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The chromatin remodelers ISWI and ACF1 directly repress Wingless transcriptional targets

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ABSTRACT

The highly conserved Wingless/Wnt signaling pathway controls many developmental processes by regulating the expression of target genes, most often through members of the TCF family of DNA-binding proteins. In the absence of signaling, many of these targets are silenced, by mechanisms involving TCFs that are not fully understood. Here we report that the chromatin remodeling proteins ISWI and ACF1 are required for basal repression of WG target genes in *Drosophila*. This regulation is not due to global repression by ISWI and ACF1 and is distinct from their previously reported role in chromatin assembly. While ISWI is localized to the same regions of Wingless target gene chromatin as TCF, we find that ACF1 binds much more broadly to target loci. This broad distribution of ACF1 is dependent on ISWI. ISWI and ACF1 are required for TCF binding to chromatin, while a TCF-independent role of ISWI-ACF1 in repression of Wingless targets is also observed. Finally, we show that Wingless signaling reduces ACF1 binding to WG targets, and ISWI and ACF1 regulate repression by antagonizing histone H4 acetylation. Our results argue that WG signaling activates target gene expression partly by overcoming the chromatin barrier maintained by ISWI and ACF1.

Introduction

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

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

In the absence of β-catenin, TCFs are thought to mediate transcriptional repression. This silencing activity is important in several development contexts. In invertebrates, these include patterning of the embryonic epidermis of *Drosophila* (Cavallo et al., 1998) and mesodermal cell fate specification in *C. elegans* embryos (Rocheleau et al., 1997; Thorpe et al., 1997). In amphibians, TCF repression is important for inhibiting dorsal cell fate in ventral blastomeres (Houston et al., 2002; Standley et al., 2006) as well as for mesoderm induction (Liu et al., 2005). In fish and mice, repression by TCF3 is important for anterior structure specification and AP axis formation (Kim et al., 2000; Merrill et al., 2004). Loss of TCF1 in mice causes spontaneous tumors in the intestine and mammary glands, consistent with inappropriate activation of Wnt/β-catenin targets (Roose et al., 1999). These findings suggest a model where TCFs act as switches, silencing Wnt target gene expression until β-catenin converts them to transcriptional activators (Parker et al., 2007).

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

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repressor from WREs upon pathway activation (Sierra et al., 2006; Wang and Jones, 2006).

While Gro/TLE is recruited to WREs through direct binding to TCFs, other factors act in parallel with TCFs to repress target gene expression. Kaiso, a protein containing BTB/POZ and zinc finger domains, represses several Wnt targets in *Xenopus* by binding to TCF and specific sites in WREs (Park et al., 2005). In *Drosophila* cell culture, the co-repressor C-terminal binding protein (CtBP) is recruited to WREs independently of TCF, where it represses expression in parallel with TCF/Gro (Fang et al., 2006). Whether these factors act in a general or gene-specific manner in repressing Wnt targets remains to be determined.

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

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

Genetic studies also reveal a role for ISWI/ACF1 in regulating chromosome architecture. Flies genetically null for *acf1* display several chromatin defects (Fyodorov et al., 2004), while *iswi* mutant flies die as pupae and display decondensation of the entire male X chromosome (Deuring et al., 2000). This *iswi* phenotype is dependent on the activity of the dosage compensation complex, with ISWI possibly acting antagonistically to the acetylation of histone H4 at lysine 16 (AcH4K16) to mediate global gene repression and chromatin compaction (Corona et al., 2002; Shogren-Knaak et al., 2006).

ISWI has also been suggested to act in more localized gene repression in flies based on the observation that the distributions of ISWI and RNA polymerase II on polytene chromosomes do not generally overlap (Deuring et al., 2000). Consistent with this, the mammalian ISWI homologue SNF2H is required for repression of thyroid hormone receptor targets in the absence of ligand (Alenghat et al., 2006). In addition, there are several reports demonstrating that the yeast ISWI homologues, Isw1 and Isw2 are directly involved in transcriptional repression (Goldmark et al., 2000; Moreau et al., 2003; Sherriff et al., 2007; Zhang and Reese, 2004).

In this report, we identify ISWI and ACF1 as important repressors in WG signaling in *Drosophila*. Loss of *iswi* and/or *acf1* causes derepression or further activation of several WG transcriptional targets in cultured cells and *iswi* is required for repression of WG targets in the wing imaginal disc. The derepression is still observed in non-dividing cells, arguing against the effect being due to a post-mitotic chromatin assembly defect. *iswi* and *acf1* are required for maximal TCF binding to WREs and to antagonize histone acetylation in the absence of signaling. ACF1 is directly associated with broad regions of several WG target loci while ISWI binding (when detectable) mirrors the localized distribution of TCF. ACF1 binding is dependent on ISWI, but not vice versa. ACF1 binding is reduced upon activation of WG signaling. These results are consistent with a model where ISWI and ACF1 silence target gene expression in unstimulated cells and modulates the switch to transcriptional activation by WG signaling.

Materials and methods

Drosophila genetics

The *iswi* mutant strains $iswi^1$ and $iswi^2$ were kindly provided by J. Tamkun (Deuring et al., 2000). For clonal analysis, $iswi^1$ was recombined onto a FRT^{42D} chromosome using standard methods (Xu and Rubin, 1993). Somatic clones of $iswi^1$ in wing imaginal discs were generated by crossing $FRT^{42D}iswi^1$ males to *yw* P[*HS-Flp*]; FRT^{42D} P [*Ubi-GFP*] females. Clones were induced by one-hour 37 °C heat shock at 48–72 h after egg laying. The null allele $acf1^5$was obtained by imprecise excision of EP(3)1181 as previously described (Zhou et al., 2003) and is described in detail in Supplemental Fig. 1.

A 2.2 kb fragment from approximately −4.1 to −1.9 kb upstream of the *Notum* TSS (Stadeli and Basler, 2005) was cloned into pH-Pelican vector (Barolo et al., 2000), and the corresponding Notum-lacZ transgenic flies were generated by BestGene Inc (Chino Hills, CA). The nkd-lacZ reporter UpE1 (Chang et al., 2008) was used to monitor *nkd* expression. Both reporters were positively regulated by WG signaling in the wing imaginal disc (Change et al., 2008; data not shown). The Decapentaplegic (Dpp)-lacZ line 3.0 (Blackman et al., 1991) was obtained from the Bloomington Stock Center.

Antibodies, immunoblot and immunostaining

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

For immunobloting, α-rabbit ACF1 (1:5000), α-ISWI (1:2000) and α-TCF (1:2000) were followed by HRP-conjugated α-rabbit IgG (1:2000). Signal was detected with the ECL kit (Amersham Bioscience). Immunostaining of wing imaginal discs was as described previously (Parker et al., 2002). The dilution factors for the primary antibodies used were: α-Sens (1:1000), α-WG (1:100), α-En/Inv (1:20), α-Spalt (1:100), rabbit α-β-galactosidase (1:200), mouse α-β-galactosidase (1:500). Cy3- and Cy5-conjugated secondary antibodies were from Jackson Immunochemicals, and Alexa 488-conjugated secondary antibody was from Molecular Probes. All fluorescent images were obtained with Olympus FV-500 Confocal microscope, and processed in Adobe Photoshop 8.0.

Drosophila cell culture

Drosophila embryonic Kc167 (Kc) cells were cultured at room temperature in Schneider's *Drosophila* media (Invitrogen) containing 5% FBS and Penicillin/Streptomycin.

Antibiotics. RNAi mediated knockdown of gene expression was performed as described elsewhere (Fang et al., 2006) with modifications. Briefly, when Kc cells approached confluent status after 4–5 days of culture (~8×10^6/ml), they were resuspended at 1×10^6/ml in standard media and seeded onto 12-well plates (1 ml/well) or T-25 flask (6 ml/well). RNA duplex was then added at a final concentration of 9 μg/ml. After 4 days, cells were resuspended, diluted into 1×10^6/ml with fresh media and reseeded onto 12-well plates or T-25 flasks. Cells were harvested after 2 additional days of incubation. dsRNAs with a typical length of 500–700 bp were synthesized using the MEGAscript T7 in vitro transcription kit (Ambion). The sequences of the PCR primers for the dsRNA synthesis are: iswi (1st duplex: 5′-CCATCAGTT

GCGGCTGCAATATGGTAA-3′ and 5′-GCGGCACGCAATAGTAATG-TAGTCGGAT-3′; 2nd duplex: 5′-CCACTTCATGACTAACAGCGCTAA-GAGT-3′ and 5′-GCAGAATCTCCG ACAGCTTCGACTTCT-3′), acf1 (1st duplex: 5′-CGACCACGTAACTCTTTGCGCCTATCTA-3′ and 5′-GCGTGTGCTGAACTTAGAACTGACAT-3′; 2nd duplex: 5′-CGATGAATG-CAA CGCTGGCACTCACAT-3′ and 5′-GGTCGCTTGAGGTGAACACATTCCA-3′). The sequences of primers for control, *arm* and *TCF* dsRNAs have been published previously (Fang et al., 2006).

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

Real-time quantitative RT-PCR (QRT-PCR)

Samples were analyzed using an iCycler iQ real-time PCR detection system. For qRT-PCR, Trizol (Invitrogen) was used to extract total RNA from $1–5\times10^6$ cells. Reverse transcription was performed using Stratascript reverse transcriptase (Stratagene) followed by qPCR analysis. *β-tubulin56D* or *arm* or *TCF* or the combination of all three were used to normalize transcript levels. qPCR primers were designed by using the online program Primer3 (http://frodo.wi.mit.edu/), and their sequences are available upon request.

Chromatin immunoprecipitation (CHIP)

ChIP analysis was performed according to the protocol of Upstate with minor modifications. An initial protein-crosslinking step was included by incubating cells in 10 mM DTBP solution (Pierce #20665) for 30 min on ice. For each immunoprecipitation, 3×10^6 cells were used and the amounts of antibodies used are as following: 5 μl guinea pig α-ACF1, 10 μl rabbit α-TCF, 5 μl rabbit α-ISWI, 3 μl rabbit α-H4, 1 μl

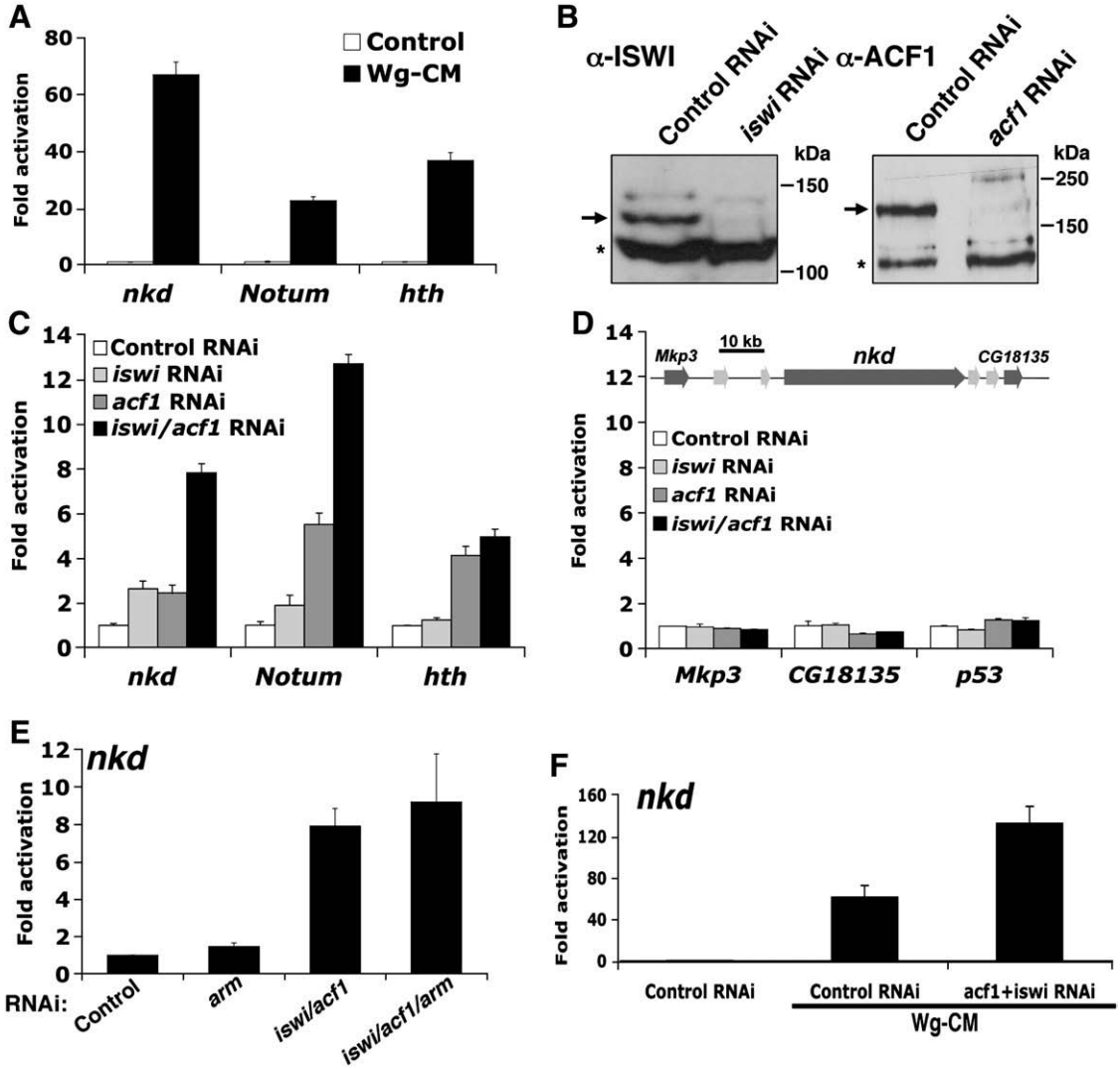

Fig. 1. ISWI and ACF1 repress WG targets in cultured cells. (A) Kc cells were treated with control media or WG-CM for 5 h prior to harvest. Transcript levels of *nkd*, *Notum* and *hth* were measured by qRT-PCR and results were normalized to *β-tubulin56D* expression. WG-CM significantly induced the expression of all three genes. (B) Western blot analysis of ISWI and ACF1 in control or corresponding RNAi-treated cells. *iswi* RNAi and *acf1* RNAi dramatically reduced ISWI and ACF1 expression, respectively. Arrows indicate the positions of ISWI and ACF1 proteins, and asterisks indicate nonspecific bands. (C) Derepression of *nkd*, *Notum* and *hth* by *iswi* or/and *acf1* RNAi. Kc cells were treated with the indicated dsRNAs as described in Materials and methods, and transcripts of WG targets were measured by qRT-PCR. Results were normalized to the average of *β-tubulin56D*, *arm* and *TCF* expression. (D) Two genes adjacent to *nkd* locus, *Mkp3* and *CG18135*, as well as *p53* were not derepressed by *iswi*, *acf1* RNAi. The same normalization strategy was used as in panel C. (E) *arm* RNAi did not affect the derepression of *nkd* by *iswi*, *acf1* RNAi. (F) Activation of *nkd* expression by WG-CM is enhanced by knockdown of *iswi* and *acf1*. Each bar in this figure represents the mean (±S.E.) of duplicate cultures with duplicate qRT-PCR reactions. All experiments have been performed at least three separate times with similar results.

rabbit α-AcH4. All ChIP samples were quantified with qPCR. The inputs refer to the samples that were not subject to immunoprecipitation. The primer sequences for ChIP sites on *nkd*, *hth* and *Notum* loci are available upon request. For the re-ChIP assay, DNA-protein complexes were eluted by incubation in 50 μl 10 mM DTT for 30 min at 37 °C. After centrifugation, the supernatant was diluted into 1 ml (20 times) with ChIP dilution buffer. Half of the eluted sample (500 μl) was saved as the secondary input, and the other half was subject to immunoprecipitation by the second antibody.

Results

ISWI and ACF1 repress WG targets in Drosophila cultured cells

WG signaling can be studied in cell culture using fly Kc167 (Kc) cells, which we have previously shown to be responsive to WG signaling (Fang et al., 2006; Li et al., 2007b). Microarrays were used to identify genes whose expression increased upon stimulation of the pathway (T. Blauwkamp and K. Cadigan, unpublished data). Three activated targets, *naked cuticle (nkd)*, *Notum* and *homothorax* (*hth)*, were chosen for further study. *nkd* and *Notum* are feedback antagonists induced by WG signaling in most fly tissues (Gerlitz and Basler, 2002; Giraldez et al., 2002; Zeng et al., 2000), while *hth* is activated by the pathway in specific tissues (Azpiazu and Morata, 2000; Casares and Mann, 2000; Wernet et al., 2003). In Kc cells, treatment with WG conditioned media (WG-CM) significantly induced the transcript levels of all three genes (Fig. 1A).

To examine whether ISWI and ACF1 play a role in regulating WG targets, cells were depleted of these factors via RNA interference (RNAi). Both ISWI and ACF1 are expressed in Kc cells, and their expression can be efficiently inhibited by the respective dsRNA (Fig. 1B). In general, inhibition of *iswi* or *acf1* caused an increased expression of *nkd*, *Notum* and *hth* transcripts in unstimulated cells, while simultaneous knockdown of both *iswi* and *acf1* led to even higher levels of derepression (Fig. 1C). To control for off-target effects of RNAi, dsRNAs targeting different regions of *iswi* or *acf1* were used, and similar results were obtained (data not shown).

For individual WG targets, differences are observed in the *iswi* and *acf1* single RNAi treatments. ISWI and ACF1 equally contribute to the repression of *nkd*, while ACF1 plays a greater role in inhibiting *Notum*. In contrast, repression of *hth* mainly depends on ACF1 (Fig. 1C). It is unclear whether these results reflect real mechanistic differences between the functions of these factors or threshold effects of the RNAi depletion.

To explore whether ISWI and ACF1 specifically repress WG targets, three genes that are not responsive to WG signaling were examined. Two of these, *Mkp3 and CG18135*, are located upstream and downstream of *nkd*, respectively, and a third one, *p53*, was picked from the non-WG-target pool of the aforementioned microarray analysis. Depletion of *iswi* and/or *acf1* had no effect on these genes (Fig. 1D). In addition to demonstrating specificity towards WG targets, these results argue against the increase in *nkd* expression being caused by a general loosening of the chromatin, since the genes adjacent to this locus are not affected.

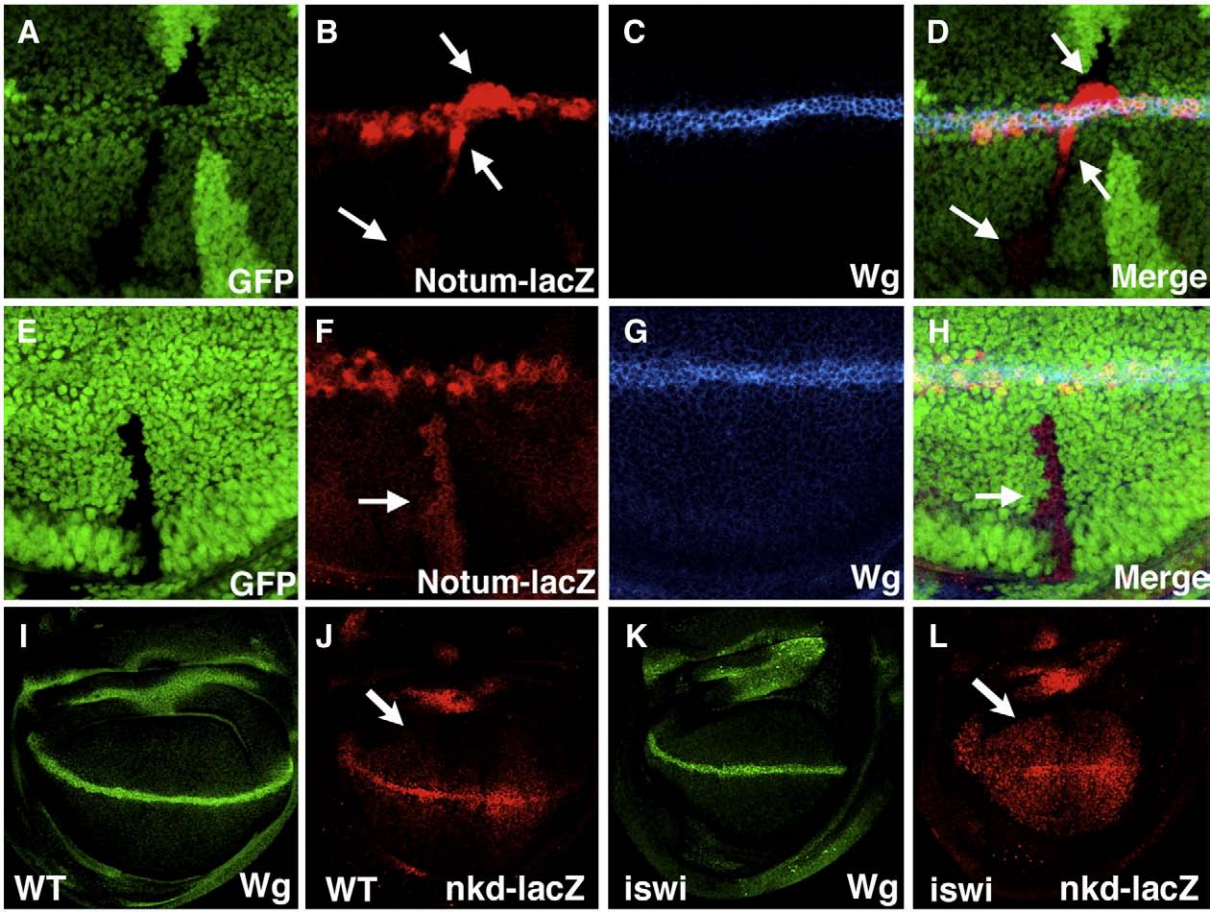

Fig. 2. Loss of *iswi* results in an expansion and/or derepression of WG targets in wing imaginal discs. Confocal images of wing imaginal discs of late third instar larva stained for WG (C, G, I, K), Notum-lacZ (B, F), and nkd-lacZ (J, L). (A–H) Mitotic clones of $iswi^1$ were marked by the absence of GFP (green in panels A and E). Notum-lacZ expression is expanded in clones along the WG expression domain (A–D, 95% penetrance, $n=39$), and is derepressed in clones far away from the D/V boundary (E–H, 64% penetrance, $n=22$). (I–L) nkd-lacZ expression is expanded in wing discs from $iswi^1/iswi^2$ transheterozygotes (L, 92% penetrance, $n=24$) while WG expression is unaffected (K).

Several negative regulators of the WG/Wnt pathway act through antagonizing Arm/β-catenin binding to TCF (Parker et al., 2007). ISWI and ACF1 repress WG targets in the absence of exogenously added WG, suggesting that they do not act by this mechanism. This was confirmed by the finding that the derepression of *nkd* expression caused by *iswi, acf1* depletion remained unchanged with the additional knockdown of *arm* (Fig. 1E). *arm* transcript levels were effectively knocked down in both *arm* single RNAi and *iswi, acf1, arm* triple RNAi cells (data not shown). Thus, ISWI and ACF1 act as *bona fide* silencers of WG targets in the absence of signaling.

To determine whether ISWI and ACF1 also repress targets upon WG stimulation, they were inhibited by RNAi in cells challenged with WG-CM. Under these conditions, induction of transcript levels of *nkd* by WG signaling was 2–3 fold greater in *iswi*, *acf1* depleted cells than controls (Fig. 1F). The data suggests that ISWI and ACF1 also play a role in regulating the degree of WG target gene activiation in addition to their role in silencing.

ISWI specifically represses WG targets in the developing wing

To determine whether ISWI or ACF1 plays a physiological role in WG signaling in flies, we analyzed the phenotype of wing imaginal disc cells lacking *iswi* or *acf1*. In this tissue, WG is expressed in a stripe at the Dorsal/Ventral (D/V) boundary of the disc (Phillips and Whittle, 1993). WG diffusing from the D/V stripe activates several genes, including *nkd* (Zeng et al., 2000; Chang et al., 2008) and *Notum* (Gerlitz and Basler, 2002; Giraldez et al., 2002). The expression of these targets could be monitored by lacZ reporters (see Materials and methods for details). Both Notum-lacZ and nkd-lacZ require WG signaling for activation in the developing wing (Chang et al., 2008; data not shown).

iswi gene activity was removed in the wing discs by inducing clones of a molecular null allele ($iswi^1$) via mitotic recombination. Two thirds of the clones had no detectable effect on the expression of WG at the D/V stripe (Fig. 2C) and no ectopic WG expression was observed in clones far away from the D/V boundary (Fig. 2G). In one third of clones at the D/V boundary, the WG stripe was kinked (data not shown), and these clones were not included in our analysis.

The removal of *iswi* gene activity resulted in a dramatic increase in Notum-lacZ expression in the developing wing. In *iswi* mutant clones near the D/V border, the Notum reporter was expanded (Figs. 2A–D). In clones further away from the WG stripe, ectopic expression of Notum-lacZ was observed in the majority of the clones (Figs. 2E–H), consistent with derepression of this WG target.

In addition to mitotic clones, $iswi^1/iswi^2$ transheterozygotes were examined. These animals often survived until early to mid pupal stages, and possessed slightly misshapen wing imaginal discs at late third larval instar (Fig. 2K, L and Fig. 3E). The width of the WG D/V stripe was normal in *iswi* mutants (Fig. 2K), but nkd-lacZ displayed a dramatic expansion that was highly penetrant (compare Fig. 2J with 2L). The pattern of Notum-lacZ was also expanded (data not shown). Together with the clonal analysis, these data confirm that ISWI represses WG target gene expression in this tissue.

To examine the role of *acf1* in wing discs, we used imprecise excision of a P-element to generate a deletion removing part of the first intron and second exon of the *acf1* gene (Supplemental Fig. 1A). This deletion ($acf1^5$) is predicted to cause a frameshift mutation after the 41st residue of the normally 1476 amino acid protein. Consistent with this lesion, $acf1^5$ embryos had no detectable ACF1 protein as judged by Western blot (Supplemental Fig. 1B). Wing discs from flies homozygous for this null alleles of *acf1* had no detectable misregulation of WG targets (data not shown). This is perhaps not surprising, since $acf1^5$ mutants were viable and fertile (data not shown). To address whether maternally contributed *acf1* mRNA could be contributing to repression of WG targets in the larval wing disc, Notum-lacZ expression was examined in $acf1^5$ mutants from homozygous $acf1^5$ mothers. No detectable expansion of Notum-lacZ expression was found (Supplemental Fig. 2), arguing against residual maternal transcript being responsible for the lack of an effect on WG targets in wing imaginal discs.

While loss of *acf1* had no detectable effect on WG targets, we did observe a genetic interaction between *acf1* and *iswi* mutants. $iswi^1/iswi^2$; $acf1^5/+$ animals had a significant developmental delay compared to $iswi^1/iswi^2$ mutants, surviving to late third larval instar/early pupation. However, no further expansion of nkd-lacZ or Notum-lacZ was observed in the compound mutants compared to *iswi* mutants

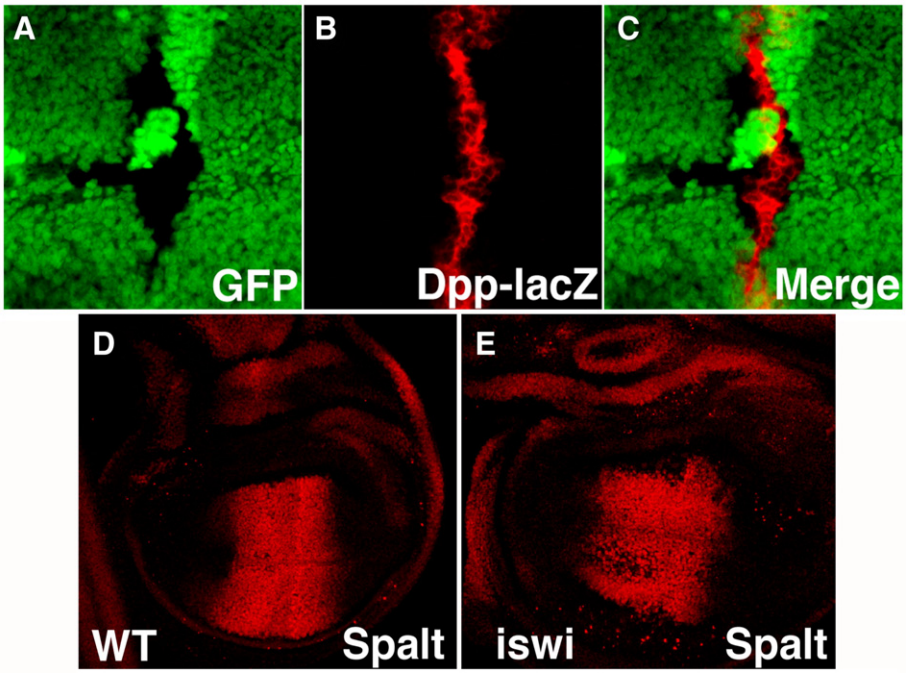

Fig. 3. *iswi* mutant cells do not affect several non-WG targets in wing imaginal discs. Confocal images of wing imaginal discs of late third instar larva stained for Dpp-lacZ (B) and Spalt (D, E). All *iswi* clones examined show normal Dpp-lacZ expression (B, $n=24$) and the width of the Spalt expression domain was unaffected in $iswi^1/iswi^2$ transheterozygotes (E, $n=12$).

(data not shown). Complete removal of *iswi* and *acf1* resulted in lethality during early to mid-larval development, precluding an examination of WG targets at later larval stages. The findings that *iswi*, *acf1* mutants are more severely affected than *iswi* mutants suggest that ACF1 has functions that are independent of ISWI in fly development.

Not all genes expressed in the developing wing are affected by the loss of *iswi*, e.g., WG expression is normal in *iswi* mutants (Figs. 2C, G, K). To extend this analysis, two other genes not regulated by WG were examined in *iswi* mutants. Dpp-lacZ is activated by Hedgehog (Hh) signaling in a stripe on the anterior side of the Anterior/Posterior (A/P) boundary, while Spalt is activated by Dpp signaling in a broad region surrounding the A/P boundary (Tabata, 2001). No significant expansion of the expression of either of these genes was observed in *iswi* mutant clones (Fig. 3B and data not shown) or in $iswi^{1}/iswi^{2}$ transheterozygotes (Fig. 3E). These results demonstrate that ISWI is not a general repressor of gene expression in the wing, displaying substantial specificity for WG targets.

ACF1 and ISWI have different distributions on WG transcriptional targets

The WG targets *nkd* and *Notum* appear to be directly activated by the pathway, based on TCF binding using ChIP. There is a major peak of TCF binding at a WRE in the first intron of *nkd* approximately 5 kb

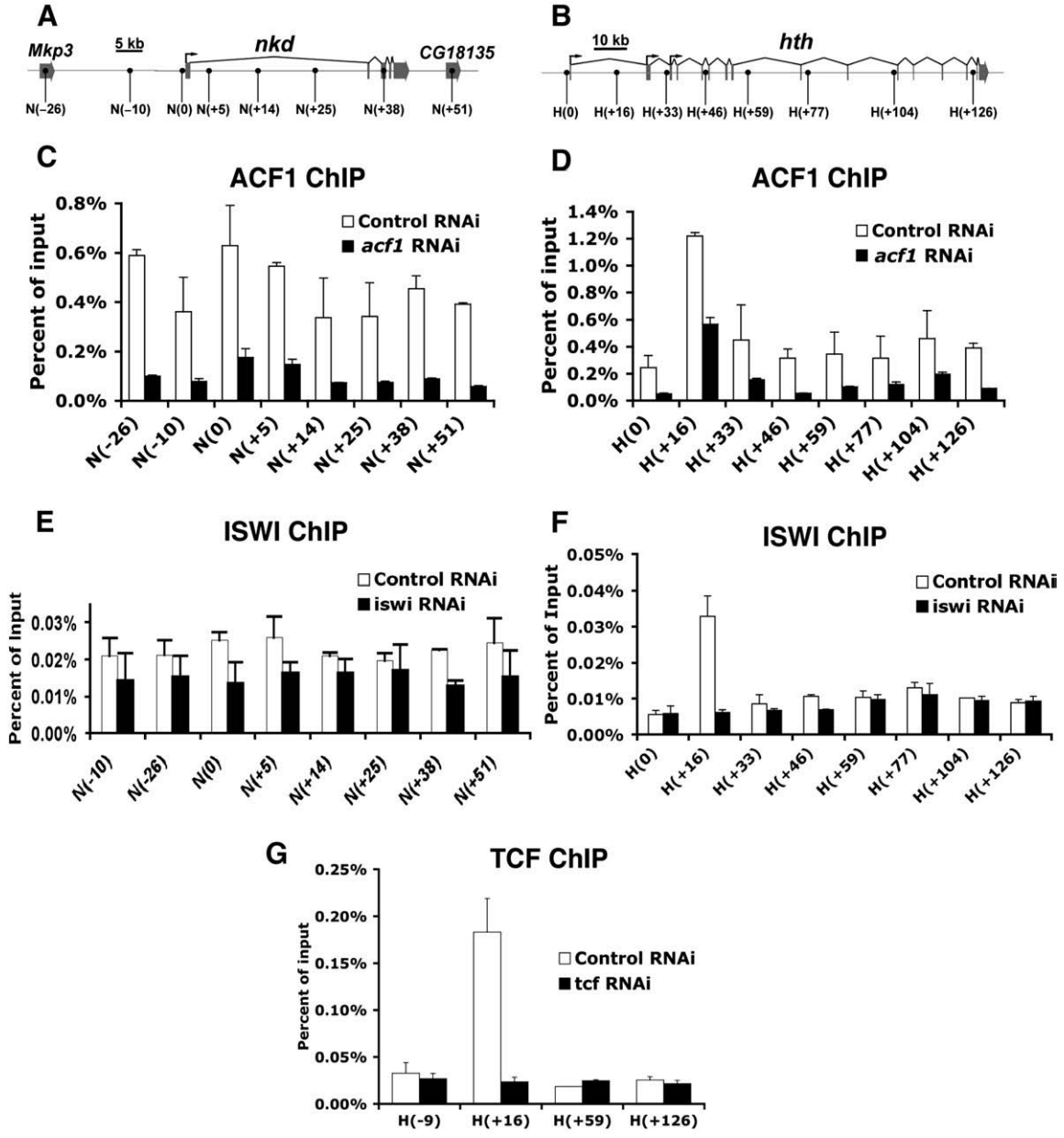

Fig. 4. ACF1 and ISWI have different distributions on WG target genes. (A, B) Schematic diagrams of the *nkd* and *hth* loci with the illustrated sites used for ChIP analysis. Arrows indicate the TSSs. The numbers in parentheses indicate the distance (in kb) from the TSS. N(−10) and N(+5) indicate the location of the two WREs of *nkd*. H(+16) is an area of *hth* bound by TCF. (C, D) ChIP analysis shows ACF1 binding to *nkd* and *hth*. Kc cells were treated with control dsRNA or *acf1* dsRNA for 6 days before they were harvested for ChIP analysis. For *nkd*, ACF1 binds to a broad region as well as the genes adjacent to it (C). For *hth*, ACF1 also bound broadly but there was a three-fold enrichment of ACF1 binding at H(+16) that was reproducibly observed (D). (E, F) ChIP analysis of ISWI binding to *nkd* and *hth*. Assays were performed as for ACF1. There is no detectable ISWI binding (i.e., signal significantly higher than that in *iswi* depleted cells) on *nkd* (E), but there is ISWI enrichment at H(+16) on *hth* (F). (G) ChIP with TCF antisera shows specific binding of TCF to H(+16). Each bar in the figure represents the mean (±S.E.) of duplicate cultures with duplicate qPCR reactions. All experiments were performed three separate times with similar results except for the TCF ChIP, which was performed twice.

downstream of the transcriptional start site (TSS) (Fang et al., 2006; Parker et al., 2008). This intronic *nkd* WRE contains several TCF binding sites required for its induction by WG signaling in a reporter gene assay (Chang et al., 2008). In addition, TCF binds to an additional area 10 kb upstream of the *nkd* TSS (Parker et al., 2008) that corresponds to the nkd-lacZ reporter repressed by ISWI in wing imaginal discs (Figs. 2J, L). TCF also binds to two areas in the *Notum* locus (Parker et al., 2008). One site is 4 kb upstream of the TSS, corresponding to the Notum-lacZ construct regulated by WG signaling and ISWI in wing imaginal discs (Figs. 2B, F). The other is in the first intron, about 6 kb downstream of the TSS.

In contrast to the binding by TCF to specific areas of the *nkd* and *Notum* genes, ACF1 was found more broadly across these loci. ACF1 binding was observed over the entire *nkd* locus, including the two aforementioned WRE sites (Fig. 4C). The ACF1 ChIP signal was significantly reduced by *acf1* RNAi, indicating that it was specfic for ACF1 (Fig. 4C). Interestingly, two genes adjacent to *nkd*, *Mkp3* and *CG18135*, were also bound by ACF1, although they were not regulated by ISWI and ACF1 (Fig. 1D). Similar to the binding profile of *nkd*, ACF1 was also bound widely across the *Notum* locus (data not shown). These results suggest that ACF1 physically associates with WG targets in the absence of WG signaling in a broader pattern than TCF.

The third WG target characterized in this report, *hth*, has a transcription unit nearly 130 kb in length (Fig. 4B) and the *cis*-acting elements controlling its expression have not been characterized. Using an online tool called Target Explorer (http://trantor.bioc.columbia.edu/Target_Explorer) (Sosinsky et al., 2003), we identified several clusters of putative TCF binding sites in the intronic regions of *hth*. ChIP analysis revealed strong TCF binding to one of these clusters, 16 kb downstream of *hth* TSS, which was reduced to background levels upon *TCF* RNAi treatment (Fig. 4G). As was seen in the other WG targets, ACF1 was bound to the entire *hth* locus (Fig. 4D). In contrast to *nkd*, greater ACF1 binding was observed at the site bound by TCF than at other areas. Although the ACF1 ChIP signal at this site was only partially abolished by *acf1* RNAi, it was more dramatically reduced when both *acf1* and *iswi* were knocked down (data not shown). We conclude from these results that ACF1 directly binds to a large portion of the *hth* locus, with enrichment at the region also bound by TCF.

Surprisingly, the pattern of ISWI binding to WG targets was distinct from that of ACF1. At the *nkd* locus, there was little detectable ISWI binding, as judged by the ratio of signal in control versus *iswi* RNAi treated cells (Fig. 4E). At *hth*, there was a peak of ISWI binding 16 kb downstream of the TSS (Fig. 4F), the same location where TCF binding

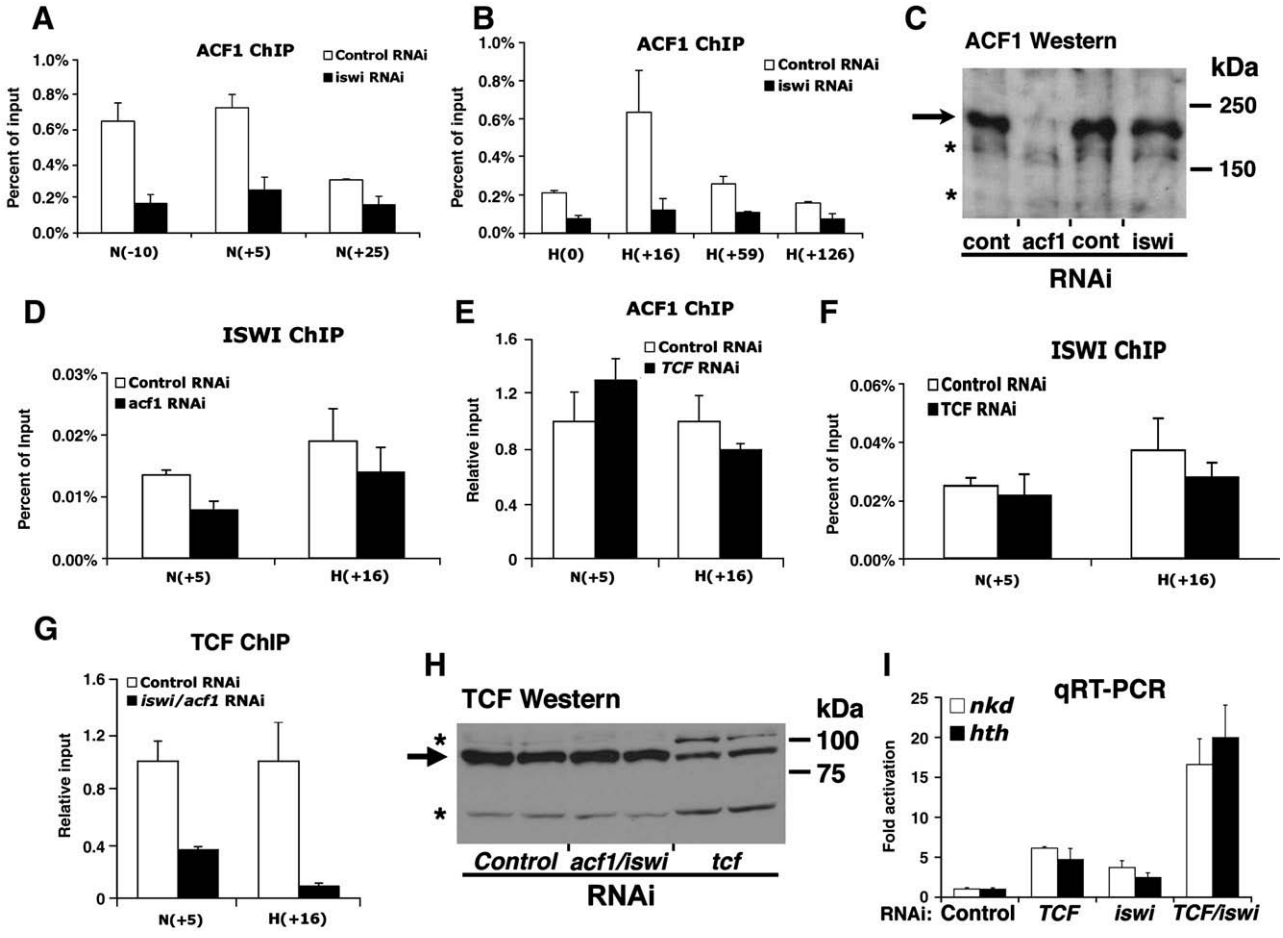

Fig. 5. Relationship between binding of ACF1, ISWI and TCF to WG target gene chromatin. (A, B) ACF1 binding is *iswi*-dependent. Kc cells were depleted of *iswi* for six days before processing for ACF1 ChIP. Marked reduction in binding was observed across the *nkd* and *hth* loci. (C) *iswi* knockdown does not reduce cellular ACF1 levels. Cells were treated with the indicated dsRNA for 6 days and processed for Western blot analysis with ACF1 antisera. Depletion of *iswi* does not affect ACF1 protein levels. (D) ISWI binds to *hth* independently of *acf1*. Depletion of *acf1* does not cause a significant and reproducible reduction of ISWI binding to N(+5) or H(+16). (E, F) Depletion of *TCF* has no effect on ACF1 (E) or ISWI (F) binding to *nkd* and *hth*. The efficiency of *acf1* and *TCF* RNAi in panels D–F was confirmed by Western blot (data not shown). (G) Depletion of *iswi* and *acf1* reduces TCF binding to *nkd* and *hth*. Depletion of *acf1* alone also reduces TCF binding to *nkd* and *hth*, but to a lesser degree compared to *iswi*, *acf1* double knockdown cells (data not shown). (H) *iswi*, *acf1* RNAi has no obvious effect on TCF expression. Western blot of TCF protein was performed on extracts treated with the indicated dsRNAs. *TCF* RNAi significantly reduced TCF protein levels but *iswi*, *acf1* RNAi did not. Asterisks indicate nonspecific bands. (I) Depletion of *TCF* and *iswi* cooperately derepresses *nkd* and *hth* transcript levels in Kc cells. All experiments have been performed three separate times (except for the ISWI ChIP, which was performed twice) with similar results. The data in panels E and G are expressed as relative input because the absolute % input values differed from the *nkd* and *hth* loci.

was detected (Fig. 4G). These data suggest that ISWI and ACF1, which are thought to form a stable complex in solution, have distinct localizations on the chromatin of WG target genes.

Interdependence between TCF, ACF1 and ISWI binding to WG targets

To determine the relationship between ACF1, ISWI and TCF binding to WG targets, ChIP for each factor was performed under various RNAi conditions. Broad ACF1 binding to *nkd* and *hth* was dramatically reduced by *iswi* depletion (Figs. 5A, B). *iswi* RNAi did not affect the expression level of ACF1 as judged by Western blot (Fig. 5C). In contrast, knockdown of *acf1* had a mimimal effect on ISWI binding to WG targets (Fig. 5D). These data suggest a model where localized ISWI promotes the broad distribution of ACF1 on the chromatin of WG targets (see Discussion for further comments).

Since ISWI and ACF1 are found at the same region as TCF on *nkd* and *hth*, their binding was examined in *TCF* depleted cells. No significant change of ACF1 or ISWI binding to the TCF-bound region of the target loci was observed upon *TCF* knockdown (Figs. 5E, F). In contrast, TCF binding to these sites was significantly reduced when *iswi* and *acf1* were knocked down (Fig. 5G). *iswi*/*acf1* depletion did not reduce TCF expression as determined by Western blot (Fig. 5H). These results suggest that ISWI and ACF1 facilitate TCF binding to WG targets in the absence of signaling.

TCF is thought to repress *nkd* expression with the transcriptional corepressor Gro (Fang et al., 2006) and the same is true for *hth* (Fig. 5I and data not shown). When *TCF* and *iswi* were depleted simultaneously, greater derepression of *nkd* and *hth* was observed than when either factor was knocked down alone (Fig. 5I). This result suggests that ISWI acts in parallel with TCF to repress WG target genes. Similar

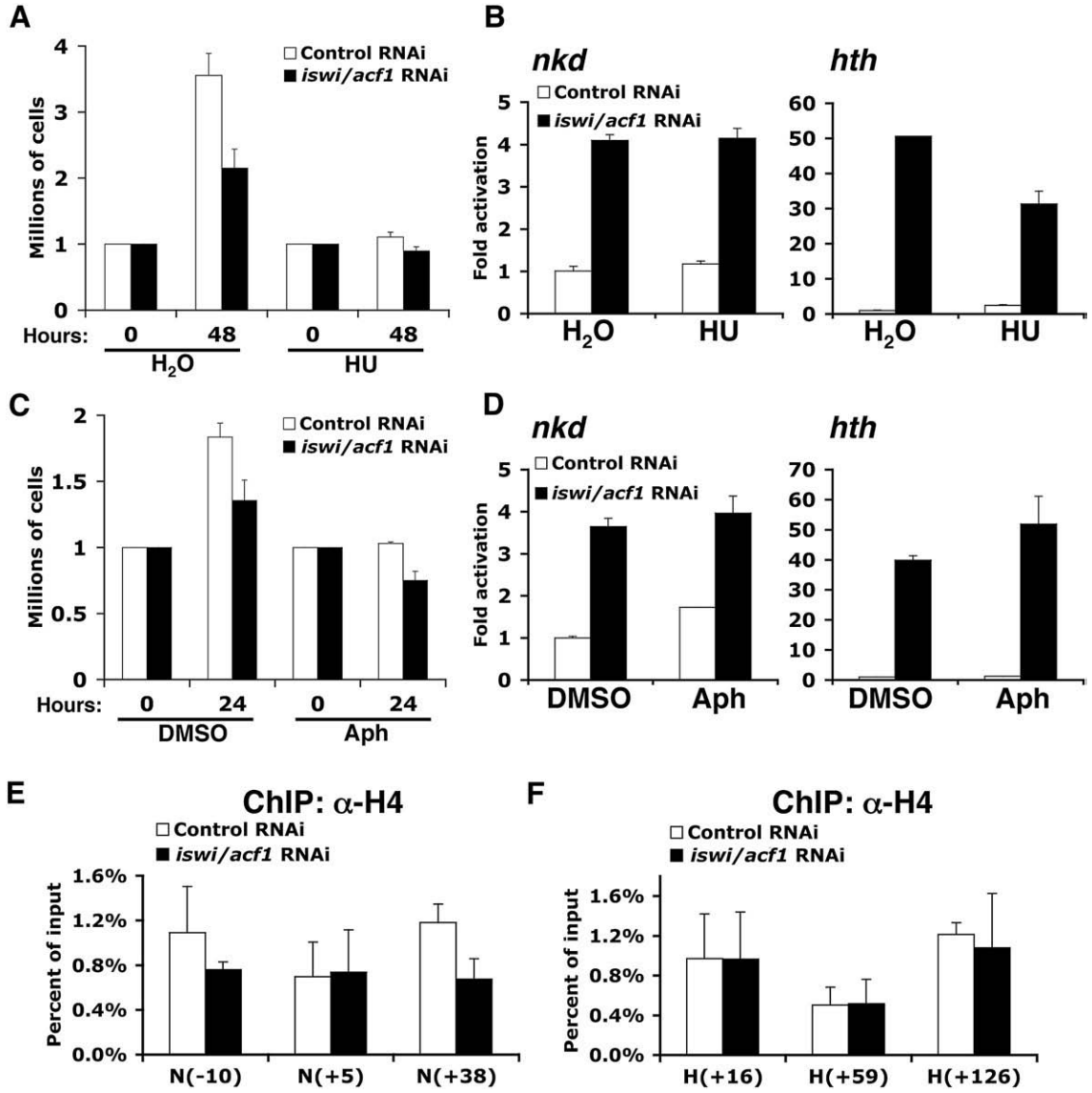

Fig. 6. ISWI and ACF1 repress WG targets independent of post-mitotic chromatin assembly. (A) Hydroxyurea (HU) effectively blocks cell division. Kc cells incubated with control or *iswi* and *acf1* dsRNA for four days were treated with H_2O or 5 mM HU for an additional 48 h. Cells stopped dividing, judged by cell number, upon HU treatment. In the control group, *iswi*, *acf1* RNAi decreases the cell division rate. A similar decrease in cell division was also observed with *acf1* RNAi, but not with *iswi* RNAi (data not shown). (B) Derepression of *nkd* and *hth* by *iswi*, *acf1* RNAi is not abolished after HU treatment. Same experimental conditions were used as in panel A, and transcript levels of *nkd* and *hth* were measured by qRT-PCR. (C) Aphidicolin (Aph) also inhibits cell division. Kc cells incubated with control dsRNA or *iswi*/*acf1* dsRNA for four days were treated with DMSO or 25 μM Aph for an additional 24 h. Effective blockage of cell division was seen in Aph treated cells. (D) *iswi*, *acf1* RNAi still derepresses *nkd* and *hth* after Aph treatment. (E, F) *iswi*, *acf1* RNAi does not significantly affect H4 binding to the *nkd* and *hth* genes. ChIP analysis for pan-H4 was performed in control dsRNA or *iswi*/*acf1* dsRNA treated cells. Multiple sites including the *nkd* WREs and TCF binding region of *hth* were tested for H4 binding, and no obvious change was observed between control RNAi and *iswi*/*acf1* RNAi. Data shown were the means of duplicates (±S.E.), and all experiments have been performed two separate times with similar results.

results were also observed upon depletion of *iswi* or *acf1* with *gro* (data not shown). These data suggest that ISWI and ACF1 have TCF-Gro-independent activities in repressing WG targets.

ISWI and ACF1 repress WG targets independently of chromatin assembly

Because of its role in chromatin assembly (Fyodorov and Kadonaga, 2002; Ito et al., 1999), it is possible that the derepression of WG targets observed in cells depleted of *iswi* and *acf1* is due to incomplete packaging of chromatin after mitotis, rather than specfic transcriptional regulation. To test this possibility, the effect of *iswi*, *acf1* depletion on WG targets in non-dividing cells was examined. Cells treated with control or *iswi*, *acf1* RNAi for four days were then treated with hydroxyurea (HU) or aphidicolin (Aph), inhibitors of DNA synthesis, for 48 or 24 h respectively. These treatments effectively blocked cell division (Figs. 6A, C), but had little or no effect on the derepression of *nkd* or *hth* observed with *iswi*, *acf1* knockdown (Figs. 6B, D). These data are complicated by the fact that *iswi*, *acf1* RNAi caused already caused some derepression of the WG targets before the HU or Aph treatment (data not shown). However, the half-life of *nkd* mRNA is 5-9 h and that of *hth* about 14 h (Supplemental Fig. 3). Therefore, it is unlikely that the elevated levels of *nkd* and *hth* observed in non-dividing, *iswi*, *acf1* depleted cells are due to the residual mRNA transcribed before the inhibition of DNA synthesis. These data argue that the repression of WG targets by ISWI and ACF1 is not due to their role in post-mitotic chromatin assembly.

It is also possible that ISWI and ACF1 are required for maintaining histone/DNA integrity of WG targets during interphase. However, no significant change of H4 density to various regions of *nkd* and *hth*, including the regions bound by TCF, was detected when *iswi* and *acf1* were knocked down (Figs. 6E, F). Taken together, these data lead us to favor a model where ISWI and ACF1 specifically repress WG target gene transcription independently of chromatin assembly or maintenance.

WG signaling reduces ACF1 binding to WG targets

ACF1 is important for maintaining the silent state of WG target gene (Fig. 1C) and is physically present at these loci in the absence of signaling (Figs. 4C, D). Therefore, we were curious to see whether ACF1 binding to WG targets was regulated by WG signaling. After Kc cells were stimulated with WG-CM for 5 h, a consistent modest reduction of ACF1 binding was observed at various regions for all three WG targets (*hth*, *nkd* and *Notum*; Figs. 7A, B; data not shown).

Although this result implies that ACF1 binding to WG targets is not significantly regulated by WG signaling, it is possible that only a portion of the cultured cells were responding to the WG-CM stimulation. The unstimulated cells would elevate the background level of ACF1 ChIP signal and obscure a greater decrease of ACF1 binding by WG signaling. Activation of WG targets is correlated with the acetylation of histones H3/H4 throughout these loci (Parker et al., 2008). This suggests that the WG-stimulated chromatin could be selected by precipitation with acetylated H4 (AcH4) antibody. An ACF1 re-ChIP can be performed on this precipitate, to determine the binding of ACF1 on activated WG targets. As a control, a pan-H4 ChIP followed by an ACF1 re-ChIP was performed. For *hth*, we observed a pronounced decrease of ACF1 binding to the AcH4 associated chromatin compared to its binding to the pan-H4 associated chromatin, with the greatest reduction observed at the location of TCF binding (H(+16)) (Fig. 7C). These results suggest that WG signaling reduces ACF1 binding to the activated WG target genes.

Our results are in agreement with previous studies that have shown that acetylation of H4 N-terminal tails may interfere with ISWI-ACF1 function (Corona et al., 2002; Shogren-Knaak et al., 2006). To further explore the relationship between ISWI and ACF1 activity and histone H4 acetylation at WG targets, we performed AcH4 ChIP on unstimulated cells depleted for *iswi, acf1*. An increase of AcH4 binding to *hth,* especially at the TCF binding site, was observed when *iswi*, *acf1* was inhibited (Fig. 7D). This result suggests that ISWI and ACF1

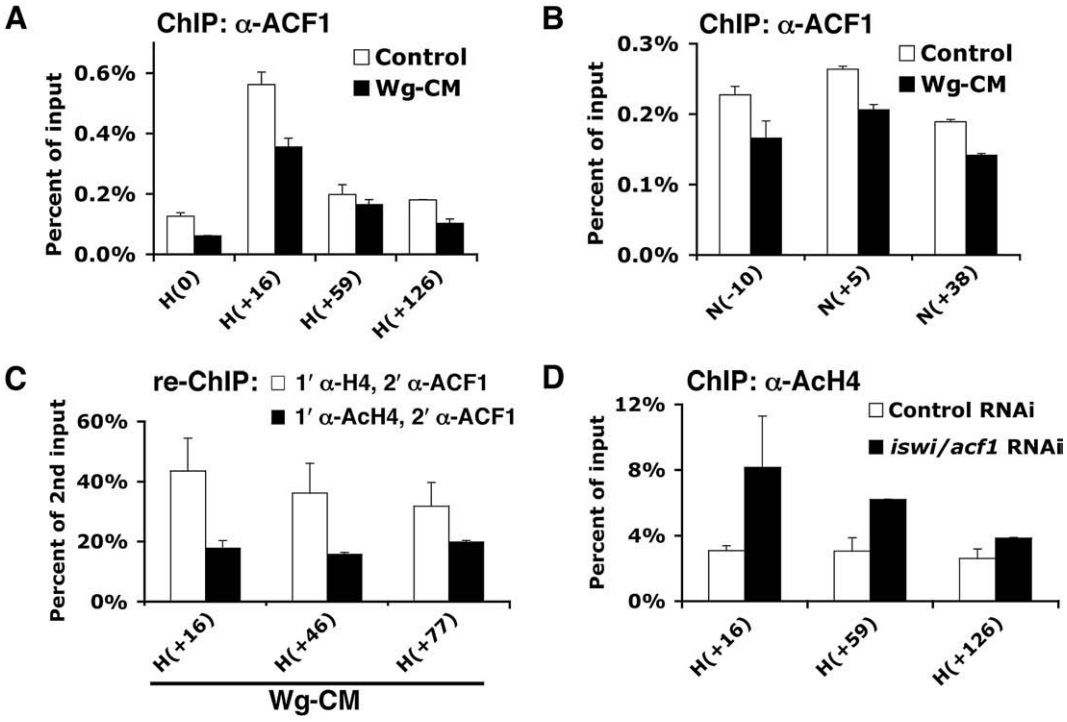

Fig. 7. ACF1 binding to WG targets is modestly reduced by WG signaling. (A, B) Cells were treated with control media or WG-CM for 5 h before harvesting for ACF1 ChIP analysis. A modest decrease of ACF1 binding was observed across the *nkd* and *hth* loci. (C) Less ACF1 binds to AcH4 on *hth* upon WG-CM treatment. Cells treated with WG-CM for 5 h underwent ChIP with either α-H4 antibody or α-AcH4 antibody, followed by a secondary ChIP with α-ACF1 antibody. The α-AcH4 antibody recognizes acetylated K5/8/12/16 on histone H4. The re-ChIP signal was normalized to the eluted solution from the first immunoprecipitate, termed the 2nd input. (D) ISWI and ACF1 antagonize AcH4 levels on *hth* in the absence of WG signaling. Cells were treated with control dsRNA or *iswi*, *acf1* dsRNA for six days before harvested for AcH4 ChIP analysis. An increase of AcH4 on *hth*, most prominently at the site bound by TCF, was observed. In general, data represent the means of duplicates (±S.E.), and all experiments have been performed at least two separate times with similar results.

antagonize the acetylation of histone H4 on WG targets in the absence of WG signaling.

Discussion

ISWI and ACF1 transcriptionally repress WG target genes

ISWI was recently found to co-purify with β-catenin, implying that it plays a positive role in Wnt signaling (Sierra et al., 2006). However, in this study, loss-of-function analyses for *iswi* and *acf1* revealed that they play negative roles in regulating WG targets in cultured cells (Fig. 1C, F). Furthermore, the expansion of Notum-lacZ and nkd-lacZ in *iswi* mutant cells in the wing imaginal disc (Fig. 2) indicates that this regulation occurs *in vivo* and at the level of transcription. Taken together, these data argue that ISWI and ACF1 act as transcriptional repressors of WG target genes.

Examination of genes not regulated by WG signaling suggests that ISWI and ACF1 are not general repressors of gene expression. In Kc cells, several genes were not affected by *iswi*, *acf1* depletion, including those adjacent to the *nkd* locus (Fig. 1D). In the wing imaginal disc, loss of *iswi* did not significantly alter WG, Dpp-lacZ or Spalt expression (Figs. 2C, G, K and 3B, E), targets of Notch, Hh and Dpp signaling, respectively. These results suggest some degree of specificity for ISWI and ACF1 towards WG targets.

Previous studies have shown that ISWI associates with ACF1 in two distinct chromatin remodeling complexes (Langst and Becker, 2001). Therefore, it is attractive to propose that ISWI and ACF1 act as a complex to repress WG targets. However, simultaneous knockdown of *iswi* and *acf1* resulted in higher derepression of targets than single RNAi treatments in Kc cells (Fig. 1C). Because RNAi does not completely abolish gene expression, these results are equivocal but could indicate that ACF1 and ISWI function independently of each other. Consistent with these proteins having non-redundant functions in WG target repression, ISWI and ACF1 have distinct distributions on the chromatin of WG targets (Fig. 4). Interestingly, a human homologue of ACF1, associates with a complex devoid of ISWI and regulates transcription (Kitagawa et al., 2003).

While ISWI and ACF1 both contribute to WG target gene repression in Kc cells (Fig. 1), the situation is different in the wing imaginal disc. In this tissue, complete removal of *acf1* had no effect on WG targets (Supplemental Fig. 2; data not shown), while cells lacking *iswi* showed ectopic expression of WG targets (Fig. 2). The lack of a detectable phenotype with *acf1* mutants in the wing disc could be due to redundancy. The closest relative to *acf1* in the fly genome is *toutatis (tou)*, which has been implicated in *Drosophila* neural development (Vanolst *et al.*, 2005). RNAi inhibition of *tou* in Kc cells had no effect on WG targets (data not shown) but it is possible that Tou and ACF1 both act with ISWI in the wing imaginal disc to repress WG targets. Deciphering the relationship between ISWI, ACF1 and Tou will require additional genetic analysis in flies.

In Kc cells, simultaneous depletion of *iswi* and *acf1* results in a greater derepression of several WG targets than depletion of either factor alone (Fig. 1C). This could be due to the fact that RNAi knockdown of gene expression is not 100% efficient. Alternatively, it could be evidence that ISWI and ACF1 have distinct functions. In this regard, it is interesting to point out that *iswi*, *acf1* double mutants have a more severe phenotype in terms of developmental rate and lethal phase than *iswi* single mutants, suggesting that they have non-redundant functions. Because of the early larval lethality of *iswi*, *acf1* double mutants, more sophisticated analysis will be required to examine the effect of removing both genes on WG targets in fly tissues.

Mechanism of ISWI-ACF1 regulation of WG targets

The repression of WG target gene expression by ISWI and ACF1 was independent of Arm (Fig. 1E). This distinguishes these factors from several other repressors that antagonize Wnt/WG signaling by interfering with β-catenin/Arm binding to TCF, such as ICAT (Tago et al., 2000), Chibby (Takemaru et al, 2003), CtBP-APC (Hamada and Bienz, 2004) and SOX9 (Akiyama et al., 2004). Rather, ISWI and ACF1 are required for silencing WG targets in the absence of pathway activation, similar to CtBP and Gro in Kc cells (Fang et al., 2006) and Kaiso in *Xenopus* embryos (Park et al., 2005).

ISWI and ACF1 are known to form a complex that can efficiently package DNA and nucleosomes into chromatin *in vitro* (Fyodorov and Kadonaga, 2002; Ito et al., 1999). However, we found that *iswi* and *acf1* were still required for silencing WG targets in non-dividing cells (Figs. 6B, D). This suggests that incomplete chromatin assembly after mitosis is not a major contributor to the derepression seen in *iswi*, *acf1* depleted cells. Consistent with this, inhibition of *iswi* and *acf1* did not alter the density of histone H4 on WG targets (Figs. 6E, F).

These data lead us to favor a model where ISWI and ACF1 act as specific transcriptional repressors of WG target genes. In line with a role as direct transcriptional repressors, ACF1 was found to be associated with the chromatin of several WG target genes (Figs. 4C, D; data not shown) while ISWI is clearly recruited to the same region of *hth* as TCF (Fig. 4F).

Although ISWI and ACF1 are thought to exist as a stable complex in solution (Langst and Becker, 2001), we found that they have distinct patterns on WG target gene chromatin. When detectable, ISWI has a localized pattern that mirrors that of TCF (Figs. 4F, G), while ACF1 is widely distributed across the entire loci (Figs. 4C, D; data not shown). This broad distribution of ACF1 is dependent on *iswi* (Figs. 5A, B), suggesting a model where ISWI-ACF1 is recruited to specific sites in WG targets, where ISWI remains, while ACF1 spreads along the chromatin.

If ACF1 binding to WG target chromatin is dependent on *iswi* (Figs. 5A, B), then why does RNAi inhibition of *acf1* and *iswi* result in a greater derepression of WG targets than *iswi* RNAi alone (Fig. 1C)? We suspect that this apparent discrepancy can be explained by the incomplete inhibition of gene activity by RNAi and the differences in sensitivity of the ACF1 ChIP assay and the qRT-PCR analysis of WG target gene expression. It is likely that in *iswi* depleted cells, there is still some ISWI (and ACF1) remaining on the chromatin that is undetectable by ChIP but still contributes to repression of transcription.

How are ISWI and ACF1 recruited to WG targets? The similar profile of ISWI and TCF localization on WG targets suggests that TCF is the transcription factor responsible. There are several examples in yeast and mammals where specific DNA-binding proteins recruit ISWI to chromatin (Alenghat et al., 2006; Bachman et al., 2005; Goldmark et al., 2000; Moreau et al., 2003; Yasui et al., 2002). However, binding of ACF1 or ISWI is not significantly reduced in *TCF* depleted cells (Figs. 5E, F).

In the absence of WG signaling, TCF contributes to target gene silencing (Cavallo et al., 1998; Fang et al., 2006). Depletion of *iswi* and *acf1* caused a marked reduction in TCF's binding to specific sites in WG targets (Fig. 5G, data not shown). While this suggests that ISWI and ACF1 act to repress these WG targets by promoting TCF binding, it is unlikely to be the whole story. Depletion of *iswi* or *acf1* with *TCF* or *gro* led to a non-additive derepression of WG targets (Fig. 5I; data not shown), suggesting that ISWI and ACF1 act independently of TCF in gene silencing. It appears that ISWI and ACF1 repress WG targets through multiple mechanisms, only some of which involve TCF.

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

decondensation (Corona et al., 2007; Deuring et al., 2000), which is correlated with a significant decrease in linker histone H1 deposition on chromatin (Corona et al., 2007). The link between linker histone H1 and transcriptional regulation is not obvious for most genes (Fan et al., 2005; Shen and Gorovsky, 1996), but we cannot rule it out as a mechanism as this time.

Regulation of ISWI and ACF1 binding to chromatin by WG signaling

Does activation of WG signaling affect ISWI or ACF1 binding to WG target genes? A modest reduction of ACF1 binding to target loci was observed after Kc cells were stimulated with WG-CM (Figs. 7A, B) and no reduction in ISWI binding was observed (data not shown). A marked decrease of ACF1 association with AcH4 was observed compared to its association with generic H4 (Fig. 7C). In addition, depletion of *iswi*, *acf1* resulted in increased AcH4 at a WG target (Fig. 7D). These results suggest that ISWI and ACF1 act antagonistically with AcH4 to regulate the transcriptional response to WG signaling. Interestingly, a similar relationship has been suggested in dosage compensation (Corona et al., 2002; Shogren-Knaak et al., 2006).

Our laboratory has recently shown that WG signaling induces a widespread increase in histone acetylation throughout WG targets (Parker et al., 2008). This chromatin modification requires CBP, a histone acetyltransferase that is recruited to WREs in a WG and Arm-dependent manner and is required for target gene activation (Li et al., 2007b; Parker et al., 2008). Therefore, it is tempting to propose that WG signaling promotes displacement or inactivation of ISWI-ACF1, by activating histone acetylation, which is necessary for transcriptional activation of WG targets.

Finally, it is possible that the residual binding of ACF1 to target genes after WG stimulation has functional relevance. In the presence of WG signaling, WG target genes were further activated upon depletion of *iswi* and *acf1* in Kc cells and in the wing imaginal disc (Fig. 1F and Fig. 2). Therefore, ISWI and ACF1 could have a dual function in regulating WG targets. In the absence of WG signaling, they help maintain the silent state of WG targets. When WG signaling is activated, the negative influence of ISWI and ACF1 may help to set the threshold for the precise activation of WG targets.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.08.011.

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