCF at other locations and the importance of this increase has not been determined. (B and C) Wg induces widespread AcH3 (B) and AcH4 (C) across the reporter construct. Notice that the increase is blocked by insulator as ChIP with the primers sets outside the insulator failed to detect increased AcH3/AcH4. Error bars indicate standard deviation from ChIP duplicates.

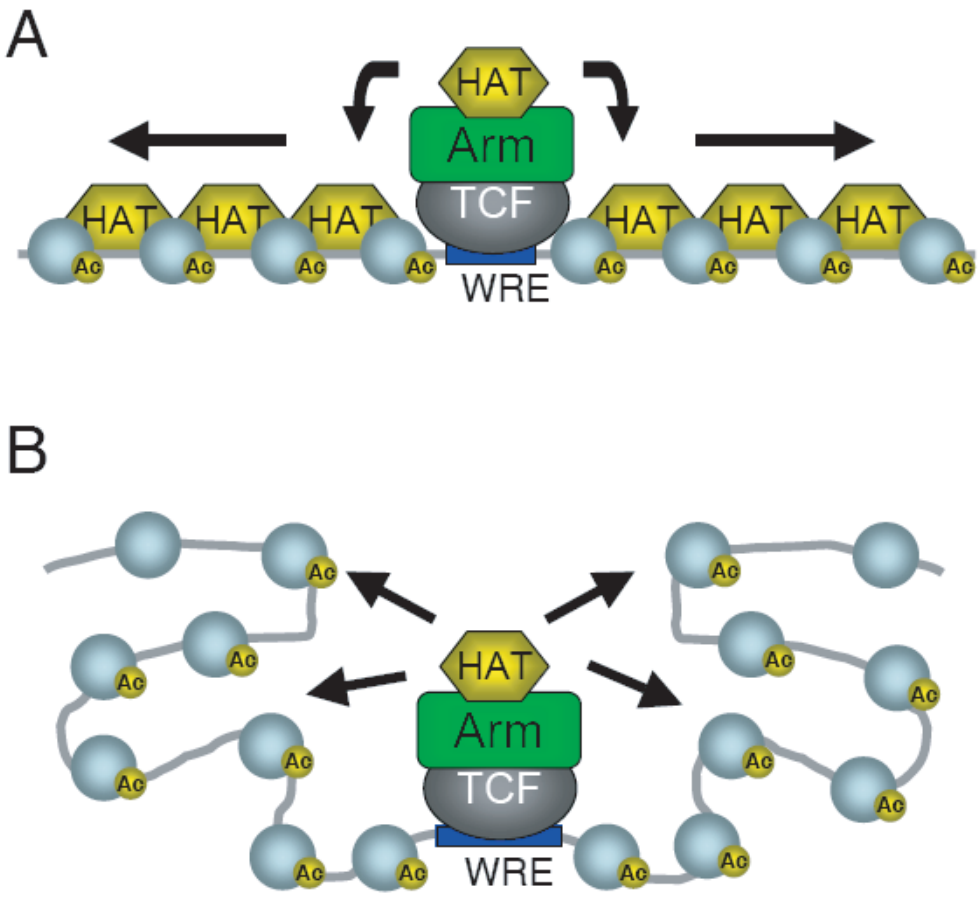


Fig 2-13 Possible models for the Wg dependant widespread histone acetylation. (A) Spreading model. In this model, the localzied HAT such as CBP recruits additional HATs to its surroundings which in turn recruits more HATs. This process propagates histone modifications across the locus. (B) The looping model. The localized activation complex induces chromosome loops which brought distant chromatin to the vicinity of HAT. The distant chromatin is acetylated through this mechanism. Figure adapted from Dave Parker.

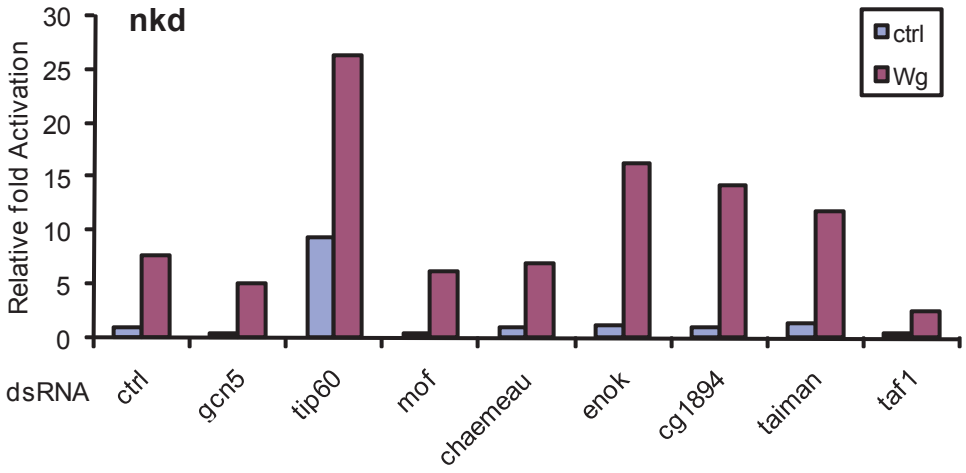


Fig 2-14 Depletion of HATs other than CBP does not cause a significant defect in nkd transcription activation. Cells were treated with the indicated dsRNA for 6 days before Wg was added. Relative transcript levels of *nkd* were shown as normalized to the transcript levels of beta-tubulin. Except for the loss of Taf1 which causes slight decrease of the activation, the loss of other HATs do not affect the activation of *nkd*. Data are represented as mean of PCR duplicates. Abbreviations: Males absent on the first (Mof), enoki mushroom (Enok), TBP-associated factor 1 (Taf1).

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Chapter III

Wg targets silencing by PcG proteins

Abstract

The Wnt/Wingless (Wg) pathway plays important roles in development and disease. How chromatin modification and remodeling is regulated in this pathway is an understudied aspect of gene regulation by Wg. In chapter II, I discussed the role of the active histone marks AcH3/AcH4 in the Wg target regulation. In chapter III, I will discuss the role of a well known repressor complex, Polycomb group (PcG) proteins, and H3K27me3 in the Wg silencing. In cells depleted of PcG subunits, the transcription of Wg target genes *naked cuticle* (*nkd*) and *homothorax* (*hth*) is elevated. PcG complexes are also needed to repress *hth* in developing fly wings. The depletion of both PcG members and TCF shows an additive effect compared with single knockdown of either suggesting that the repression by PcG complexes is in parallel with the repression mediated by the Wg transcription factor TCF. Consistent with the parallel repression model, the binding of PcG member Enancer of Zeste (E(z)) and that of TCF is not interdependent. Although the levels of H3K27me3 on *nkd* and *hth* clearly depend on PcG, the regulation of H3K27me3 by Wg is more complicated. I will also discuss the

involvement of H3K27 demethylase UTX, H2A ubiquitilation and H3K27 dimethylation in the Wg pathway in this chapter.

Introduction

The Wnt/Wg signaling pathway is required throughout the animal kingdom for a wide variety of developmental processes (Cadigan and Nusse 1997; Logan and Nusse 2004; Grigoryan et al. 2008; Holstein 2008). This pathway revolves around the stability and subcellular localization of □-catenin, which is known as Armadillo (Arm) in *Drosophila*. In the absence of signaling, □-catenin/Arm is constitutively targeted for proteosome degradation by a phosphorylation-ubiquitination cascade. These events are mediated by a destruction complex consisting of the scaffolds adenomatous polyposis coli (APC) protein and Axin, the protein kinases GSK3 and CKI and the E3 ubiquitin ligase TrBP/Slimb (Cadigan and Peifer 2009; Kennell and Cadigan 2009). Upon activation of Wg/Wnt signaling, the activity of the destruction complex is compromised, allowing hypophosphorylated □-catenin/Arm to accumulate and translocate into the nucleus (MacDonald et al. 2009).

Once in the nucleus, □-catenin/Arm can interact with several transcription factors, most notably members of the TCF family (Parker et al. 2007). In *Drosophila*, a single TCF (sometimes called Pangolin) is thought to mediate the vast majority, perhaps all of Wg signaling (Brunner et al. 1997; van de Wetering et al. 1997). In the absence of Wg signal, TCF represses target gene expression (Bienz 1998; Cavallo et al. 1998; Schweizer et al. 2003; Fang et al. 2006). Several factors participate with TCF to silence Wg targets, including the co-repressors Groucho and CtBP (Bienz 1998; Cavallo et al. 1998;

Schweizer et al. 2003; Fang et al. 2006) and the Brahma and ACF chromatin remodeling complexes (Collins and Treisman 2000; Liu et al. 2008). Arm binding to TCF either displaces (Daniels and Weis 2002; Liu et al. 2008) or somehow inactivates these repressive factors. In addition, Arm recruits several transcriptional co-activators to Wg targets, promoting activation of target gene expression. These factors are thought to form a TCF transcriptional complex that turns on target gene expression (Parker et al. 2007; (Mosimann et al. 2009).

The transcriptional switch of TCF is also known to act at the level of chromatin modifications of Wg target loci. Wg signaling causes dramatic increases in Histone 3 and Histone 4 acetylation (H3Ac and H4Ac) and H3K4 trimethylation (H3K4me3) at a Wg target, *nkd* (Parker et al. 2008). The Wg induced increase in H3K4me3 was localized at the promoter and was dependent on transcription, while the increase in H3 and H4 occurred throughout the entire target gene (Parker et al. 2008). This widespread histone acetylation may be required to counteract repressive chromatin modifications that are also broadly located across Wg target gene loci, although such a repressive chromatin mark has not been reported.

One potential candidate for a chromatin modification contributing to Wg target gene silencing is H3K27 trimethylation (H3K27me3). This histone modification is mediated by a methytransferase called Enhancer of zeste E(z) (Czermin et al. 2002; Muller et al. 2002). E(z) belongs to a class of proteins collectively known as Polycomb group (PcG) proteins. PcG proteins are found in several distinct protein complexes that mediate transcriptional repression (Muller and Verrijzer 2009; Simon and Kingston 2009). E(z) is a subunit in a complex known as PRC2, which is responsible for mono (H3K27me)

and dimethylation (H3K27me2) of H3K27 (Nekrasov et al. 2007). PRC2 can also be bound by Polycomblike (Pcl), forming the Pcl-PRC2 complex, which is required for H3K27me3 in flies (Nekrasov et al. 2007). Some studies indicate that H3K27me and H3K27me2 are found on 50% of the histone H3 in flies (Ebert et al. 2004) but its role in transcription repression is not clear. In constrast, H3K27me3 is highly correlated with gene silencing in plants, invertebrates and vertebrates (Schwartz and Pirrotta 2007; Hennig and Derkacheva 2009; Schuettengruber and Cavalli 2009).

In addition to the PRC2 complexes containing the E(z) methyltransferase, several PcG proteins exist in a complex known as PRC1 (Shao et al. 1999; Francis et al. 2001; Mohd-Sarip et al. 2005). One of the mechanisms by which PRC1 contributes to gene silencing is through the histone H2A ubiquitylase activity of the Sce/Ring subunit (Cao et al. 2005; Lagarou et al. 2008) . Distinct from this activity, PRC1 can cause chromatin compaction in vitro, which could contribute to transcriptional silencing (Francis et al. 2004). PRC1 and PRC2 are often thought to act together to achieve transcriptional repression, though a recent study in the mouse indicates that they can also repress gene expression independently of each other (Leeb et al.).

PcG proteins were originally discovered in flies as being required for repression of Hox gene expression throughout development (Schwartz and Pirrotta 2007). The relationship between PcG and Hox genes initially suggested that PcG proteins were only involved in static long-term repression of gene expression (Ringrose and Paro 2004). However, more recent data suggests that PcG repression of genes can be very dynamic. For example, the binding profile of PcG members change dramatically throughout fly development (Negre et al. 2006; Kwong et al. 2008; Oktaba et al. 2008). In particular,

testis-specific genes have been shown to promote terminal differentiation of male germ cells by removing PcG proteins from target promoters (Chen et al. 2005). In mammals, genome wide mapping of PcG subunits revealed an important function of PcG protein in stem cell maintenance and PcG proteins have been found to directly repress biologically important targets in mouse and human embryonic stem cells (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). The repression by PcG is released upon differentiation which is accompanied by an induction of the target genes although the removal of PcG proteins from those genes is controversial (Lee et al. 2006; Pasini et al. 2007). However, the signals involved in regulating PcG protein in these interesting biological contexts are not known.

In this study, we tested the hypothesis that PcG proteins negatively regulate transcription of Wg target genes. We provide evidence that PcG proteins are required for silencing of the Wg targets *nkd* and *hth* in fly cell culture and the developing fly eye. The derepression observed when PcG proteins are depleted does not depend on Arm, indicating that PcG is not repressing *nkd* and *hth* expression through inhibition of TCF-Arm complex formation. Both the PRC1 and PRC2 complexes are required for repression of these Wg targets. High levels of E(z)-dependent H3K27me3 are widely distributed (40-120 kB) across both genes in the absence of Wg signaling. These data demonstrate the importance of PcG proteins in maintaining repression of target genes in the absence of Wg signaling. They also provide a model for how a developmental signaling pathway can influence PcG activity in a dynamic manner.

Material and Methods

Cell culture

Kc cells were grown in the Drosophila Schneider media with 10%FBS. S2 cells stably transformed with a tubulin-Wg construct were used to produce Wg-conditioned medium (WCM). S2-Wg cells were grown in the presence of hygromycin until a concentration of at least 6 million/ml was reached. Cells were then precipitated and medium was removed. Cells were then resuspended in fresh medium with no hygromycin without dilution and allowed to grow for another 4-6 days. After palleting and discarding the cells, the conditioned medium was filtered and concentrated (optional) before storage in the -80c freezer.

dsRNA was added directly to the culture medium at the concentration of $10ug/10^6$ cells. 4 days of addition of the dsRNA, cells were diluted 1:4 and allowed to grow for another 2-3 days before harvesting for assay.

Fly Genetics and immunostaining

For clonal analysis in fly eye discs, ywFLP/Y;GFP2A/TM6 were crossed with $E(z)^{731}$2A/TM6 in which $E(z)^{731}$ is a molecularly characterized E(z) null allele (Muller et al. 2002). The progeny were heat shocked and 72 hours later female non-tubby larvae or pupae were collected and eye imaginal discs were dissected and stained with anti-*Hth*. The anti-*Hth* antibody was a gift from Dr. Richard Mann.

Chromatin Immunoprecipitation

About 3×10^7 KC cells were cross linked with 1% final concentration of formaldehyde for 20 minutes at room temperature. After washing in PBS, cells were

lysed and sonicated for 3 pulses of 10 seconds at the power output of 4 on a sonicator (Fisher scientific Model 100). 3×10^6 cells were used for each pulldown and ChIP was performed according to the Millipore ChIP protocol. Antibodies used in this study include: anti-TCF generated by the Cadigan lab (Fang et al. 2006), H3K4me3 (Millipore 07-473), H3K27me3 (Millipore 07-449), H3K27me2 (Millipore 07-421 or Cell Signaling 9728), H2Aub (Millipore 05-678) and anti-E(z) which was generously provided by Dr. Richard Jones. Immunoprecipitates were analysed with quantitative PCR.

RNA extraction and RT-qPCR

RNA was prepared with trizol (Invitrogen) according to the manufacturer's instructions. cDNA was made by reverse transcription using reverse transcriptase from Invitrogen (Superscript RT) and oligo(dT) as primers. Quantitative PCR was performed using iCycler IQ real-time detection system (Bio-Rad).

Data analysis

Individual treatments in each experiment were performed in duplicate. Some experiments were repeated 2-13 times. Some duplicate data points are presented as is (n=2). Other data are represented as the combination of multiple duplicate experiments (n=3 or more). In the latter cases, data points from all control cells are normalized to 1 and the mean of relative fold differences from control cells in multiple independent experiments is represented. The relative fold differences were then log transformed and statistically significant differences were determined with a p-value less than 0.05 based on the transformed numbers using Student's t-test (unpaired) with Microsoft Excel.

Results

PcG complex represses the transcription of *nkd*

nkd is one of the best studied Wg direct targets in flies. In order to study the function of PcG complex in the Wg pathway transcription regulation, we used RNAi to knock down PcG complex member E(z) in fly KC cells and then measured the *nkd* transcript levels by RT-qPCR. When E(z) is depleted alone, we observed a slight derepression of the *nkd* transcription (1.8 fold compared with control cells), similar to when the transcription factor TCF is knocked down. Interestingly, when E(z) was knocked down together with TCF, we saw a further derepression (7.5 fold, Fig 3-1B). This collaborative behavior has been observed for several other repressors of the Wg pathway in KC cells (Fang et al. 2006; Liu et al. 2008).

Genome wide mapping of PcG complexes in flies have identified *wg* as a target gene, so the derepression of *nkd* in cells depleted of PcG complexes could be an indirect effect of elevated Wg expression. Furthermore, as opposed to direct repressors of the Wg targets, a class of Wg antagonist functions as Wg buffers by interrupting the Arm-TCF interaction (reviewed in Parker et al. , 2007). In order to differentiate from these indirect repression mechanisms, cells were incubated with Arm RNAi together with E(z)/TCF RNAi. No loss of derepression was seen with Arm RNAi. In contrast, Arm knockdown causes a slight but statistically significant increase of the derepression of *nkd* (from 4 fold derepression in E(z)/TCF depleted cells to 6 fold derepression in E(z)/TCF/Arm depleted cells). On the other hand, the Axin RNAi induced activation of *nkd* is highly dependant on Arm (Fig 3-1C).

As a general repressor, PcG complex may repress a lot of genes (Bracken et al. 2006; Schwartz et al. 2006; Squazzo et al. 2006; Tolhuis et al. 2006). To rule out the possibility that PcG may repress the whole *nkd* genomic region, rather than repressing *nkd* in a Wg specific way, we examined transcription of two genes (*mkp3* and CG18135) next to *nkd* in the cells where PcG subunits were knocked down. Figure 3-1D and 3-1E show that the transcription of these two genes was not significantly affected by the RNAi treatments.

E(z) directly represses *nkd* transcription by maintaining high levels of H3K27me3

As our lab has shown before, TCF binds to the *nkd* Wg response element (WRE at 5.4 kb downstream of the transcription start site, Fig 3-2 A and B). When E(z) is depleted with dsRNA, the binding of TCF on *nkd* is not decreased, and on the contrary a slight increase at the WRE by 10% compared with control cells was observed. This increase may be an indirect result of changed chromatin structure in E(z) depleted cells (Fig 3-1B).

E(z) is a histone methyltransferase that catalyzes all three states of methylation on the histone 3 lysine 27(ie, H3K27me1,2,3). H3K27me3 is the best characterized epigenetic mark among the three that represses transcription. We tested whether the high levels of H3K27me3 across the *nkd* region in the absence of signaling are regulated by E(z). Figure 3-2C shows that the levels of H3K27me3 are dramatically reduced to 20%-60% of the levels in control cells when cells are treated with E(z) RNAi.

E(z) directly represses *hth* transcription by maintaining high levels of H3K27me3

To extend our study of the PcG repression of the Wg targets to other genes, we examined another Wg direct target *hth*. Similarly, when E(z) was knocked down, we saw a slight derepression of 3.8 fold. We also observed an additive effect between E(z) and TCF(Fig 3-3 A) and the derepression of *hth* increases from 3.8 fold in E(z) depleted cells to 19 fold in E(z)/TCF depleted cells. The repression of *hth* by E(z)/TCF is downstream of Arm, as cells depleted of Arm can still be derepressed by E(z)/TCF knockdown (Fig 3-3 B).

TCF binds to the *hth* WRE as shown before (Fig 3-4B). TCF RNAi treatment decreases the enrichment of TCF to 10% of the control cells. Similar to *nkd*, E(z) RNAi causes an increase of TCF binding (5.5 fold) at the potential WRE of hth, probably due to the chromatin structure change as a result of E(z) depletion. Conversely, E(z) also binds to the same region of *hth* and its binding is not altered by TCF knockdown (Fig 3-4C).

We tested whether H3K27me3 levels are regulated by PcG on *hth*. H3K27me3 levels are high across the whole *hth* region and when E(z) was depleted, its levels were reduced across the whole region(Fig 3-4D). The amplitude of change is between 0-5 fold.

hth is regulated by Wg signaling in multiple fly tissues (Zirin and Mann 2004; Benchabane et al. 2008). We wanted to test whether PcG also negatively regulates *hth* in vivo. E(z) mitotic clones were induced in fly eye imaginal disks with $E(z)^{731}$ allele at two developmental stages (larval and 30 hours after pupation). E(z) clones which are patches of cells that are homozygous E(z) mutants are marked by the lack of GFP signal (Fig 3-5B,E). *hth* expression is elevated inside the E(z) clones in both stages(Fig3-5A,D).

Are PcG proteins displaced from the chromatin by Wg activation?

Although PcG is a well documented repressor, how the repression is relieved is less understood, probably due to the long-time belief that PcG complexes are long-term repressors (Ringrose and Paro 2004). Recent research especially in the stem cell field completely reversed this belief and established PcG proteins as dynamic repressors. A group of differentiation genes are thought to be repressed by PcG in ES cells (Boyer et al. 2006; Bracken et al. 2006; Lee et al. 2006). Surprisingly however, the activation of these developmental regulators during differentiation is not accompanied by the loss of PcG proteins or H3K27me3 on these targets (Pasini et al. 2007). Furthermore, another study in flies show that Pol II recruitment and transcription can happen even when Pc still binds and H3K27me3 levels are still high on the target genes (Schwartz et al.). These results put the idea that PcG binding and H3K27me3 are mutually exclusive with transcription activation in doubt. In fact, when Wg is activated, we also observed that the binding of E(z) is not removed (Fig3-6). Although the transcription of *hth* which is activated by *axin* dsRNA is much stronger than the derepression caused by E(z) dsRNA (Fig2-6 B, 38 fold with *axin* dsRNA compared with 5.6 fold with *E(z)* dsRNA), the E(z) binding is only slightly reduced by 20% by *axin* dsRNA whereas E(z) dsRNA completely abolished the E(z) binding (Fig 2-6 A).

Are H3K27me3 decreased by Wg activation?

We then asked if the levels of H3K27me3 are decreased with Wg activation. The results turn out to be quite variable. Sometimes, we observed a Wg dependant decrease of H3K27me3 across the *nkd* region as shown in Fig 3-7A. For example, at 16kb

downstream of the *nkd* TSS, H3K27me3 enrichment decreases from 0.6% to 0.15%. The fold difference of the H3K27me3 enrichment in control and E(z) depleted cells ranges from 0-4fold (Fig 3-7A). But Wg activation sometimes did not have detectable effect on H3K27me3 enrichment on *nkd* (Fig 3-7B) although the transcription activation was generally comparable in all experiments (data not shown). Fig3-7C shows a summary of all the experimental results. This result suggests that the removal/decrease of H3K27me3 is not the primary requirement for Wg target gene activation.

Depletion of Pcl has opposite effect on H3K27me3 and H3K27me2

Previous reports have shown that there is a Pcl-PRC2 complex specifically required for H3K27me3 but not H3K27me2 (Nekrasov et al. 2007). We tested whether Pcl demonstrates the same substrate specificity in our system. Fig3-8A shows that cells depleted of Pcl have much lower levels of H3K27me3 on *hth* than wild type cells. 20%-35% H3K27me3 levels were observed in Pcl depleted cells compared with control cells (Fig 3-7A). Surprisingly, when we examined H3K27me2 levels in the Pcl depleted cells, we saw a dramatic increase of H3K27me2 on *hth* (7-8 folds over control cells, Fig3-8 B). Similarly for *nkd*, loss of Pcl decreases the level of H3K27me3 while it increases the level of H3K27me2 (Fig 3-9). The increase of H3K27me2 in Pcl depleted cells could be due to of cells inability to catalyze trimethylation which results in the accumulation of H3K27me2 as a substrate. Alternatively, there may be a competition between Pcl containing PRC2 (PRC2-Pcl) which can trimethylate H3K27 and PRC2 without Pcl (PRC2) which only catalyzes the mono and di methylation. Pcl like depletion favors the formation of PRC2 and results in high levels of H3K27me2.

H3K27 acetylation is controlled by CBP

A recent report showed that CBP can mediate acetylation of H3K27 and this modification antagonizes the polycomb mediated H3K27me3 and silencing (Tie et al. 2009). Although the global levels of H3K27me3 and H3K27ac change during embryogenesis in complementary fashion, the antagonism between the two markers on endogenous Polycomb targets is more subtle (Tie et al. 2009). Similar antagonism has also been reported in ES cells and the level of H3K27me3 and H3K27ac is regulated oppositely during differentiation (Pasini et al.). Whether similar antagonism also contributes to dynamic transcription regulation in a signaling pathway is not known. Previously, our lab has shown that CBP is required for Wg activation as well as Wg dependant increase of histone acetylation on *nkd* (Li et al. 2007; Parker et al. 2008) and I confirmed the positive role of CBP on *hth* in this study (Fig 3-10 B). *hth* is activated 11 folds by *axin* dsRNA and the activation is decreased significantly to 4.2 folds when CBP is depleted together with Axin. H3K27ac on *nkd* is also controlled by CBP as CBP depletion results in a decrease of H3K27ac on *nkd* (Fig3-11 C) both in the absence (ctrl dsRNA) and in the presence (*axin* dsRNA) of Wg activation. The decrease of H3K27ac is accompanied by a subtle but statistically significant increase of H3K27me3 (Fig3-11 B). H3K27me3 levels are increased by 1.4-1.7 folds when CBP is depleted. It is also interesting to note that H3K27ac levels are not significantly regulated by Wg activation (Fig3-11 C) although in the same cells AcH3 is dramatically increased with *axin* dsRNA (Fig3-11 A).

Discussion

Widespread K27me and widespread acetylations

We have previously shown that Wg activation induces widespread acetylation of H3 and H4 across two Wg targets *nkd* and *notum* when the pathway is turned on. This phenomenon is surprising because acetylated histones are believed to localize to the promoter regions (Wang et al. 2008). One possible explanation is that there is a widespread repressive mark across the Wg targets, so that in order for the transcription to proceed, the repressive marks have to be removed and the widespread acetylated histones are one of such mechanisms.

In this study, we reported that methylated H3 lysine 27 plays a negative role in the Wg pathway regulation. Consistent with the previous reports showing that H3K27me3 occupies a large chromatin domain (Papp and Muller 2006; Schwartz et al. 2006), H3K27me3 spreads across two Wg targets (*hth* and *nkd*, Fig3-2 C and Fig3-4 D). Interestingly, the pattern of H3K27me3 on *nkd* in the absence of the signaling is roughly the mirror image of the pattern of H3Ac and H4Ac in the presence of the signaling (Fig3-2 C and (Parker et al. 2008). This relationship may reflect a situation in which H3K27me3 and acetylated histones counteract each other.

Although our results have clearly shown that polycomb silencing is important to keep Wg targets off in both KC cells and fly wing discs, it is clearly not the only repression mechanisms involved in the Wg targets repression. For example, we have shown that E(z) depletion greatly reduced the level of H3K27me3 across *nkd* and *hth* (Fig 3-2C and Fig3-4D), but this reduction is not sufficient to relieve all the repression of these two genes because *nkd* and *hth* transcription is only modestly derepressed in E(z)

depleted cells but the derepression becomes much stronger in TCF/E(z) double knockdown cells (Fig3-1B and Fig3-3 A). This additive effect between TCF and E(z) is not due to further decrease of H3K27me3, as TCF depletion does not affect H3K27me3 on its own (Fig 3-12).

Polycomb silencing also does not always need to be released upon activation. Both E(z) and H3K27me3 can be found on active Wg targets (Fig3-6 and Fig 3-7). The PcG proteins on active Wg targets may still negatively affect transcription to prevent maximum activation. Alternatively, the activity of PcG may be inhibited by the activator/co-activator complexes; therefore the physical removal of PcG and H3K27me3 may not be necessary. Similar retention of PcG and H3K27me3 on active targets has also been reported by other studies (Schwartz et al. ; Pasini et al. 2007), but the functional importance of this phenomenon is not clear.

Is UTX, the H3K27 demethylase involved in Wg activation?

The JmjC-domain proteins UTX and JMJD3 were first identified in mammals as H3K27 demethylases (Swigut and Wysocka 2007). One homolog in flies, dUTX, also possesses H3K27 demethylase activity (Smith et al. 2008). Whether dUTX participates in transcription activation is unknown. We tested whether the activation of Wg targets require dUTX in cell culture. Fig3-13 shows that while the activation of *nkd* by *axin* dsRNA is not consistently affected by dUTX depletion, the activation of another Wg target *notum* is reproducibly lowered by dUTX knockdown. However, when we tested whether the H3K27me3 levels at the *notum* gene are upregulated in dUTX depleted cells, we did not see any upregulation (data not shown). We also attempted dUTX ChIP to test

whether dUTX binds to *nkd* or *notum* and was unable to show significant binding (data not shown). So far, our data suggests that dUTX might play a role in the activation of *notum*, but the directness of this regulation is in question.

Comparable derepression by different PcG subunits

Although different biochemical properties have been linked with different PcG subunits (Muller and Verrijzer 2009; Simon and Kingston 2009), dissection of subunit-specific roles in transcription regulation remains understudied. For example, the relationship between the ubiquitylation mediated repression by dRing/PRC1/dRAF and the methylation mediated repression by E(z)/PRC2 is unclear. A recent study suggests that PRC1 and PRC2 complexes are redundant in stem cell differentiation (Leeb et al.). In our study, we found that the derepression caused by the single knockdown of E(z) or dRing alone is not significantly lower than derepression caused by double knockdown of E(z) and dRing, suggesting that PRC1 and PRC2 do not act in parallel in our system (data not shown).

The role of H3K27me2 in transcription regulation

Previous studies show that the binding profile of H3K27me3 is very different from that of H3K27me2 (Ebert et al. 2004). H3K27me2 has a broader pattern occupying about 50% of the genome (Peters et al. 2003; Ebert et al. 2004) compared with the more localized pattern of H3K27me3. It is not clear whether H3K27me2 plays a role in transcription regulation. However, a recent study showed that E(z) dependant H3K27 dimethylation but not trimethylation is involved in the transcription repression of the E2F/RB pathway in flies (Lee et al.). In contrast, the role of H3K27me2 in transcription

repression has been challenged in other studies. For example, loss of Pcl in embryos causes an expansion of some polycomb targets expression but this derepression is accompanied by an increase of H3K27me2 on those targets (Nekrasov et al. 2007). Our results agree with the latter study in which Pcl loss of function derepresses *nkd* and *hth* but the levels of H3K27me2 are increased on the chromatin (Fig3-8 and Fig3-9).

Looping?

Our results as well as many others have found the broad enrichment of H3K27me3 in genomic regions where the binding of PcG proteins is more localized. In addition, the localized E(z) is absolutely needed for the widespread pattern of H3K27me3 in flies (Fig4; (Papp and Muller 2006; Schwartz et al. 2006). This is similar to the situation with CBP histone acetylation. While CBP is required for the widespread H3Ac and H4ac, it is localized only to the enhancer region (Parker et al. 2008). One possibility to explain the difference of localization is a looping mechanism where parts of the chromatin is brought together by some protein complexes and the closeness of the chromatin to the chromatin modifying enzymes make the modification possible. Analysis of the higher chromatin structure with techniques such as chromosome conformation capture (3C) (Vassetzky et al. 2009) may shed light on this issue.

Transcriptional balance maintained by H3K27me3

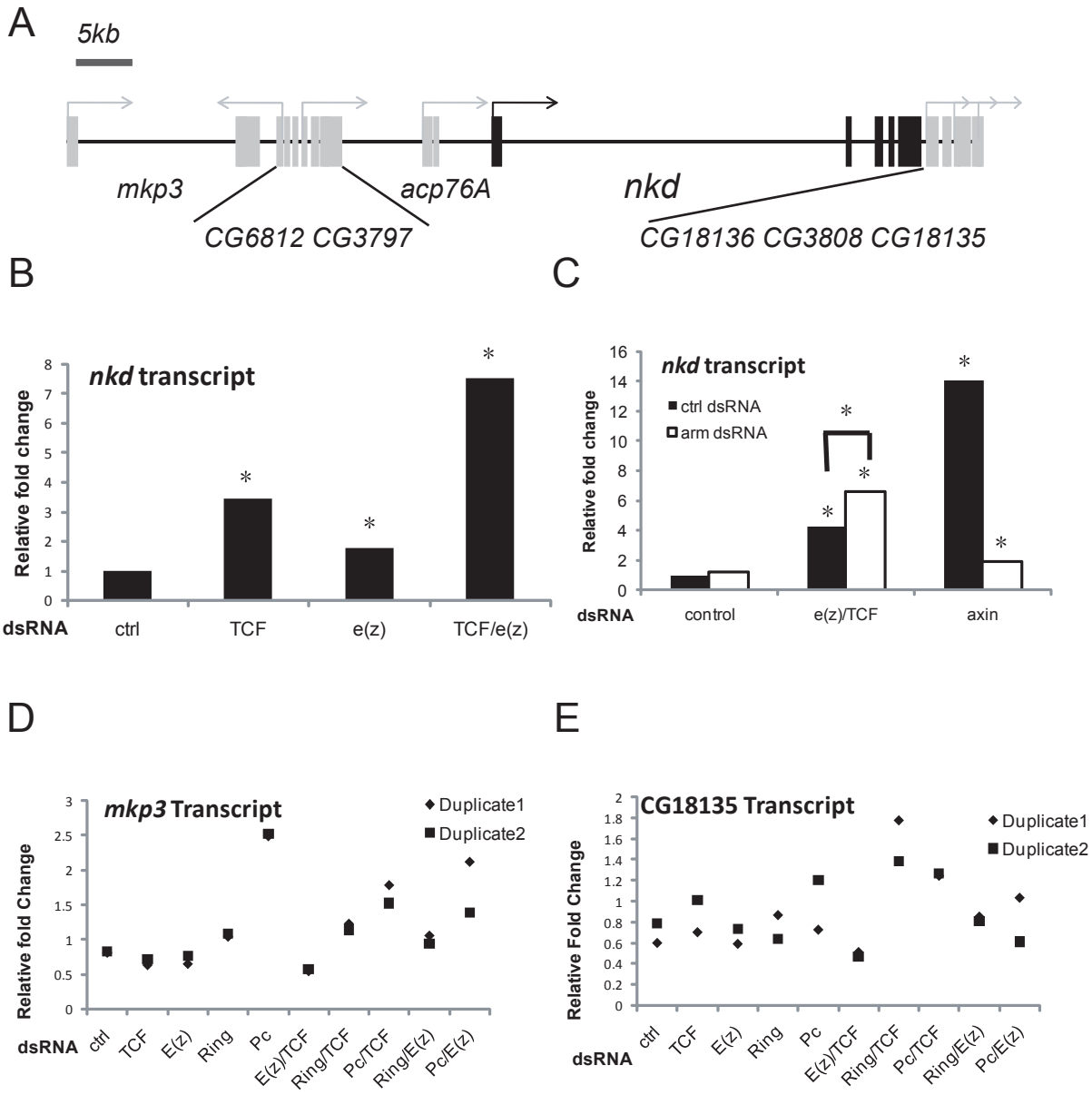
In this chapter, we have shown that H3K27me3 is required to repress Wg targets in the absence of signaling. In chapter II, increased histone acetylation is connected with Wg target gene activation. Interestingly, even when Wg targets are activated, PcG proteins and H3K27me3 are still present at Wg target genes and appear to be still

functional. Why do cells maintain the repressive mark at activated genes? One possible answer is that maximal targets activation is not always desirable for cells. It takes time to turn on or turn off the transcriptional switch and the maintenance of repressive marks at target genes may provide a strategy for cells to switch off transcription quickly when needed. So instead of a switch, transcription regulation is more like a balance which could be shifted by activators and repressors.

Is Polycomb mediated repression dynamic?

Since PcG proteins were discovered as repressors for Hox genes, they have been thought to be static epigenetic repressors, an idea based on the long-term repression of Hox genes observed. Recently, this belief has been abandoned by most researchers mainly due to following two pieces of evidence. Firstly, many developmental regulators are found to be Polycomb targets in genome wide ChIP assays (Boyer et al. 2006; Lee et al. 2006). Because the activation of these genes is necessary for development, the repression by Polycomb proteins must be reversed. Secondly, the discovery of histone demethylases capable of removing H3K27 methylation provided the biochemical mechanism for reversing transcription repression (Swigut and Wysocka 2007). Although there is strong biochemical evidence for the removal of H3K27 methylation and the reversal of Polycomb mediated silencing, whether this process happens in normal development is still unknown. I have demonstrated that Polycomb is required to repress Wg targets both in cell culture and in developing fly tissues. Since Wg regulates many targets throughout development, this finding provides a great system where the developmental regulation of Polycomb silencing can be examined.

Fig 3-1. PcG proteins act together with TCF to silence *nkd* expression in the absence of Wg signaling. A. Schematic of the *nkd* genomic region. Transcription units are represented by boxes, with the exons in black and the TSSs are indicated with arrows. B. Kc cells were treated with the indicated dsRNA for 6 days prior to RNA extraction and qRT-PCR detection of *nkd* transcript levels. *β-tub 56D* was used to normalize the data. The *nkd* transcript levels in control cells are normalized to 1 and asteriks above data points represent significant changes of transcript levels in dsRNA treated cells compared with that in control cells with $p<0.05$ and $n=4$, Student's T-test. C. Kc cells were incubated with *arm* dsRNA together with *e(z)* and *TCF* dsRNA or *axin* dsRNA and *nkd* transcripts were measured as described above. The derepression of *nkd* observed by simultaneous E(z) and TCF depletion was not affected by *arm* dsRNA. In contrast, depletion of Arm dramatically reduced the activation of *nkd* expression observed with *axin* dsRNA. Asteriks above data points indicate significant changes of *nkd* transcript levels over control cells for which the nkd transcript levels were normalized to 1. The asterik between E(z)/TCF and E(z)/TCF/Arm depleted cells indicate a significant increase of *nkd* transcript levels when Arm is depleted together with E(z)/TCF. Asteriks denote $p<0.05$ with $n=4$, Student's T-test. D & E. *mkp3* and *CG18135*, which are adjacent to *nkd*, were not significantly regulated by depletion of PcG proteins and/or TCF. Two data points from the duplicated experiments are shown.


A
5kb
mkp3
acp76A
nkd
CG6812 CG3797
CG18136 CG3808 CG18135
B
nkd transcript
Relative fold change
dsRNA
ctrl
TCF
e(z)
TCF/e(z)
C
nkd transcript
ctrl dsRNA
arm dsRNA
Relative fold change
dsRNA
control
e(z)/TCF
axin
D
mkp3 Transcript
Duplicate1
Duplicate2
Relative fold Change
dsRNA
ctrl
TCF
E(z)
Ring
Pc
E(z)/TCF
Ring/TCF
Pc/TCF
Ring/E(z)
Pc/E(z)
E
CG18135 Transcript
Duplicate1
Duplicate2
Relative Fold Change
dsRNA
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Ring/E(z)
Pc/E(z)

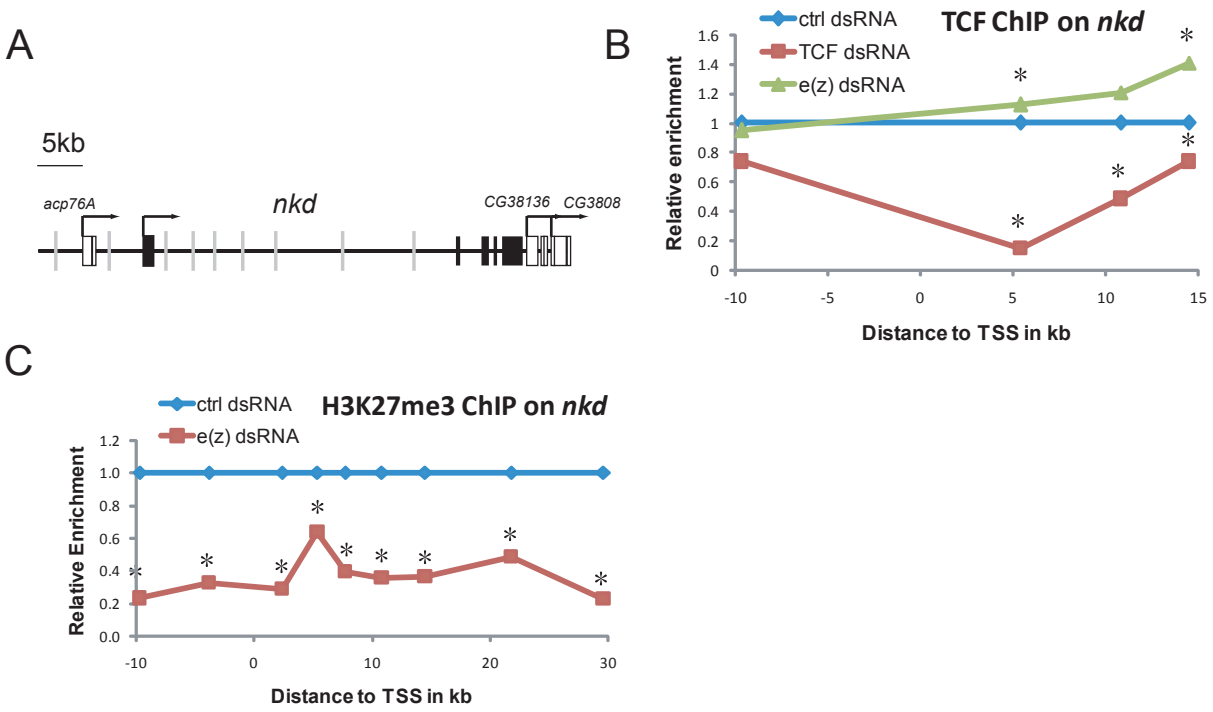


Fig. 3-2. E(z) and TCF act on different regions of the nkd locus. A. Schematic of the *nkd* locus. Black boxes indicate *nkd* exons and open boxes the exons of adjacent genes. The location of primers used in the ChIP experiments are shown as dashed lines, with the approximate position in relation to the *nkd* TSS indicated below. B. Kc cells were treated with control, *TCF* or *e(z)* dsRNA for 6 days after which ChIP using a TCF antibody was performed. TCF binding in control dsRNA treated cells were normalized to one at all locations. Asterisks above each data points represent a significant change in TCF binding in TCF or E(z) dsRNA treated cells compared with control dsRNA treated cells with $P<0.05$. (n=3) C. H3K27me3 levels in the *nkd* genomic region are regulated by E(z). Kc cells were treated with either control or e(z) dsRNA. TCF binding in control dsRNA treated cells were normalized to 1 at all locations. Asterisks above each data points represent a significant change in H3K27me3 levels in E(z) dsRNA treated cells compared with control dsRNA treated cells with $P<0.05$. (Student's T-test, n=4)

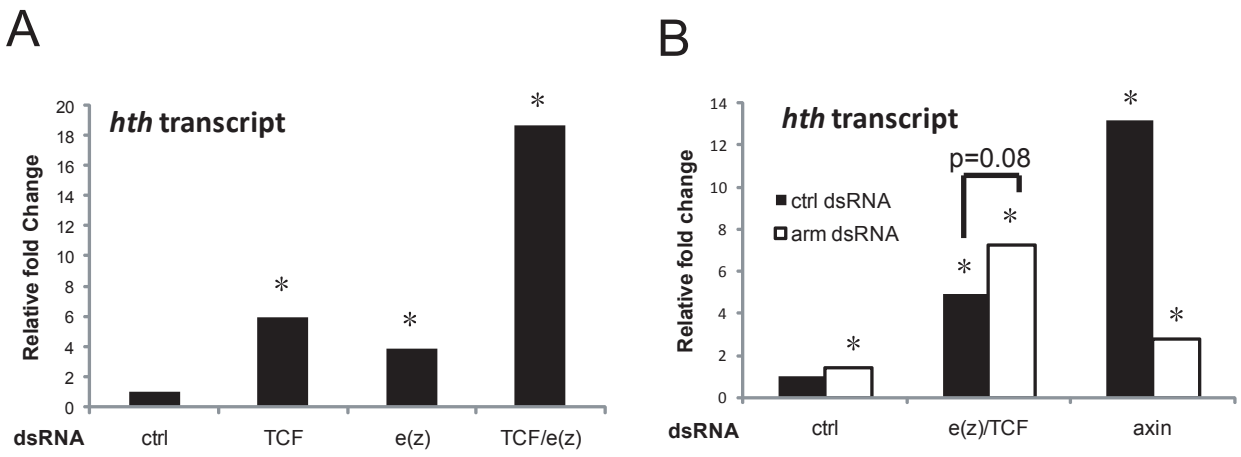


Fig 3-3. PcG proteins act together with TCF to silence *hth* expression in the absence of Wg signaling. A. Kc cells were treated with indicated dsRNA and relative transcript levels of hth were represented by normalizing to the transcript levels of *beta-tubulin*. Relative *hth* transcript levels are normalized to 1 in control cells and asterisks above data points represent significant changes compared with control cells with $p<0.05$ and $n=4$. B. Kc cells were incubated with *arm* dsRNA together with *e(z)* and *TCF* dsRNA or *axin* dsRNA and *hth* transcripts were measured as described above. The derepression of *hth* observed by simultaneous E(z) and TCF depletion was not affected by *arm* dsRNA. In contrast, depletion of Arm dramatically reduced the activation of *hth* expression observed with *axin* dsRNA. Asteriks above data points indicate significant changes of *hth* transcript levels over control cells for which the *hth* transcript levels were normalized to 1. Asteriks denote $p<0.05$ with $n=4$, Student's T-test.

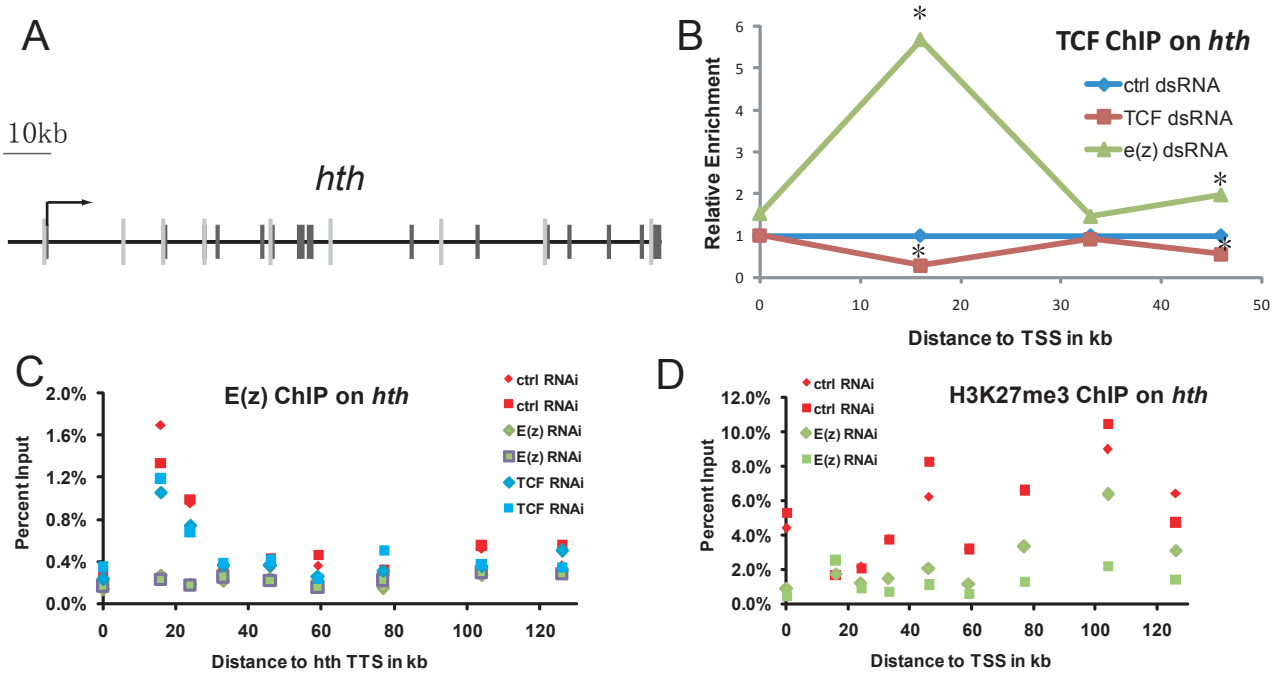


Fig. 3-4. H3K27me3 levels in the *hth* genomic region are directly regulated by E(z). A. Schematic of the *hth* genomic region. Exons are indicated in black and the location of the primers used for the ChIP assays are represented by gray bars (approximate distance from the *hth* TSS indicated below). B. Kc cells were depleted with E(z) or TCF as described in Fig. 1 and subject to ChIP analysis with antibodies against TCF. The TCF binding levels in control cells were normalized to 1 at all positions. Asterisks above the data points in TCF and E(z) depleted cells represent significant changes of the TCF binding compared with that in control cells with $P<0.05$ and $n=3$ (Student's T-test). C. E(z) is enriched at the *hth* region and its binding is TCF independant. Data points from duplicate experiments are shwon. D. Kc cells were depleted for E(z) and analyzed for H3K27me3 association. Data points from duplicate experiments are shown.

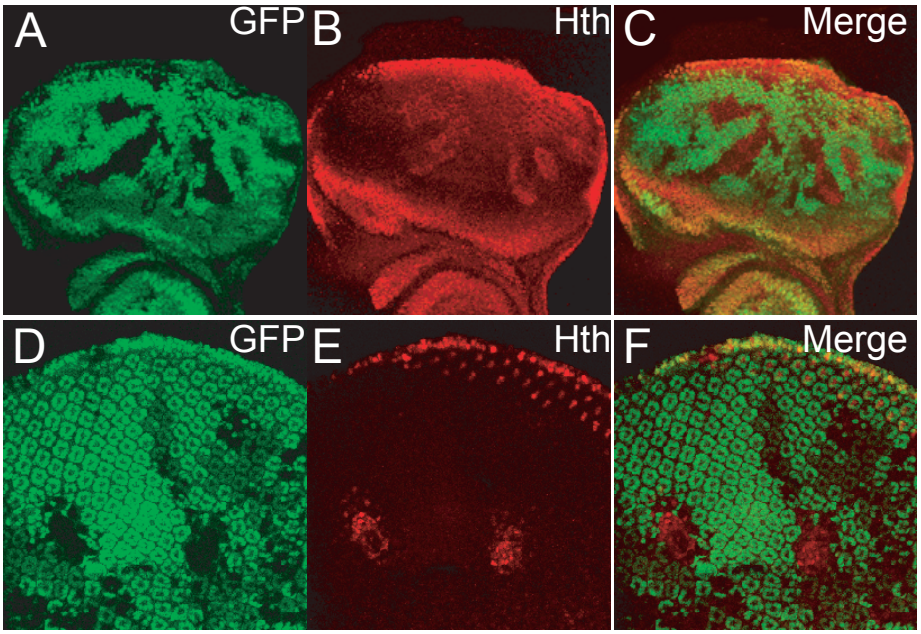
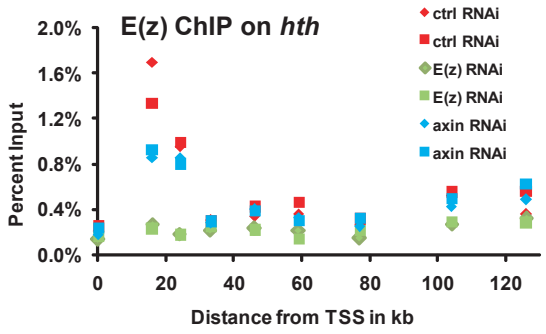


Fig 3-5. E(z) represses *hth* expression in the developing fly eye. Mitotic clones homozygous for the mutant allele E(z)731 were induced by heat shock. Larval eye-antennal imaginal discs (A-C) and mid-pupal eyes (D-F; 30 hr APF) were stained with Hth antibody (A, D). The boundary of the clones is marked with GFP (B, E).

A



B

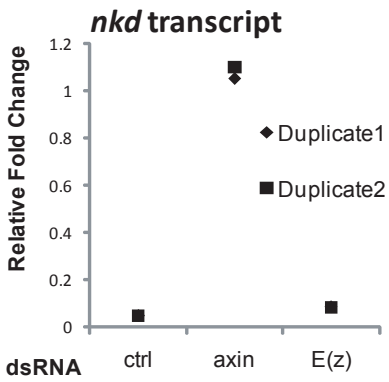
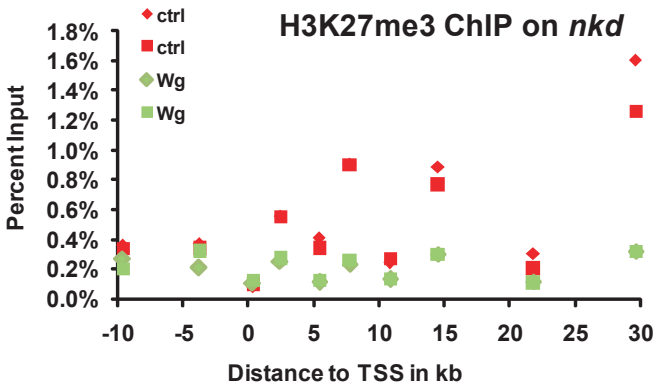


Fig 3-6. Wg activation does not displace E(z) from *hth*. (A) Cells were treated with indicated dsRNA. While *e(z)* dsRNA completely abolished E(z) binding to *hth*, showing that the ChIP signal is real, the *axin* dsRNA treatment does not significantly decrease the E(z) binding to *hth*. (B) *hth* transcription is greatly activated by *axin* dsRNA and *e(z)* dsRNA causes a slight depression of *hth*. Data points from duplicate experiments are shown.

A



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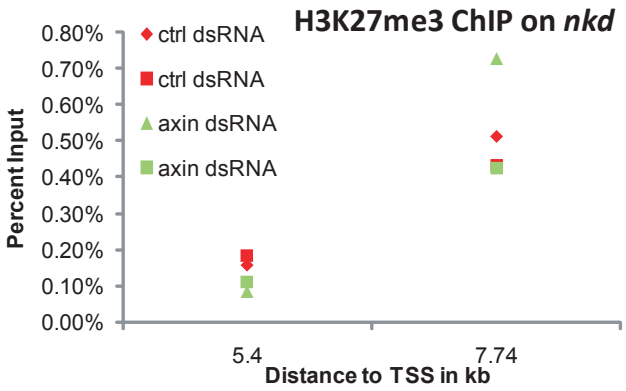
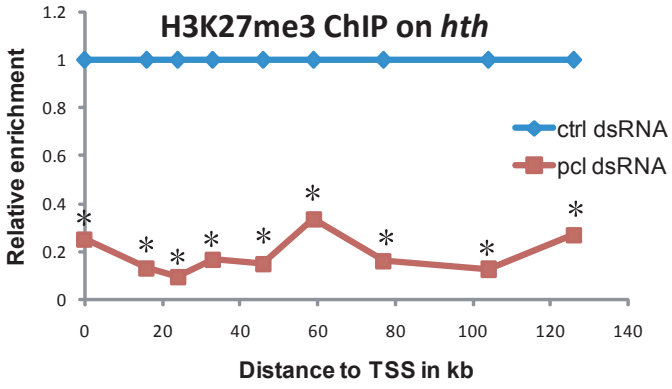


C

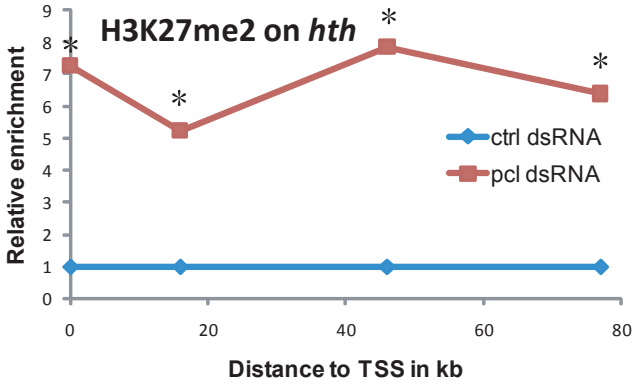
	Activated by axin dsRNA	Activated by WCM	Total
Widespread decrease of H3K27me3	4	2	6
Localized/ partial decrease of H3K27me3	3	0	3
No decrease of H3K27me3	4	2	6

Fig 3-7 Variable results regarding the removal of H3K27me3 from *nkd* upon Wg activation. (A) Example of Wg dependant widespread decrease of H3K27me3 on *nkd*. Kc cells were treated with control or axin dsRNA and the H3K27me3 levels on nkd were measured. Data points in duplicate experiments are shown. (B) Example of unchanged H3K27me3 levels on *nkd* with Wg activation. Data points in duplicate experiments are shwon. (C) Summary of all results. Experimental results are divided into 3 categories. Numbers represent the number of experimental results that belong in each category.

A



B



C

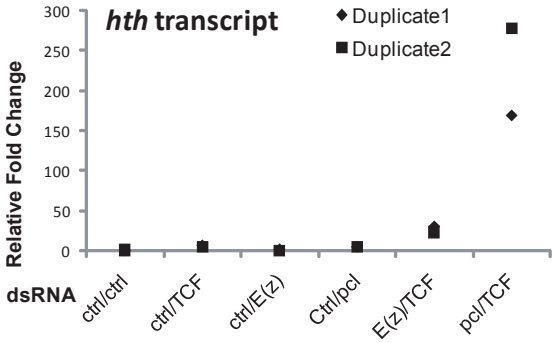
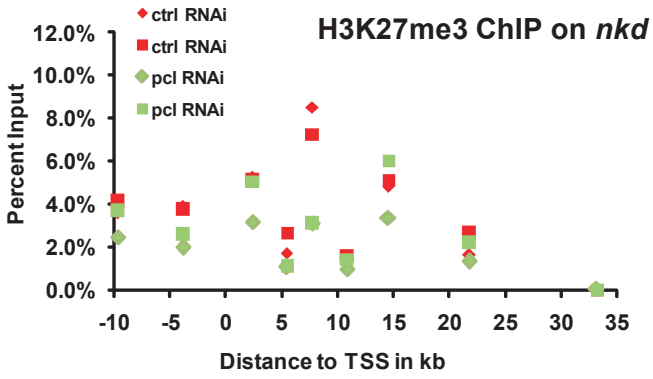


Fig3-8 Pcl depletion has opposite effects on H3K27me3 and H3K27me2. (A) Pcl depletion causes a decrease of H3K27me3 levels on *hth*. H3K27me3 levels in control cells are normalized to 1 and asteriks above data points represent significant changes in H3K27me3 levels compared with control cells with $p<0.05$ and $n=3$. (B) Loss of Pcl results in a dramatic increase of H3K27me2 on *hth*. H3K27me3 levels in control cells are normalized to 1 and asteriks above data points represent significant changes in H3K27me3 levels compared with control cells with $p<0.05$ and $n=4$. (C) Pcl depletion derepresses *hth* transcription, synergistically with TCF. Data points from duplicate experiments are shown.

A



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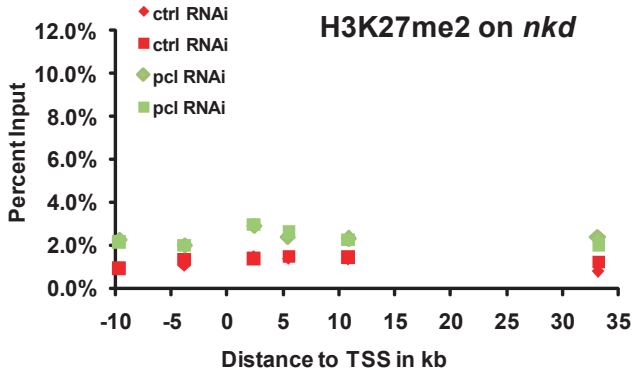

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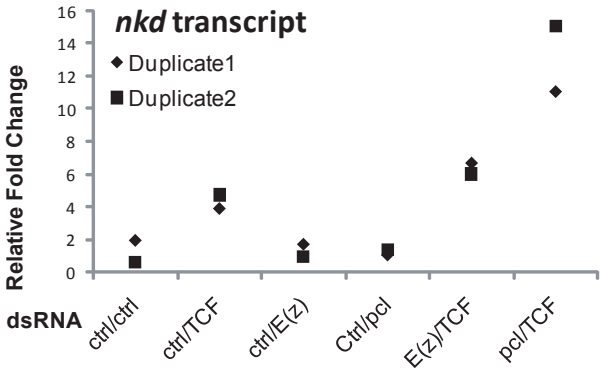


Fig 3-9 Pcl depletion has opposite effects on H3K27me3 and H3K27me2. (A) Pcl depletion causes a decrease of H3K27me3 levels on *nkd.* (B) Loss of Pcl results in an increase of H3K27me2 on *nkd*. (C) Simultaneous knockdown of Pcl and TCF depresses *nkd* transcription more dramatically than single knockdown of each. Data points from duplicate experiments are shown.

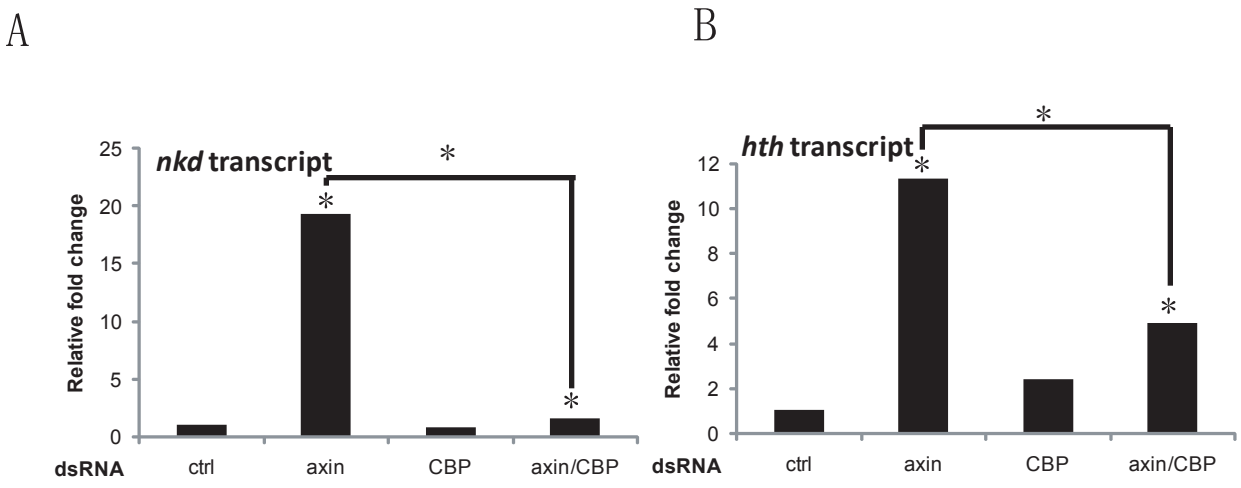
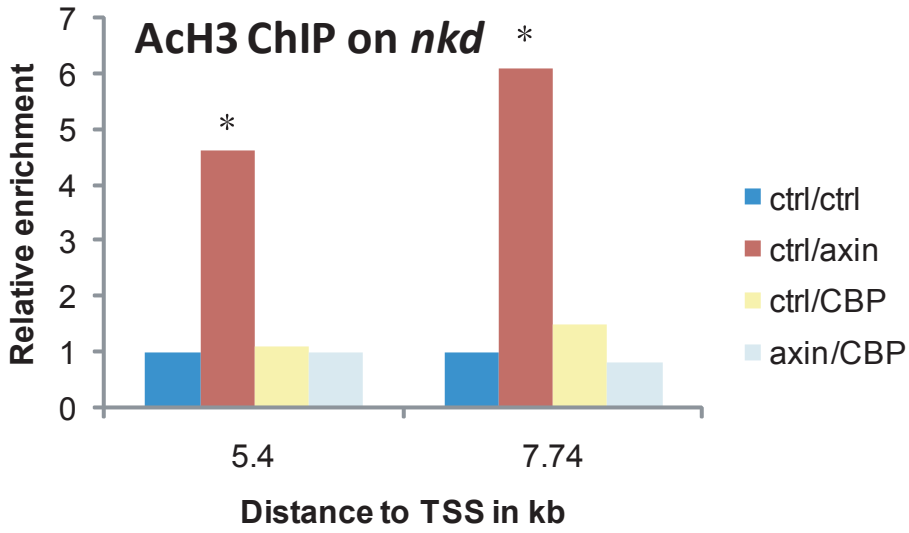


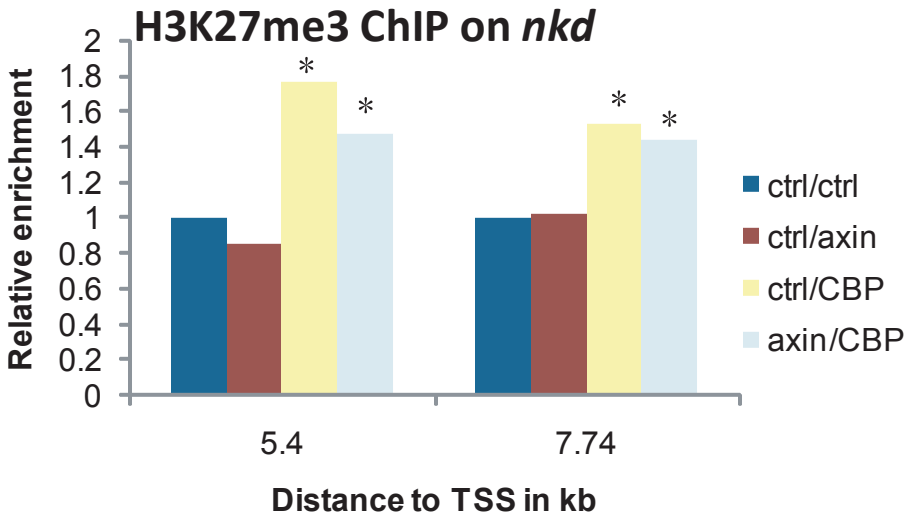
Fig 3-10 CBP is required for the activation of *nkd* and *hth.* The relative transcript levels of nkd and hth are calculated by normalizing to the transcript levels of *beta-tubulin*. Relative transcript levels of *nkd* and *hth* are then normalized to 1 in control dsRNA treated cells. Asteriks indicate significant changes in transcript levels compared with control cells with $p<0.05$ and $n=3$. In addition, there are significant differences in transcript levels of *nkd* and *hth* between Axin and Axin/CBP depleted cells.

Fig3-11 Relationship of H3K27me3 and CBP mediated H3K27ac. (A) Wg activation by *axin* dsRNA causes increased recruitment of AcH3 to *nkd* and CBP depletion completely abolishes the increase. Asteriks denote significant changes in AcH3 enrichment levels compared with control cells in which the AcH3 enrichment levels are normalized to 1. $p<0.05$ and $n=4$. (B) H3K27me3 enrichment is slightly increased with CBP depletion. Asteriks denote significant changes in H3K27me3 enrichment levels compared with control cells in which the H3K27me3 enrichment levels are normalized to 1. $p<0.05$ and $n=4$. (C) CBP regulates H3K27ac both in the absence and presence of the Wg signaling.While activation of the Wg signaling by *axin* dsRNA has no detectable effect on H3H27ac levels, *CBP* dsRNA decreases the H3K27ac both in the absence and in the presence of the Wg signaling. Data points from duplicate experiments are shown.

A


AcH3 ChIP on nkd
Relative enrichment
0
1
2
3
4
5
6
7
*
*
5.4
7.74
Distance to TSS in kb
ctrl/ctrl
ctrl/axin
ctrl/CBP
axin/CBP

B


H3K27me3 ChIP on nkd
Relative enrichment
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
*
*
*
*
5.4
7.74
Distance to TSS in kb
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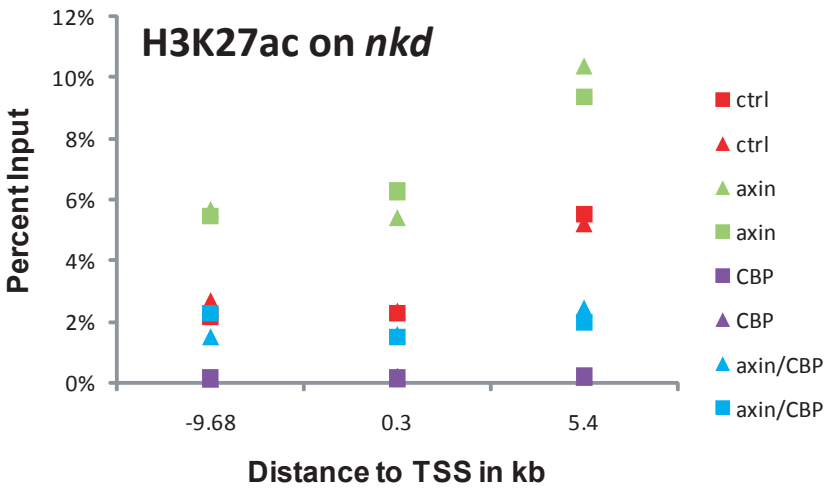
ctrl/axin
ctrl/CBP
axin/CBP

C


H3K27ac on nkd
Percent Input
0%
2%
4%
6%
8%
10%
12%
-9.68
0.3
5.4
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axin
axin
CBP
CBP
axin/CBP
axin/CBP

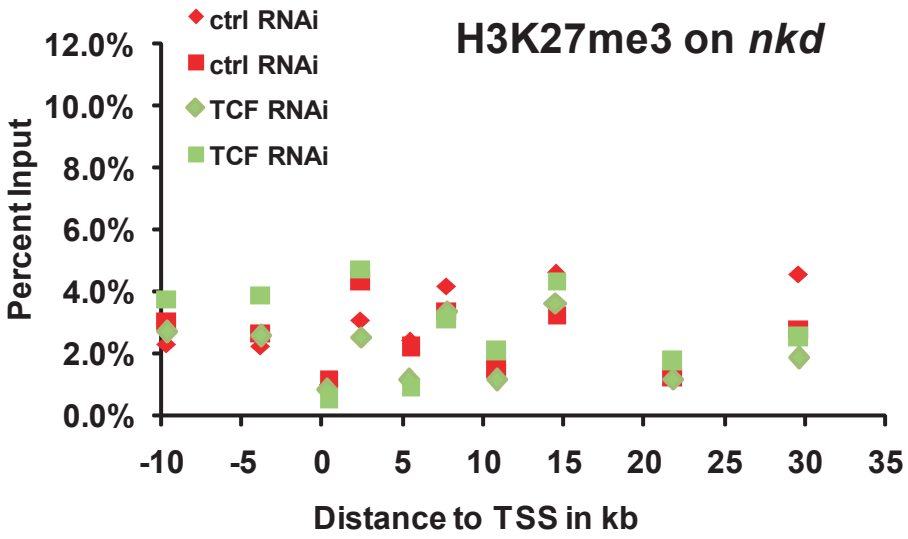
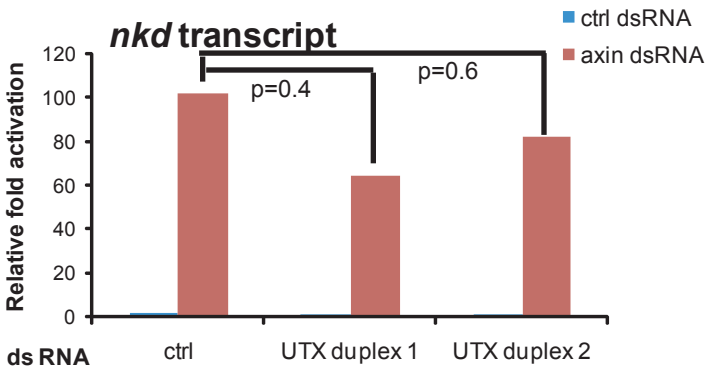


Fig 3-12 TCF depletion does not affect H3K27me3 enrichment levels on *nkd*. Cells are treated with control or TCF dsRNA and the enrichment of H3K27me3 is represented as percent input. Data points from duplicate experiments are shown.

A



B

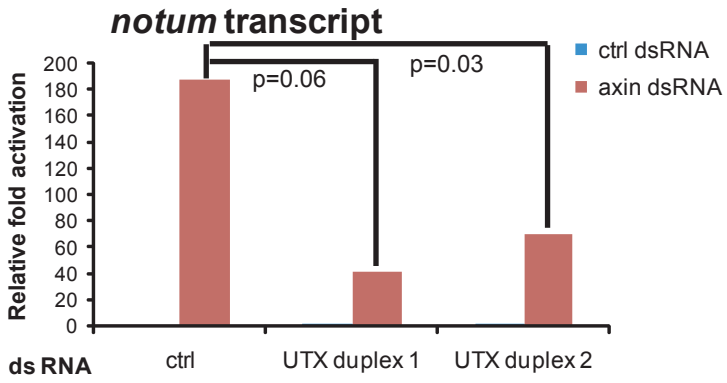


Fig 3-13 dUTX is required for the activation of *notum* but not *nkd.* Cells are treated with indicated dsRNA and the relative fold change of *nkd* and *notum* is represented. *nkd* and *notum* relative transcript levels in control cells are normalized to 1. P values comparing the *nkd* and *notum* transcript levels in Axin and Axin/UTX depleted cells are shown. n=3.

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Chatper IV
Possible dual roles of the Brm complexes in the Wg pathway regulation

Abstract

Transcription is regulated on multiple levels. At the level of chromatin, post-translational modification of histones and ATP-dependant alteration of histone-DNA interactions can both remodel the structure of chromatin. In the previous two chapters, I discussed the role of active and repressive histone modifications in the Wg pathway regulation. In this chapter, I will discuss the potential dual function of the ATP-dependant chromatin remodeler Brahma (Brm) complexes in the Wg regulation. dsRNA depletion of Brm complexes subunits in Kc cells caused either derepression or loss of activation of Wg targets. This phenomenon could be explained by different chromatin environment of different targets. Furthermore, the different activities of the Brm complexes could be explained by different subunit composition. Before further investigation of the mechanism of this dual function, we have to confirm that both the repression and the activation are direct. I will discuss our attempts in this regard in this chapter.

Introduction

The packaging of eukaryotic DNA into chromatin serves as an important mechanism in transcription regulation. Tight DNA-histone interactions generally prevent transcription. Therefore, cells have evolved two major mechanisms to regulate the DNA-histone interaction: post translational modifications of histones and ATP-dependent chromatin remodeling. ATP-dependant chromatin remodeling is crucial for both the assembly and dissolution of chromatin, and is therefore capable of positively or negatively regulating transcription (Ho and Crabtree). ATP-dependant chromatin remodelers are grouped based on their sequence and the structure of the ATPase subunit. The Swi/Snf type of chromatin remodeler is the best studied class. Brm or BAF complexes belong to the Swi/Snf family (Trotter and Archer 2008) with Brm in flies or Brg1 in mammals as the ATPase subunit. Fly Brm was first identified as a gene related to the yeast transcription activator Snf2/Swi2 (Tamkun et al. 1992).

As is the case in mammals, fly Brm has been reported in multiple complexes differing in subunit composition (Mohrmann et al. 2004). At least two complexes exist in flies that contain Brm and they share most of their subunits with only subtle differences (Fig4-1). The BAP complex contains Osa while the PBAP complex contains Polybromo(PB) and BAP170 as its unique subunits. Polytene chromosome staining showed that Osa and PB occupy distinct but overlapping regions (Mohrmann et al. 2004; Mohrmann and Verrijzer 2005). Similar BAP and PBAP complexes have also been identified in mammals as well as yeast (Trotter and Archer 2008).

Mammalian Brahma-related gene 1 (Brg1) is also found to be associated with transcription factors and histone modifying enzymes. Those associated proteins may

modulate the biochemical function of the Brg1 core components to either activate or repress transcription. For example, the histone arginine methyltransferase CARM1 is found to be in the same complex with Brg1 and this association plays a role in the activation of estrogen receptor targets, probably through histone arginine methylation (Xu et al. 2004). Brg1 has also been shown to interact with histone deacetylases and a corepressor KAP-1 to form a repression complex (Underhill et al. 2000).

Brm/Brg1 and its associated proteins have been linked with Wnt/Wg signaling. Osa has been reported to genetically interact with multiple Wingless components in flies (Collins and Treisman 2000). For example, overexpression of a constitutively active Arm in Wing discs caused a misexpression of a potential Wg target *nub* and a disc morphological change. Coexpression of Osa with the active Arm restore the normal disc morphology and *nub* expression. Furthermore, Osa mutant results in the expansion of the expression of a mid gut enhancer of ultrabithorax (UbxB), the normal expression of which depends on both Wg and Dpp signaling (Thuringer et al. 1993; Collins and Treisman 2000) in embryos. The expansion of UbxB reporter expression in Osa mutants does not depend on TCF as the same expansion is seen even when dominant negative TCF is expressed. Mutating one of the Wg response element also does not abolish the expansion in Osa mutants, suggesting that Osa does not directly function on the affected Wg response elements (Collins and Treisman 2000). The repressive function of Osa in the Wg pathway was also observed in adult wings and larval eye discs in this report and two other subunits Brm and Mor also seems to participate in the repressive function. Although this paper presented convincing genetic evidence showing a repressive function of the Brm complex in flies, it is not demonstrated whether the regulation is direct.

Mammalian Brg1 on the other hand has been linked with Wnt activation through interactions with β-catenin (Barker et al. 2001). Overexpression of Brg1 activates the Wnt responsive *siamois* reporter in a β-catenin dependant manner and dominant negative Brg1 represses the transcription of endogenous Wnt targets in a colorectal cancer cell line (Barker et al. 2001).

The two reports discussed above presented contradicting results in the Brm/Brg1 function in Wnt/Wg pathway. The difference could be due to different organisms used in the two studies. Alternatively, Brm/Brg1 complex could have two different functions in the same organism in a context dependant manner. This chapter will examine whether Brm complexes both positively and negatively regulate Wg targets and whether the regulation is direct.

Material and Methods

Cell culture, RNA prep, Q-PCR, Chromatin immunoprecipitation and data analysis were performed as described in chapter III. Anti-Osa antibody was from Developmental Studies Hybridoma Bank.

Co-immunoprecipitation was performed using the Nuclear Complex Co-IP Kit from Active Motif (54001).

Results

Several targets respond to Wg activation in KC cells

In Kc cells, Wg can be activated by adding Wg conditioned media or knocking down the negative regulator Axin by dsRNA. Fig4-2A shows that Axin depletion robustly actives the transcription of *nkd*, *dfz3*, *crm* and *notum* 20-200 folds. Among the four genes, our lab has previously identified *nkd*, *dfz3* and *notum* as direct Wg targets.

Fig4-2B shows that TCF is preferentially recruited to the potential or identified Wingless response elements in *nkd*, *dfz3* and *notum* compared with a control region. The enrichment of TCF at the WREs is between 2-7 folds over control regions.

nkd and _crm_ are repressed by Brm complex in the absence of the signaling

In the attempt to find chromatin remodelers involved in the Wg pathway regulation, I did a small scale dsRNA screen (data not shown) and Brm depletion displayed an interesting phenotype. When cells are depleted of Brm complex members, the *nkd* transcript is highly elevated with Brm or Osa depletion with the amplitude of 60 and 30 folds respectively (Fig 4-3A). The level of derepression is comparable with the activation achieved by *axin* dsRNA (Fig4-2A). Brm depletion also derepressed the *crm* transcription by 8.5 folds, but *osa* dsRNA did not cause a significant change (Fig4-3B).

If the repression function of Brm is upstream of Arm/TCF interaction or an indirect effect, the derepression by Brm depletion would be Arm dependent. To test this, we performed the depression assay in the presence of *arm* dsRNA. In contrast to the Wg induced activation of *nkd* and *crm*, which is highly dependent on Arm, the derepression of *nkd* and *crm* by Brm depletion is not affected by Arm knockdown (Fig4-4).

Brm complex subunits play complicated roles in the presence of the signaling

We first tested whether Brm complexes continue to repress *nkd* and *crm* when the signaling is turned on. For *nkd*, compared with control cells activated by *axin* dsRNA treatment, the activation is 2.5-3 folds stronger in cells depleted of Brm or Osa (Fig4-5A) suggesting that Brm and Osa may continue to repress *nkd* even when the signal is on to prevent maximum activation, thus fine tuning the *nkd* expression. In the case of *crm*, Brm depletion enhanced the *crm* activation by *axin* dsRNA by 5 folds. But surprisingly, Osa

knockdown decreased the activation strength (Fig4-5B) to a level significantly lower than control cells, which may be an indirect effect.

Brm complex is required for the activation of *dfz3* and *notum*

In contrast to the repression function of Brm on *nkd* and *crm*, the dominant function of Brm for the other two targets *dfz3* and *notum* is activation. *axin* dsRNA treatment results in an 18 fold activation of *dfz3* transcription but the activation is reduced to less than 5 fold when Brm or Osa is depleted. Polybromo (PB) depletion caused a more modest effect on *dfz3* activation and the statistical significance of this effect has not been tested (Fig4-6 A). Similarly, *notum* transcription is 220 fold activated by *axin* dsRNA and this strong activation is completely abolished in Brm or Osa depleted cells. PB depletion also results in a less severe activation defect which could be due to inefficient knockdown.

Discussion

We have demonstrated that Brm complex both positively and negatively regulates Wg induced transcription in a target specific manner. In the absence of the Wg stimulation, loss of Brm caused an increase in the transcript of *nkd* and *notum* (Fig4-3). At the same time, Brm depletion almost abolished the Wg induced activation of *dfz3* and *notum* (Fig4-6). We made several attempts to investigate the directness of the regulation of Wg targets by Brm complexes. I will discuss them below.

Does Osa bind to *nkd* region?

We have shown that the derepression of *nkd* and *crm* by Brm is Arm independent which suggests that the repression is likely direct. To formally test this, we performed ChIP with antibody against Osa, the unique subunit of BAP complex (Fig4-1). Our

preliminary results suggest that Osa binds to *nkd* WRE and the binding is abolished with *osa* dsRNA (data not shown).

Possible physical interaction between Brm and Arm

To test whether the activation function of Brm complexes is direct, we ask whether Osa also binds to activated targets. However, we failed to detect Osa binding on *dfz3* whose activation by Wg is Brm dependant. At the time of the study, anti-Osa was the only available antibody among all Brm complex subunits. As an alternative, we overexpressed Flag tagged full length Brm and attempted ChIP with Flag antibody. Highly inconsistent results were obtained probably due to low and variable transfection efficiency (data not shown).

Mammalian Brg-1 has been shown to directly interact with β-catenin (Barker et al. 2001) and promote Wnt pathway activation. We want to test whether the same interaction exists between fly Brm and Arm which can support a direct activation by Brm. To this end, we used Flag antibody to pulldown the over-expressed Flag-Brm in KC cells and examined whether the over-expressed active Arm (Arm*) was in the precipitate. Fig4-7 shows that Flag antibody can efficiently pull down Flag-Brm and Arm is also slightly enriched in the precipitates where Flag-Brm is expressed (compare Arm blot in lane 1 and lane2).

If Brm can directly activate as well as repress Wg targets, it is interesting to speculate what the mechanism is. One appealing model is that the different functions of Brm are contributed by two Brm containing complexes, BAP and PBAP (Fig4-1, (Mohrmann et al. 2004). For example, *nkd* is derepressed by the loss of Brm or Osa

whereas the depletion of PB has no effect (Fig4-3A). Osa is the unique subunit of BAP and PB is the unique subunit of PBAP, so this result is consistent with BAP being required for *nkd* repression. Interestingly, although the loss of the PBAP specific unit PB has no effect on the *nkd* transcription alone both in the absence and presence of the signaling (Fig4-3A and Fig4-5A), double depletion of the two unique subunits of PBAP caused an activation defect of *nkd* (Fig 4-8). This data suggests that BAP is required for *nkd* repression and PBAP is required for *nkd* activation. How BAP and PBAP are selectively recruited to *nkd* when the signal is off and on respectively needs further investigation.

Although dsRNA provides a convenient way to knockdown the desired gene in Kc cells, it also has the caveat of being inefficient which complicates the interpretation of some results. For example, in the absence of Wg, *crm* is derepressed by Brm depletion (Fig4-3B), but this derepression is not seen with either *osa* or *PB* dsRNA, making it difficult to assign the repression of *crm* to BAP or PBAP. The lack of phenotype with *osa* or *PB* dsRNA may have a true biological implication, or it could simply be a result of insufficient knockdown.

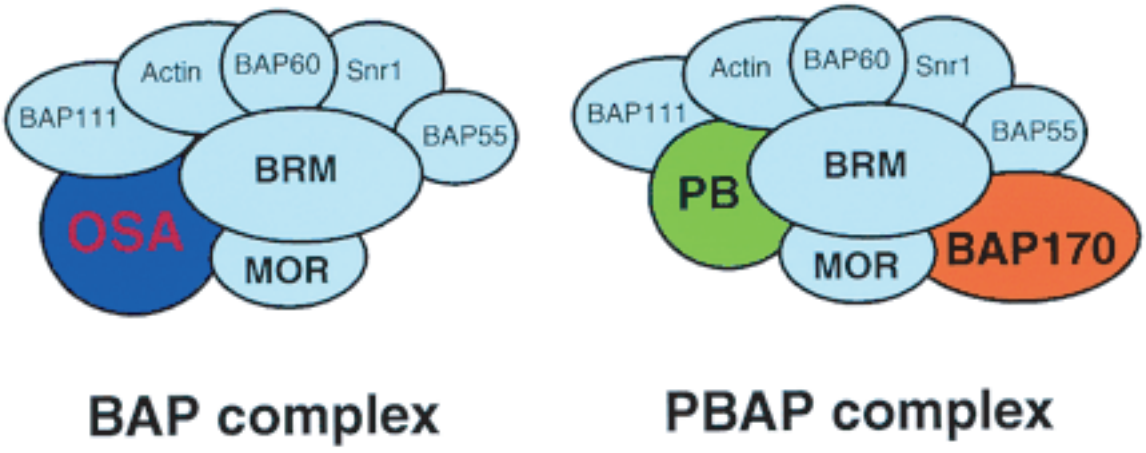
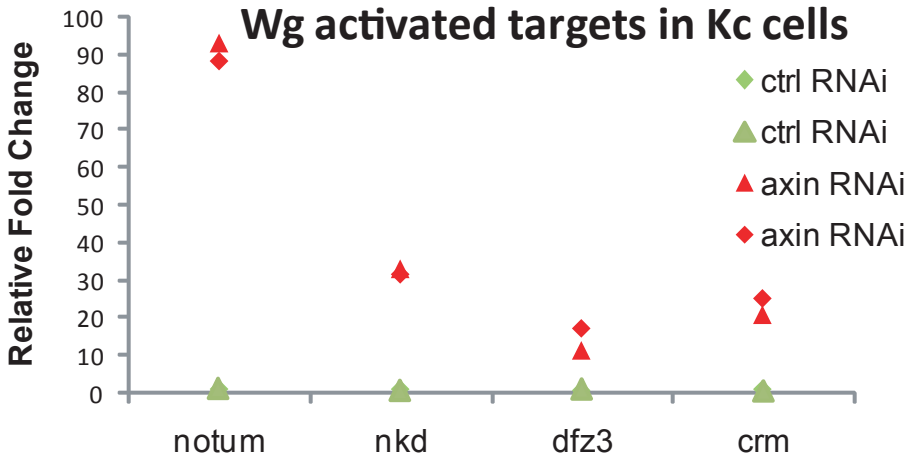


Fig 4-1 Distinct fly Brm complexes. The two Brm complexes share most of their subunits. The BAP complex is characterized by containing Osa and the PBAP contains Polybromo(PB) and BAP170 as its unique subunits. Figure adapted from Mohrmann et al., 2004.

A



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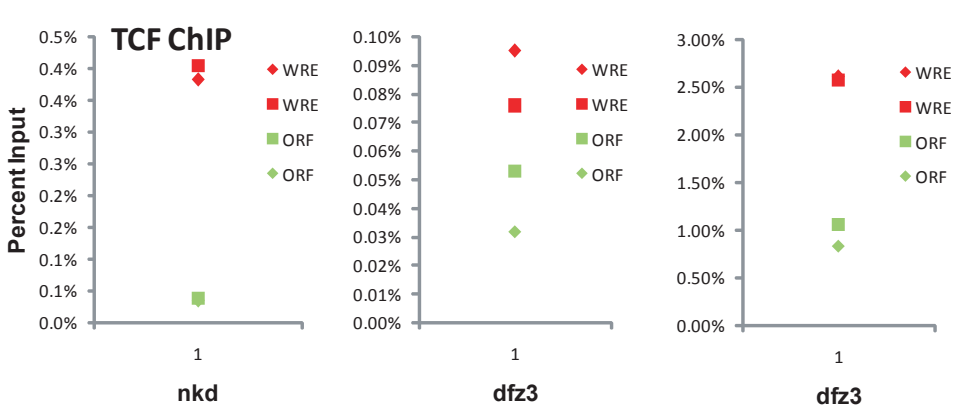
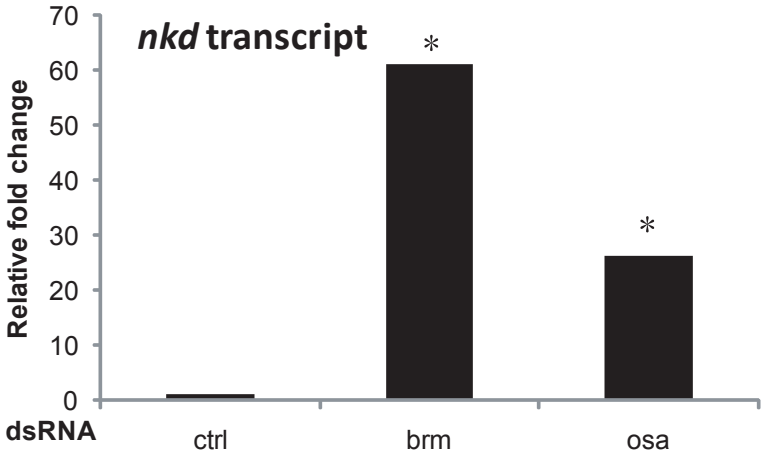


Fig 4-2 Wg activated targets in Kc cells. (A) Cells were treated with control or *axin* dsRNA and the relative transcript levels of the indicated genes are measured. *beta-tubulin56D* is used as normalization. (B) TCF ChIP on indicated genes. Compare the TCF recruitment to WRE and a control region of *nkd, dFz3 and notum*. Data points from duplicate experiments are shown.

A



B

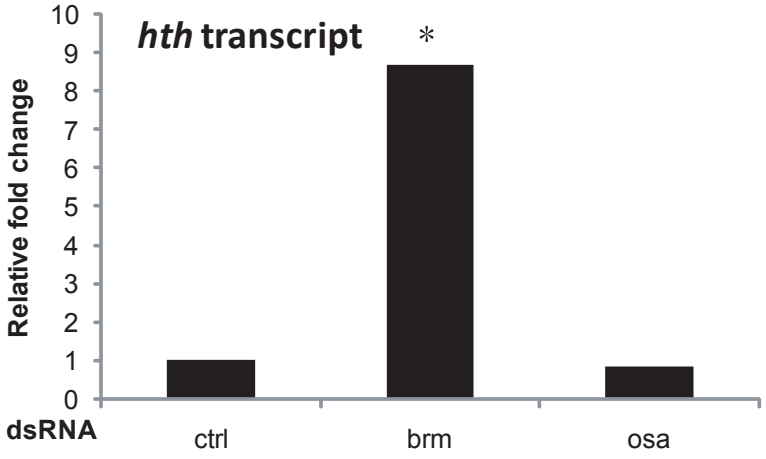
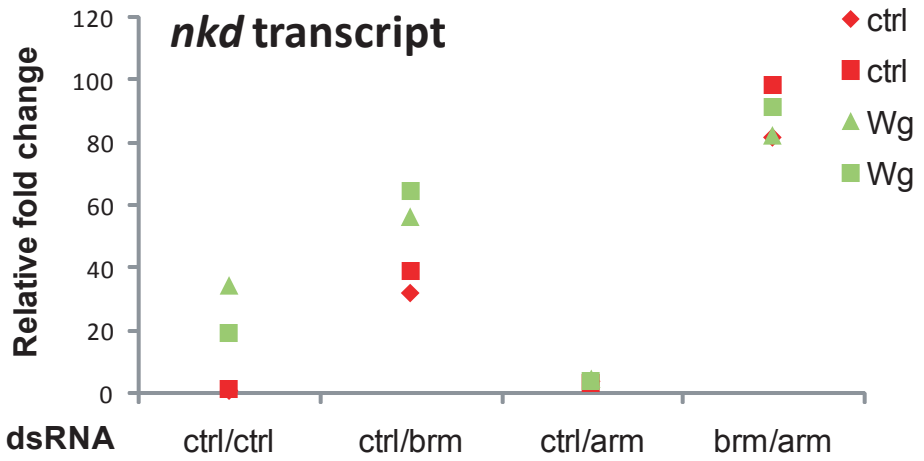


Fig4-3 *nkd* and *crm* are repressed by Brm complexes. (A) Cells were treated with the indicated dsRNA and the relative transcript levels of *nkd* were measured. Brm and Osa depletion greatly increased the *nkd* transcript levels. Asteriks represent significant changes in the transcript level compared with control cells with $p<0.05$ and $n=4$. (B) Brm depletion derepressed *crm* transcription. Asteriks represent significant changes in the transcript level compared with control cells with $p<0.05$ and $n=4$.

A



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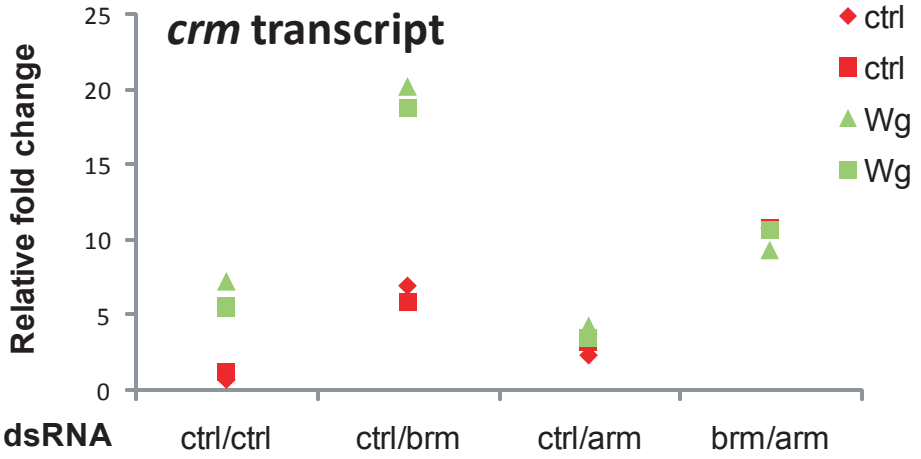
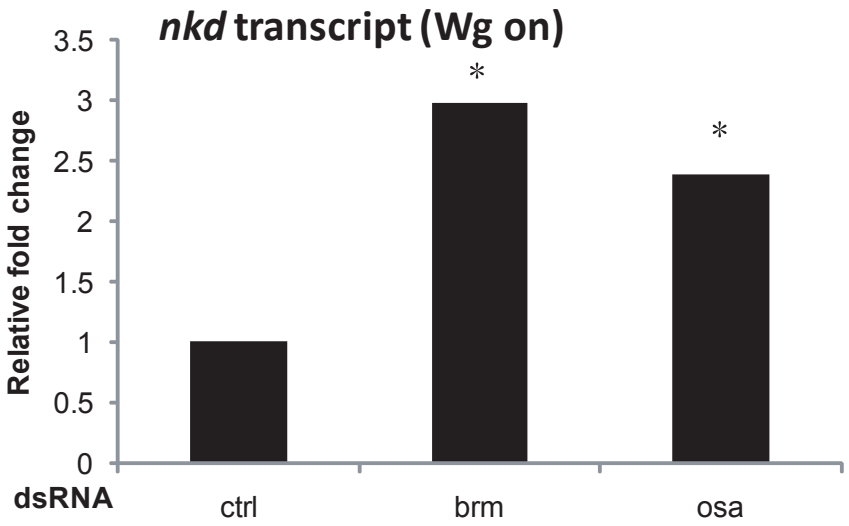


Fig4-4 The derepression of *nkd* and *crm* by Brm depletion is Arm independant. While the activation by Wg of *nkd*(A) and *crm*(B) is abolished when Arm is depleted, the derepression by *brm* dsRNA is not affected by loss of Arm. Data points from duplicate experiments are shown.

A



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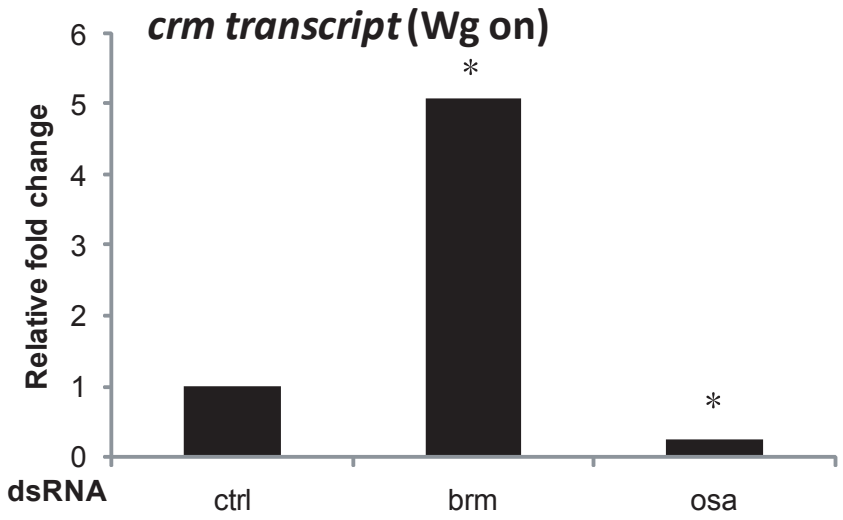
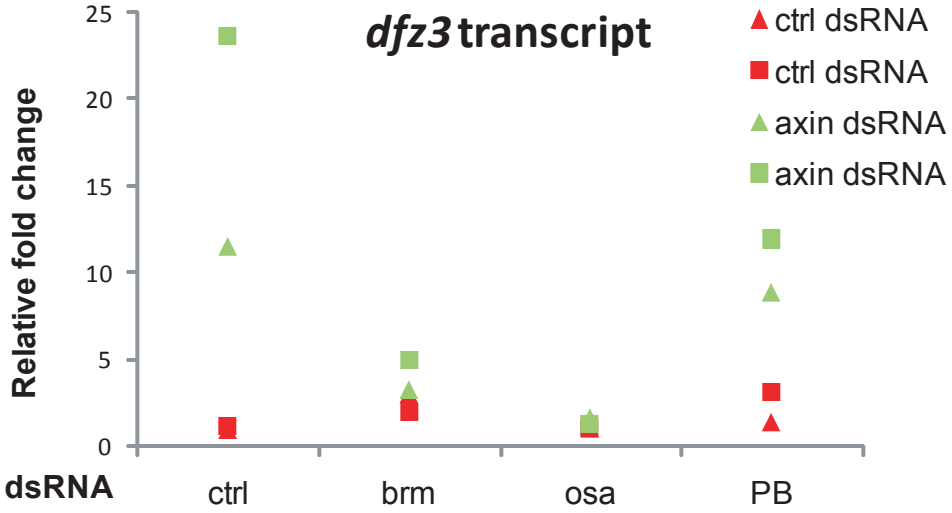


Fig4-5 The role of Brm complex in the activation of *nkd* and *crm*. All cells were treated with *axin* dsRNA together with the indicated dsRNA. (A) Depletion of Brm or Osa enhanced the activation of *nkd*. Asteriks indicate significant changes in *nkd* transcript levels compared with control dsRNA treated cells with $p<0.05$ and $n=4$. (B) Depletion of Brm further potentiated the activation of *crm* but Osa knockdown caused an activation defect. Asteriks indicate significant changes in *nkd* transcript levels compared with control dsRNA treated cells with $p<0.05$ and $n=4$.

A



B

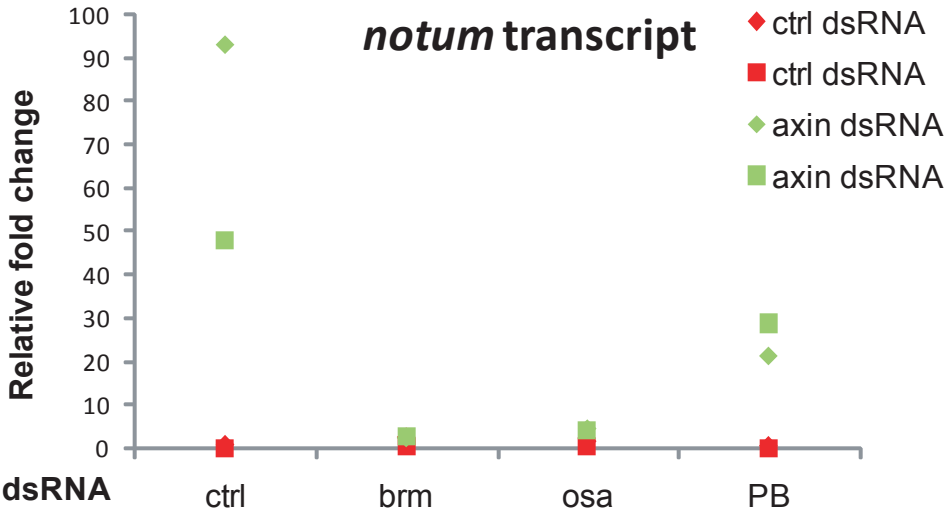


Fig4-6 Wg dependant activation of *dfz3* and *notum* requires Brm complex. The Wg pathway was activated by axin dsRNA(red columns) and indicated Brm complex subunits were depleted with dsRNA. The transcription activation of *dfz3*(A) and *notum*(B) is abolished with Brm or Osa depletion whereas the PB depletion has a more subtle effect on activation. Data points from duplicate experiments are shown.

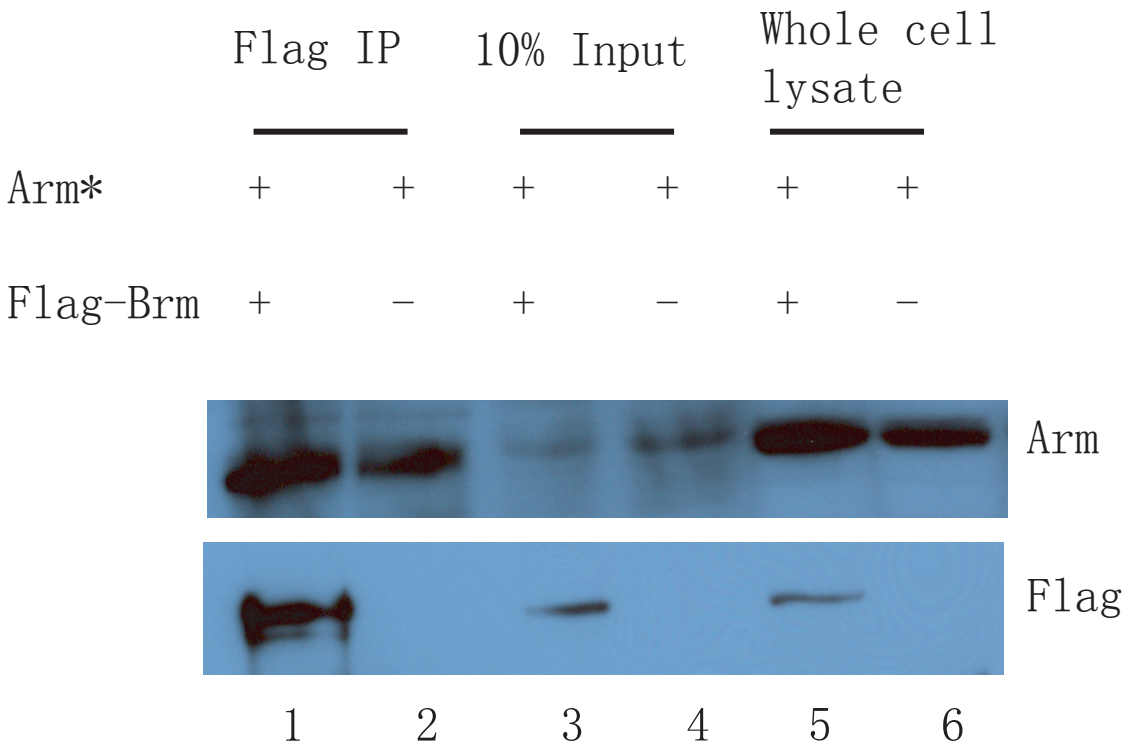


Fig 4-7 Possible interaction between Brm and Arm. Constitutively active Arm* is expressed in all cells with or without Flag-Brm. Blots probed with antibodies specific to Arm and Flag are shown. Whole cell lysate (lane 5 and 6) and 10% input (lane 3 and 4) contain proteins from 1 million cells and Flag IP (lane 1 and 2) contains proteins from 10 million cells. Flag antibody pulls down more Arm in the cells transfected with Flag-Brm (lane1) compared with control (lane2). Representative results of 2 experiments are shown.

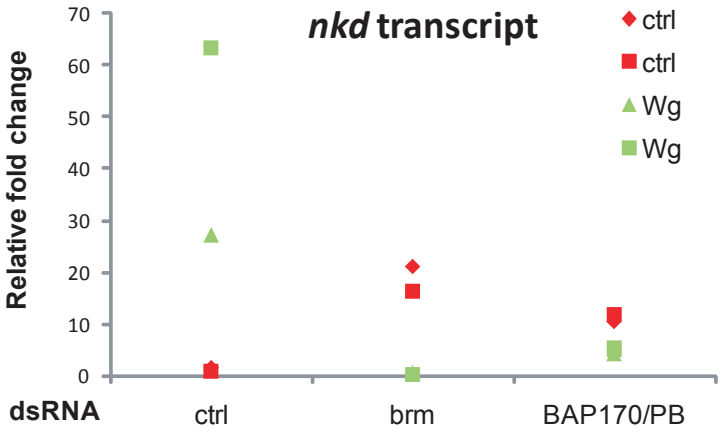


Fig 4-8 nkd activation is decreased with BAP170/PB depletion. Kc cells are treated with indicated dsRNA before Wg activation. Relative fold change of nkd transcript normalized to beta-tubulin transcript is shown. Data points from duplicate experiments are shown.

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Chapter V

Conclusions and future directions

Transcription is a very complicated and highly regulated cellular process. Conserved developmental pathways such as the Wnt/Wg pathway execute their biological functions primarily by controlling target gene expression. Generally, a target gene needs to be repressed in the absence of the signaling and activated in the presence of the signaling. Both the repression and the activation are controlled on multiple levels. My thesis work has focused on the chromatin remodeling and its role in the Wg transcription regulation. I have explored the role of histone post translational modifications as well as ATP dependant chromatin remodeling in the repression and activation of Wg targets.

Conclusions

Wg induces widespread histone acetylations

Histone acetylations are generally connected with transcription activation and acetylated histones are typically found in sharp peaks around the transcription start site (Wang et al. 2008). Our lab has found that Wg induced a widespread increase of AcH3 and AcH4 across *nkd* and *notum*, two Wg direct targets in fly Kc cells. Similar Wg dependent widespread AcH3 and AcH4 are seen on the *notum* gene in fly embryos. The increase of AcH3 and AcH4 is transcription independent as inhibiting transcription initiation/elongation does not affect the increase of AcH3/AcH4. Although the

widespread increase of AcH3/AcH4 depends on the HAT CBP, CBP itself is only localized to the Wingless response element in a Wg dependant manner. The Wg dependant widespread AcH3/AcH4 happens on Wg targets both in fly cell culture and in fly embryos, however it seems to be Wg specific as the constitutively active genes in fly cells have only a sharp peak of AcH3/AcH4 around their promoters.

Polycomb complexes and H3K27 trimethylation negatively regulates Wg transcription

Polycomb group (PcG) complexes were first identified as repressors for Hox genes and the repression was thought to be stable throughout the animal development (Ringrose and Paro 2004). Recent work especially in the stem cell field revealed that Polycomb can dynamically regulate developmental genes although signaling pathways involved in this regulation are largely unknown (Schuettengruber and Cavalli 2009). In this study, I demonstrated that PcG complexes and its associated histone marker H3K27me3 play a role in the Wg target repression. Depletion of Polycomb subunits leads to a derepression of Wg targets *nkd* and hth in the absence of the signaling in fly Kc cells. Loss of E(z) also derepressed *hth* in developing eye discs. The repression is direct which is shown by its independence from Arm levels and by the binding of the HMTase E(z) to a Wg target. The repression is also in parallel with the repression mediated by the transcription factor TCF. Consistent with this, the binding of TCF and E(z) is independent of each other. H3K27me3 on *nkd* and *hth* is greatly reduced when E(z) is depleted. However, Wg activation does not always decrease the level of H3K27me3. Consistent with this, E(z) is also not displaced even when Wg target is highly activated. Similar presence of H3K27me3/Polycomb at activated targets has also been reported in

both flies and mammals (Schwartz et al. ; Pasini et al. 2007). Whether H3K27me3/Polycomb is still functional when targets are activated is unknown.

Possible dual roles of the Brm complexes in the Wg pathway regulation

The Swi/Snf type ATP-dependant chromatin remodeling complexes have been implicated in both transcription activation and repression (Ho and Crabtree ; Trotter and Archer 2008). Fly Brm and mammalian Brg-1, the ATPases, have been shown to play negative and positive roles respectively in the regulation of Wg targets (Collins and Treisman 2000; Barker et al. 2001). In fly KC cells, depletion of Brm with dsRNA results in derepression of Wg targets *nkd* and *crm* and impaired activation of *dfz3* and *notum*. Brm complex subunit Osa binds to *nkd* suggesting that *nkd* is likely directly regulated by the Brm complex. We have not been able to physically place Brm complex on the activated genes but the interaction between Brm and Arm is promising, suggesting that the activation by Brm could also be direct. We have attempted to assign activation and repression to two distinct Brm containing complexes BAP and PBAP through dsRNA knockdown experiments, but the complication from the possible insufficient knockdown by dsRNA has hindered this process.

Future Directions

Is WRE the origin of the widespread AcH3/AcH4?

Widespread chromatin remodeling has been documented in several cases including heterochromatin silencing (Danzer and Wallrath 2004) and polycomb silencing (Papp and Muller 2006; Schwartz et al. 2006). But whether the widespread chromatin remodeling is nucleated from a central region has not been rigorously tested. I have

shown that widespread AcH3/AcH4 happens on a lacZ reporter construct driven by *notum* enhancer integrated into fly genome (Fig2-12). There are insulators on both sides of the reporter construct, so the increase of AcH3/AcH4 does not cross the insulator. It is reasonable to expect that the genomic regions around the integration site will also have increased AcH3/AcH4 if the insulators are removed. Since both the lacZ and the surrounding genome contains no bona fide Wingless response element, any increase of AcH3/AcH4 in these regions should have spread from the *notum* enhancer. To test the nucleated spreading model even more rigorously, one can mutate the WRE in the *notum* enhancer and examine whether the increase of AcH3/AcH4 on lacZ and the surrounding genomic regions is affected. The results of these experiments would provide convincing evidence that spreading of chromatin remodeling is nucleated from enhancers, an assumption often made but not tested in the literature.

Is there a chromosomal loop?

We have observed widespread AcH3/AcH4 and H3K27me3 across several Wg targets, but the corresponding modifying enzymes CBP and E(z) are both localized to a small region. Fig2-13 illustrated two possible models to explain how the localized enzyme can promote the widespread modifications using HAT and histone acetylations as an example. If the spreading model in Fig2-13A is correct, we would expect a delay between the initial increase of AcH3/AcH4 near the WRE and the increase of AcH3/AcH4 far away which we did not see with time course experiments (data not shown). Although we can not exclude the spreading model completely, we can directly test whether chromosome loop forms on the Wg targets. Chromosome conformation capture (3C) is a technique designed to study long range chromosome interactions

(Vassetzky et al. 2009). Briefly, cells are fixed and chromatin is digested with a restriction enzyme. Fragmented chromatin is then ligated at a very low concentration so that the ligation between fragments brought together by the chromosome looping is favored over ligation between random pieces. PCR is then performed to test whether two remote fragments are brought together by looping. In our cell culture system, we can test whether loop forms and if Wg regulates loop formation. We can also ask whether CBP or E(z) is important for the loop formation.

The role of H3K27me2 in transcription regulation

The best studied biochemical function of PcG complexes is trimethylating H3K27. Whether H3K27me1 and H3K27me2 also repress transcription is unknown. In fact, a PRC2 variant containing Pcl has been shown to specifically catalyze tri-methylation, but not di- and mono-methylation (Nekrasov et al. 2007). Interestingly, both we and others found that loss of Pcl results in an increase of H3K27me2 on PcG targets (Nekrasov et al. 2007), but at the same time derepresses PcG targets (Fig3-8 and 3-9). These results argue that H3K27me2 may not repress transcription, but is H3K27me2 just a transit state that has no biological function, at least not in all cases. Lee et al showed that H3K27me2 but not H3K27me3 is required for the repression by RB proteins, providing the first evidence for H3K27me2 biological function (Lee et al.). Also in our hands, although *pcl* dsRNA results in derepression of *hth* and increase of H3K27me2 (Fig3-8), a much stronger derepression is achieved when Pcl and TCF are depleted together. It is possible that the elevated H3K27me2 serves as a back up to prevent maximum transcription derepression when H3K27me3 is decreased. There is a balance between PRC2 and Pcl-PRC2(Fig1-5) in wild type cells and the presence of Pcl makes the Pcl-PRC2 the dominant form of the

two which explains the relatively high level of H3K27me3 on *nkd* and *hth* compared with H3K27me2 (Fig3-8 and 3-9). Loss of Pcl results in a decrease of H3K27me3 but at the same time favors the formation of PRC2 which promotes H3K27me2. The downregulation of H3K27me3 and the upregulation of H3K27me2 in Pcl depleted cells allow low level of transcription. It would therefore be interesting to test whether the simultaneous depletion of Pcl and E(z) abolishes the increase of H3K27me2 and causes a greater derepression.

Genome-wide pattern of TCF and histone modifications

In this thesis we have discussed the Wg dependant chromatin remodeling on a handful of targets. While we have produced informative data concerning how chromatin conformation is regulated on some Wg targets, it is impossible to generalize our findings to all Wg targets with the type of small-scale experiments described so far. To help identify more Wg targets and advance our understanding of Wg transcription regulation, genome-wide mapping of TCF and histone markers will be performed.

Technique: Chromatin Immunoprecipitation followed by massive parallel sequencing with Genome Analyzer from Illumina (ChIP-Seq). Briefly, ChIPed DNA is size selected and ligated with an adapter. The DNA library is then amplified by PCR before fixed to a flow cell through the adapters. Each DNA fragment is then amplified again to generate a cluster on the flow cell. Millions of clusters are then sequenced simultaneously with a fluorescence based technique. The sequences are aligned to the genome to generate the binding pattern of the concerned protein.

As a pilot experiment, I performed ChIP with TCF antibody in cells treated with axin dsRNA or axin/TCF dsRNA. TCF recruitment has been shown to increase on several (Fang et al. 2006; Parker et al. 2008) WREs by Wg activation, so I used cells with activated Wg pathway (axin dsRNA treated cells) to achieve a higher signal. Fig5-1 shows that in axin dsRNA treated cells, TCF is strongly enriched at three WREs compared with the control region. The signal is completely abolished when TCF is depleted with dsRNA. To get enough material for the library preparation, DNA precipitated from 8 pulldowns (~25 million cells) was combined and dissolved in 20ul of water. Library preparation was performed with the Illumina kit (IP-102-1001) following the Kit instruction. After library preparation, DNA was sent to the DNA sequencing core and from each sample about 5×10^8 base sequences were obtained, enough to cover the entire fly genome 5 times.

Orientation/spacing constraints of HMG/helper sites inside TCF recognition motif

Besides discovering more Wg dependant enhancers/targets, the enhancer sequences can be very informative as well. Previously, the Cadigan lab has reported a bipartite recognition model by TCF protein where the HMG domain of TCF recognizes the traditional TCF site (will be called HMG site from now) and the C-Clamp domain of TCF recognizes a novel site called helper site (Chang et al. 2008b). Although both HMG and helper sites are functionally required in most WREs, there seem to no constraints on how far the two sites need to be (spacing) and the relative orientation between the two (Chang et al. 2008b). In our quest for the spacing/orientation rule, we took an alternative bioinformatics approach. We searched the fly genome for the appearance of HMG sites

and helper sites within 10bp of each other and recorded the orientation and spacing between the two sites. Fig5-2 shows that there appears to be a slight enrichment of HMG/helper pairs with 1 or 2bp in between the two sites, however all 4 orientations are equally represented. Although we used very stringent criteria in selecting HMG and helper sites, it's still not guaranteed that all the sites we identified are real TCF binding sites. True TCF binding sites may also not have the perfect sequences for HMG or helper sites. Therefore, the *in silico* approach can not be relied on solely for the purpose of finding the spacing/orientation rule. ChIP-seq has been used to accurately predict functional enhancers (Visel et al. 2009), therefore the enhancers identified by TCF ChIP-seq are likely to give us a better dataset to understand the spacing/orientation rule. Similar analysis as seen in Fig5-2 will be applied to this dataset.

Is widespread chromatin remodeling a general feature of Wg targets?

We have shown that both the positive AcH3/AcH3 and negative H3K27me3 histone markers are widespread on Wg targets. We ask whether the widespread chromatin remodeling is a general feature of Wg targets. To test this, genome-wide mapping of AcH3 or AcH4 as well as H3K27me3 can be performed in the absence and presence of the signaling. DNA fragments showing a change of histone markers will be correlated with DNA fragments bound by TCF and the pattern of acetylated histones and H3K27me3 will be examined.

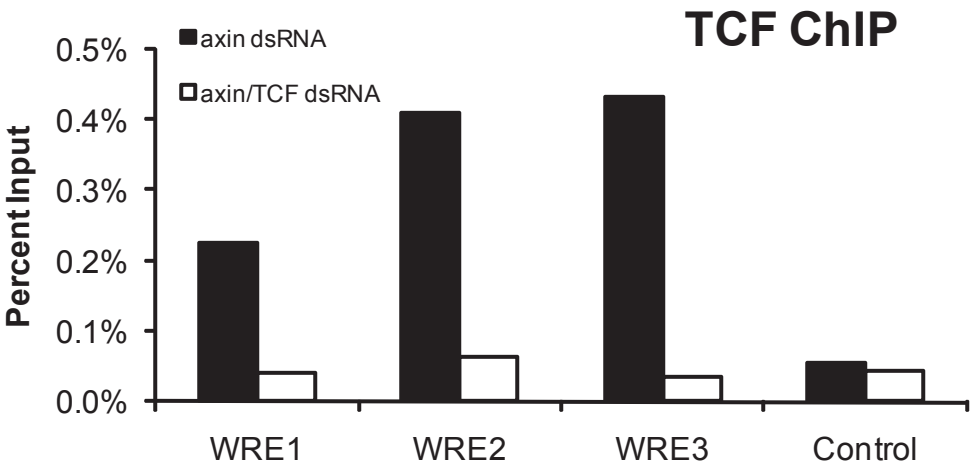

Fig5-1 TCF is strongly enriched at WREs compared with control regions and TCF depletion abolished the signal completely. Kc cells were treated with either *axin* or *axin/TCF* dsRNA and immunoprecipitation was performed with antibody against TCF. Data are represented as the mean of PCR duplicates.

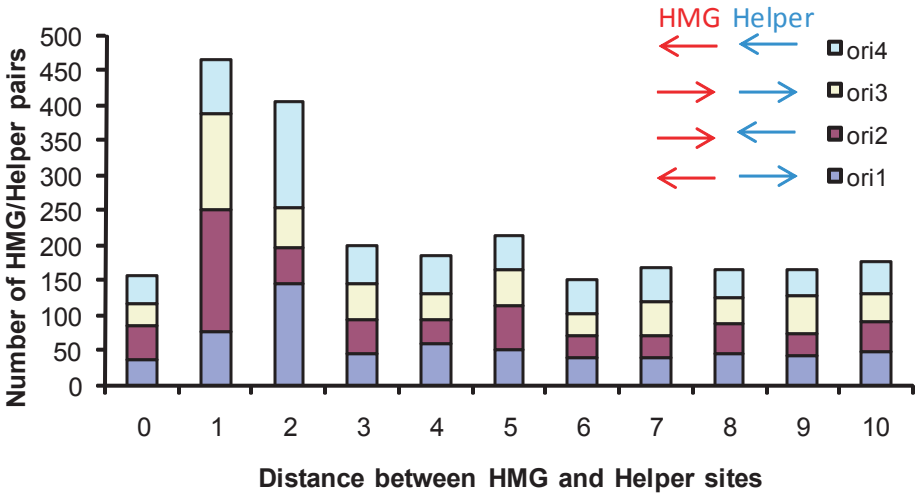

Fig5-2. Predicted HMG/Helper pairs. We searched the fly genome for HMG sites and helper sites within 10bp and their spacing/orientation is represented. The height of each bar represents the number of pairs assuming the indicated spacing and the colors within each bar represent 4 orientations.

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