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The ISWI-containing NURF complex regulates the output of the canonical Wingless pathway

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Wingless (Wg) signalling regulates the expression of its target genes through Pangolin, Armadillo and their interacting co-factors. In a genetic screen for Wg signalling components, we found that imitation switch (ISWI), a chromatin-remodelling ATPase, had a positive role in transducing the canonical Wg signal, promoting the expression of the Wg target *senseless*. ISWI is found in several chromatin-remodelling complexes, including nucleosome remodelling factor (NURF). The effect of interfering with the function of other components of the NURF complex *in vivo* mimics that of ISWI. The NURF complex is also required for the efficient expression of other Wg target genes. Armadillo interacts directly with the NURF complex *in vitro* and recruits it to Wg targets in cultured cells. Together, our results suggest that the ISWI-containing NURF complex functions as a co-activator of Armadillo to promote Wg-mediated transcription.

Keywords: ISWI; NURF; signal transduction; Wingless
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INTRODUCTION

β-Catenin/Armadillo has an important role in regulating the expression of Wnt/Wingless (Wg) target genes. In the absence of the Wnt/Wg ligand, the cytosolic pool of β-catenin/Armadillo is constitutively targeted for degradation (Aberle *et al*, 1997; Pai *et al*, 1997). In the presence of Wnt/Wg, β-catenin/Armadillo is stabilized, accumulates and then enters the nucleus where it competes with co-repressors bound to T-cell factor/Pangolin (Tcf/Pan). β-Catenin/Armadillo then recruits various co-factors to form a complex that activates the expression of Wnt/Wg target genes (Stadeli *et al*, 2006).

The carboxy-terminal region of β-catenin/Armadillo (including the tail and the Armadillo repeats 11 and 12) recruits numerous transcriptional co-factors. Many of these, including the histone acetyltransferase CREB binding protein (CBP)/p300 (Hecht *et al*, 2000; Takemaru & Moon, 2000), the histone methyltransferase mixed lineage leukaemia 1 (MLL1)/SET1 (Sierra *et al*, 2006) and the peroxisomal assembly factor 1 (PAF1) complex component Hyrax/Parafibromin (Mosimann *et al*, 2006), are part of complexes involved in chromatin remodelling. In addition, the region of β-catenin comprising Armadillo repeats 7–12 recruits brahma related gene-1 (BRG-1)/Brahma, a chromatin-remodelling ATPase of the switch/sucrose nonfermentable (SWI/SNF) class (Barker *et al*, 2001). Here, we show that the nucleosome remodelling factor (NURF) complex (Xiao *et al*, 2001; Langst & Becker, 2001a), which contains another class of chromatin-remodelling ATPase, imitation switch (ISWI; Tsukiyama *et al*, 1995), is also recruited by the C-terminal region of Armadillo and promotes the transcription of a subset of Wg target genes.

RESULTS AND DISCUSSION

Genetic assays show ISWI is a Wg pathway component

Legless (Lgs) is a core component of the nuclear Wg signalling complex (Kramps *et al*, 2002; Hoffmans & Basler, 2004; Thompson, 2004; Hoffmans *et al*, 2005). Expression of a dominant-negative form of Lgs (Lgs^{17E}) in wing discs by *spalt* enhancer Gal4 (*salE*-Gal4) results in a notched-wing phenotype in the adult (Fig 1A,B; Mosimann *et al*, 2006), presumably owing to the compromised transcriptional output of the Armadillo activation complex. To uncover new components of the Wg signal transduction pathway, we performed an enhancer P element (EP) overexpression screen for genes that rescue this notched-wing phenotype. Hyrax, an essential co-activator of Armadillo, was identified using this set-up (Mosimann *et al*, 2006). We also found that the Lgs^{17E} phenotype could be rescued by an EP insertion driving the overexpression of ISWI, a chromatin-remodelling ATPase (Fig 1C,D). A qualitatively similar rescue was obtained by using a *UAS-iswi* transgene (Deuring *et al*, 2000), confirming that ISWI was responsible for the reversion (data not shown).

The expression of Lgs^{17E} generates a sensitized system in which Wg signalling is compromised. Reducing the functions of other positive regulators in this background should further impair the Wg signal. To confirm that ISWI does have a positive role, we tested whether the expression of a dominant-negative form ($ISWI^{K159R}$; Deuring *et al*, 2000) enhances the Lgs^{17E} phenotype. Consistent with a positive role for ISWI, the expression of $ISWI^{K159R}$ augmented the notched wing phenotype (Fig 1E,F).

To investigate further the function of ISWI in Wg signalling, we examined the effect of overexpressing ISWI on another sensitized system—the *sevenless*-Wingless (*sev*-Wg) assay. In this set-up,

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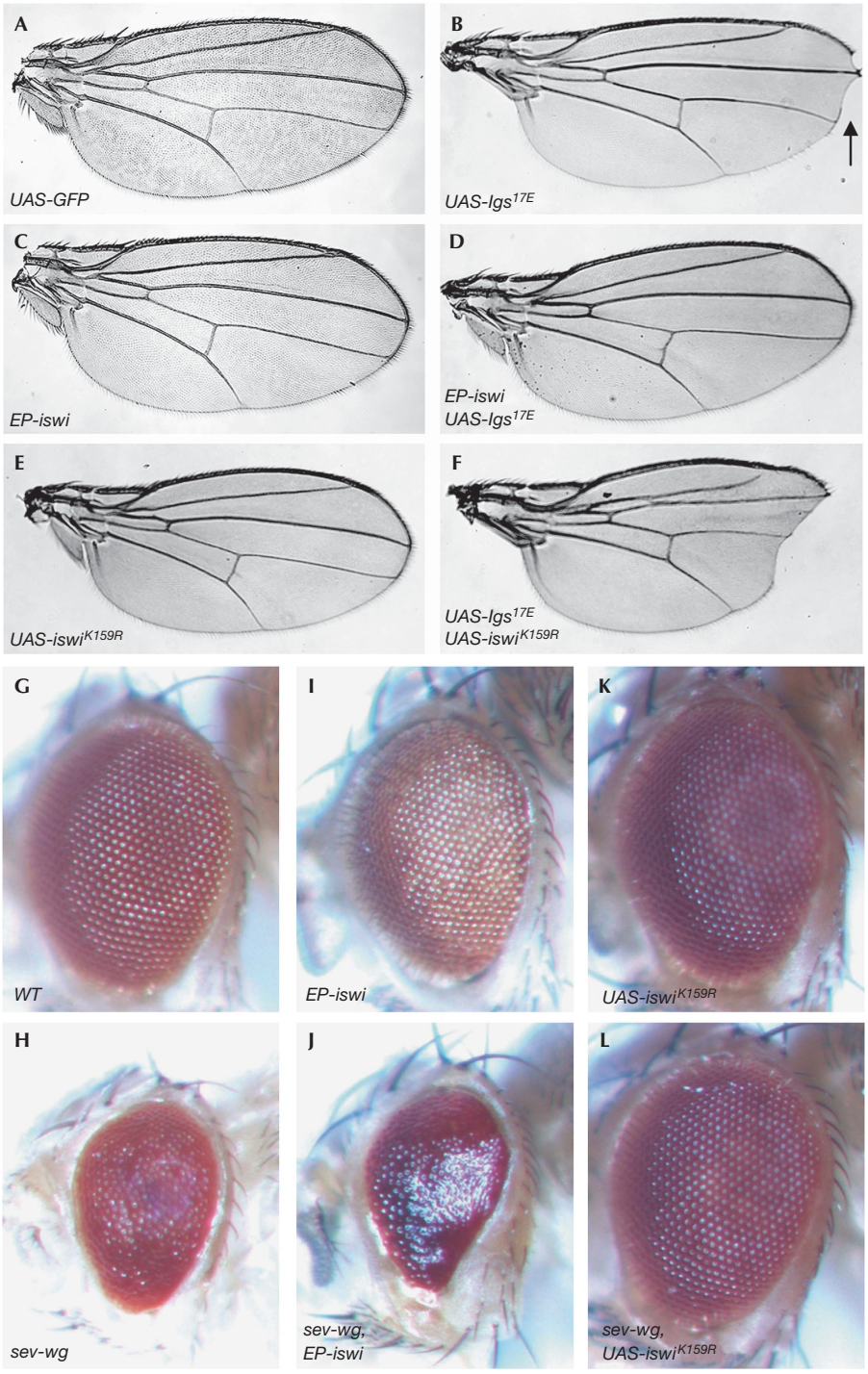

Fig 1 | The activity of ISWI is crucial when Wingless signalling is compromised in the wing and eye of *Drosophila*. *salE*-Gal4 drove the expression of (**A**) *UAS-GFP*, (**B**) *UAS-lgs*^{17E}, (**C**) *EP-iswi*, (**D**) *UAS-lgs*^{17E} and *EP-iswi*, (**E**) *UAS-iswi*^{K159R}, and (**F**) *UAS-lgs*^{17E} and *UAS-iswi*^{K159R}. The arrow in (**B**) indicates the notched-wing phenotype caused by Lgs^{17E}. (**G–L**) The rough eye phenotype generated by *sev*-Wg was affected by ISWI activity. (**G**) *GMR-Gal4/+*, (**H**) *GMR-Gal4/+; sev-wg/+*, (**I**) *GMR-Gal4/EP-iswi*, (**J**) *GMR-Gal4/EP-iswi; sev-wg/+*, (**K**) *GMR-Gal4/+; UAS-iswi*^{K159R}*/+*, and (**L**) *GMR-Gal4/+; UAS-iswi*^{K159R}*/sev-wg*. It has been reported that universal or strong expression of ISWI^{K159R} can affect cell viability (Deuring *et al*, 2000). However, on expressing ISWI^{K159R} alone using *salE*-Gal4 or *GMR*-Gal4, we did not observe any wing or eye phenotypes (**E,K**). We believe that the lack of an obvious effect on viability is due to the moderate expression levels achieved with these drivers and possibly because of the timing of the expression. EP, enhancer P element; GFP, green fluorescent protein; ISWI, imitation switch; Lgs, legless; *sev*, *sevenless*; Wg, wingless; WT, wild type.

the ectopic expression of Wg (*sev*-Wg) resulted in a rough eye phenotype (Fig 1G,H), which could be suppressed by mutations in essential components of the Wg pathway, including Pan, Armadillo, Lgs and Pygopus (Brunner *et al*, 1997; Kramps *et al*, 2002). The use of *GMR*-Gal4 to drive the expression of *EP-iswi* or *UAS-iswi* enhanced the rough eye phenotype (Fig 1I,J; data not shown), whereas the expression of *UAS-iswi*^{K159R} suppressed the rough eye phenotype (Fig 1K,L). Thus, through an independent series of assays, we confirmed that ISWI is a new positive regulator of Wg signalling.

ISWI promotes the expression of the Wg target *senseless*

To confirm that the expression of Lgs^{17E} at the centre of the wing discs by *salE*-Gal4 specifically weakened the transcriptional output of the Armadillo activation complex, and to test whether the co-expression of ISWI could specifically restore it, we monitored the expression of Wg and Notch targets (Giraldez & Cohen, 2003). Expression of the low-threshold Wg target *Distal-less* (*Dll*) was not affected by Lgs^{17E} (supplementary Fig S1A online). By contrast, the high-threshold Wg target *senseless* (*sens*) was clearly repressed (supplementary Fig S1B online). This is probably because Lgs^{17E} only reduced, but did not abolish, the transcriptional activity of the Armadillo activation complex. The residual activity was able to maintain the expression of *Dll*, but not that of *sens*, which require higher levels of Wg signal transduction. Expression of the Notch targets *wg* and *cut* at the dorsal–ventral (DV) boundary of the wing discs was not affected by Lgs^{17E} (supplementary Fig S1B′,C online). When ISWI was co-expressed with Lgs^{17E} at the center of the wing discs, *sens* expression was rescued (supplementary Fig S1E online); expression of *Dll*, *wg* and *cut* was not altered (supplementary Fig S1D,E′,F online). Interestingly, in a wild-type background, expression of ISWI by *salE*-Gal4 did not alter the expression of *Dll*, *sens*, *wg* or *cut* in wing discs (supplementary Fig S1G–I online) and caused no phenotype in adult wings (Fig 1C), suggesting that increased ISWI activity was not sufficient to enhance or ectopically induce Wg signalling during wing development. These results show that over-expression of *iswi* is able to ameliorate the effect of impaired transcriptional Armadillo activity, as caused by expressing Lgs^{17E}.

Proper expression of *sens* requires ISWI function

Next we investigated whether ISWI function was required for expression of the Wg target *sens*. We created *iswi* null clones in the wing disc using the *iswi*^{1} allele (Deuring *et al*, 2000). Consistent with previous reports that the loss of ISWI affects cellular viability, *iswi*^{1} clones had a growth disadvantage. To facilitate clonal analysis, we induced clones in a *Minute* background. To ensure that an effect on *sens* expression was specific, we monitored *wg* expression in the same wing disc. Generally, we found that 3 days after clone induction the expression of *wg* was not detectably altered in the clones (Fig 2E‴). However, *sens* expression was reduced in the *iswi* clones but not in wild-type clones (Fig 2E–E″,F–F″). *sens* expression started at the centre of the wing disc in mid-third instar and then extended to the periphery of both antero-posterior compartments (Fang *et al*, 2006). Interestingly, the reduction of *sens* expression was only detectable in discs when *sens* was not fully expressed. When Sens staining extended to the periphery of the wing disc, the expression of *sens* was no longer detectably altered in clones (supplementary Fig S2A–A″ online). These observations suggest that the ISWI function promotes, but is not essential for *sens* expression. Other transcriptional co-activators might compensate for the loss of ISWI at late stages. We also attenuated ISWI function in the posterior compartment of the wing discs by driving the expression of the dominant-negative ISWI^{K159R} with *en*-Gal4 (Fig 2A–D). Although we observed no effect on the levels of *wg* (Fig 2B,C‴) or *Dll* expression (Fig 2A–A″) in wing discs from third-instar larvae, the expression of *sens* was weakened. In contrast to the null clones, expression of ISWI^{K159R} could weaken the expression of *sens* even when Sens staining was fully extended (Fig 2C–C″). This difference could be due to the interference with other co-activators by the dominant-negative ISWI^{K159R} or the residual ISWI in null clones (Deuring *et al*, 2000).

NURF is required for expression of Wg target genes

There are at least four ISWI-containing chromatin-remodelling complexes in *Drosophila*: NURF, ATP-utilizing chromatin remodelling and assembly factor (ACF), chromatin remodelling and assembly factor (CHRAC) and a TATA binding protein related factor 2 (TRF2)-associated complex (Langst & Becker, 2001a; Hochheimer *et al*, 2002). ACF and CHRAC contribute primarily to the formation of repressive chromatin (Fyodorov *et al*, 2004), whereas the NURF complex (Fig 3A) is involved in both transcriptional activation and repression, and directly associates with histone H3 trimethylated at lysine 4 (H3K4me3; Wysocka *et al*, 2006). It has been reported that the loss of NURF301—the largest subunit of the NURF complex—results in phenotypes that are indistinguishable from those of *iswi* alleles (Badenhorst *et al*, 2002). We were therefore interested in examining whether the positive function of ISWI in Wg signalling was mediated through recruitment of the NURF complex. To test this, we examined the effect of *nurf301* loss of function on *sens* expression using the *nurf301*^{8} null allele (Badenhorst *et al*, 2005). Similar to *iswi* clones, *nurf301* clones have a growth disadvantage and were thus generated in a *Minute* background. At 3 days after clone induction, as for *iswi*, *sens* expression was repressed in slightly younger larvae, whereas it was only weakened in older larvae in which *sens* expression was fully extended (Fig 3D–D″; supplementary Fig S2C–C″ online). The expression of *wg* was not affected in *nurf301* clones (Fig 3D‴; supplementary Fig S2C‴ online). We also reduced NURF301 function in the posterior compartment of wing discs by RNA-mediated interference (RNAi). Consistent with our results so far, knockdown of NURF301 weakened *sens* expression without reducing Wg or Dll levels (Fig 3C–C‴; supplementary Fig S2B–B″ online). These flies survived to adulthood and showed notches in the posterior wing compartment—a phenotype seen when Wg signalling is impeded (Fig 3B).

Next we investigated whether the NURF complex was required for the expression of Wg targets other than *sens*. We addressed this question by knocking down NURF complex function in *Drosophila* Kc cells. To target the NURF complex specifically, we selected NURF301 as it is not present in other chromatin-remodelling complexes. NURF301 RNAi had no effect on the stability of ISWI (Fig 4E), ruling out an effect on other ISWI-containing complexes. The effect of NURF301 RNAi on the transcription of Wg target genes was examined by reverse transcription–PCR, in both unstimulated and stimulated cells (Axin RNAi; Blauwkamp *et al*, 2008). Four Wg target genes were tested: *naked cuticle* (*nkd*; Zeng *et al*, 2000; Chang *et al*, 2008),

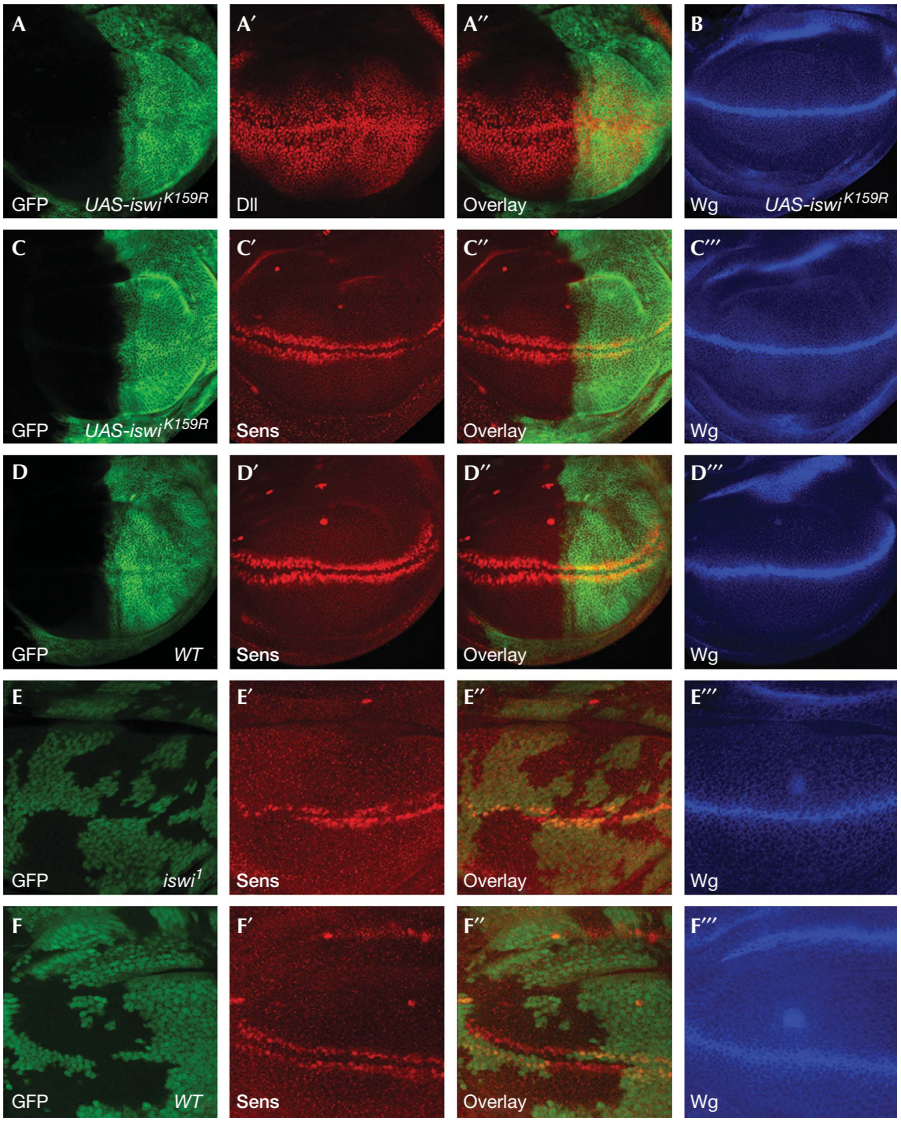

Fig 2 | ISWI activity is required for *sens* expression in the wing disc. (**A–C‴**) Expression of *UAS-iswi^{K159R}* in the posterior compartment of the wing disc was controlled by *en-Gal4, Gal80^{ts}* (second-instar larvae were preserved at 29 °C temperature for 2 days). Expression of (**A–A″**) *Dll* and (**B**) *wg* was not affected by ISWI^{K159R}. (**C–C‴**) The expression of *sens*, but not *wg*, was weakened by ISWI^{K159R} in the same wing disc. (**D–D‴**) The control disc expressing *en-Gal4, Gal80^{ts}* showed wild-type expression of *sens* and *wg*. (**E–E‴**) Expression of *sens*, but not *wg*, was weakened in *iswi* clones induced by *hs-flp; FRT42 ubi-GFP Minute/FRT42: iswi^{1}*. (**F–F‴**) The expression of *sens* and *wg* were unaltered in wild-type clones induced by *hs-flp; FRT42 ubi-GFP Minute/FRT42 +*. *Dll*, *Distal-less*; FRT, flippase recognition target; GFP, green fluorescent protein; hs-flp, heat shock-flippase; ISWI, imitation switch; *sens*, *senseless*; Wg, wingless; WT, wild type.

CG5895 (Mosimann *et al*, 2006), *frizzled 3* (*fz3*; Sivasankaran *et al*, 2000) and *CG6234* (Fang *et al*, 2006). NURF301 RNAi had no effect on the basal expression of these genes (Fig 4A–D). In stimulated cells, the induction of *CG5895*, *fz3* and *CG6234* was reduced, but not abolished: the induced transcription of *CG5895* was reduced by 30% (Fig 4B); the induction of *fz3* or *CG6234* was reduced by ⩾50% (Fig 4C,D). Interestingly, the induction of *nkd* was unaffected by NURF301 RNAi (Fig 4A). We observed similar results on using Wg-conditioned medium to stimulate the pathway (data not shown). Consistent with these results in wing discs from *nurf301* mutant larvae, the expression of several Wg targets was clearly reduced, whereas that of *nkd* was not (supplementary Fig S3 online).

Together with our finding that *Dll* expression is unaffected by reducing ISWI or NURF301 function (Fig 2A–A″; supplementary Fig S2B–B″ online), these data suggest that the NURF complex is needed to promote the expression of some, but not all, Wg targets.

Recruitment of the NURF complex by Armadillo

Is the NURF complex recruited to nuclear Armadillo? We investigated this question by testing for a direct interaction between Armadillo and components of the NURF complex.

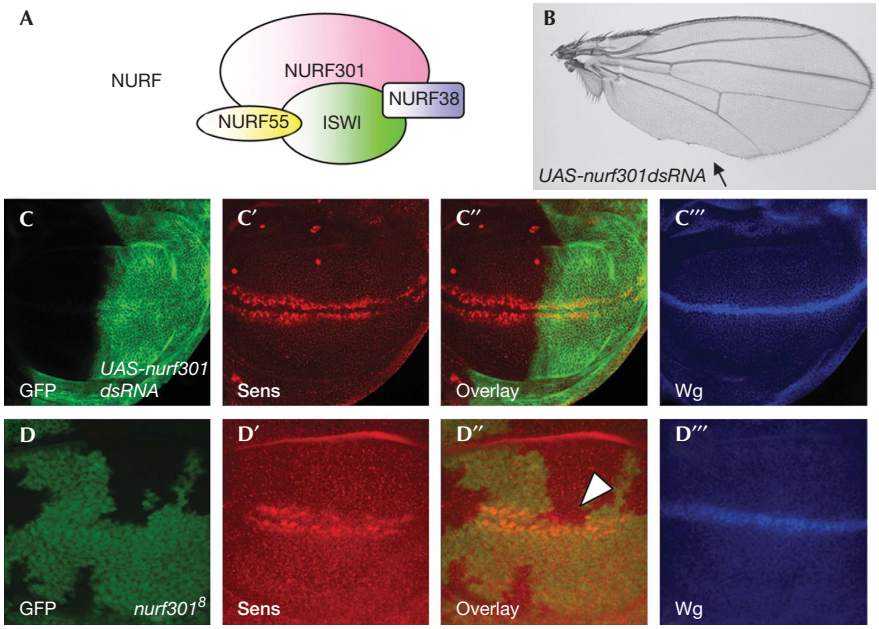

Fig 3 | The NURF complex is required for *sens* expression in the wing disc. (**A**) The NURF complex comprises four subunits. (**B–C″**) Expression of *UAS-nurf301dsRNA* in the posterior compartment of the wing disc by *en-Gal4*, *Gal80^{ts}* resulted in (**B**) a notched-wing phenotype in the posterior wing (arrow), and (**C–C″**) weakening of *sens* expression without affecting *wg* expression in the posterior wing disc. (**D–D″**) The expression of *sens*, but not *wg*, was weakened in *nurf301* clones induced by *hs-flp; ubi-GFP Minute FRT80/nurf301^{8} FRT80* (arrowhead). dsRNA, double-stranded RNA; GFP, green fluorescent protein; hs-flp, heat shock-flippase; NURF, nucleosome remodelling factor; *sens*, *senseless*; Wg, wingless.

Armadillo contains an amino-terminal region, 12 Armadillo repeats and a C-terminal tail (Fig 5A). The N-terminal region is phosphorylated by the destruction complex (Peifer & Polakis, 2000), whereas the Armadillo repeats and the C-terminal tail are known to act as interaction platforms for various co-factors (Hecht *et al*, 2000; Takemaru & Moon, 2000; Barker *et al*, 2001; Mosimann *et al*, 2006; Sierra *et al*, 2006). We expressed Armadillo repeat 1–8 (ArmR1–8), ArmR1–10, ArmR9–C and ArmR11–C as glutathione *S*-transferase (GST) fusion proteins and used them to pull down *in vitro* translated components of the NURF complex (Fig 5A). Unfortunately, we could not obtain *in vitro* translated NURF301, presumably owing to its large size (>300 kDa). Of the smaller NURF components tested, neither NURF55 nor NURF38 bound to Arm; however, we found that ISWI interacted with the C-terminal part of Arm, being pulled down by ArmR11–C and ArmR9–C (Fig 5B; data not shown). To test further whether Arm interacts with the NURF complex, we investigated whether an intact, purified NURF complex (Xiao *et al*, 2001) could be pulled down by the above Arm proteins. Consistent with the above results, we found that the ISWI subunit of the NURF complex was specifically pulled down by ArmR11–C. The NURF55 subunit, which does not interact directly with Arm, was co-immunoprecipitated (Fig 5C).

To test whether Arm can recruit the NURF complex to the Wg target genes, we performed chromatin immunoprecipitation (ChIP) experiments and monitored the binding of ISWI to the loci of *CG6234* and *nkd*, which contain validated clusters of Pan binding sites (Fang *et al*, 2006). At Pan sites in the *CG6234* locus, we observed a moderate ISWI signal in unstimulated cells that was not affected by NURF301 RNAi, suggesting the binding of another ISWI-containing complex. Wg stimulation increased the binding of ISWI at the Pan sites. This binding was greatly reduced by both Arm and NURF301 RNAi, confirming that on Wg signalling ISWI, in the context of the NURF complex, was recruited through Arm (Fig 5D). At the *nkd* locus, we did not observe the ISWI signal in unstimulated cells, whereas a moderate signal, which also depended on Arm and NURF301, was detected at the Pan sites on stimulation (supplementary Fig S4 online).

A recent report has found that, in the absence of Wg signalling, ISWI and the ACF/CHRAC component, ACF1, are required for the Pan-mediated basal repression of Wg target genes (Liu *et al*, 2008). Our data suggest that, in the presence of Wg signalling, the activation of Wg target genes also requires ISWI activity but in the presence of another complex, the NURF complex, which is recruited by Arm. Interestingly, it has been reported that SNF2H, a mammalian homologue of ISWI, also interacts with β-catenin in an equivalent domain (Sierra *et al*, 2006); in this case the functional relevance has not been noted.

Concluding remarks

The finding that two types of chromatin-remodelling ATPases—Brahma, of the SWI/SNF type (Barker *et al*, 2001), and ISWI (this report)—can be recruited by Armadillo to enhance target gene expression suggests the existence of more complex regulatory mechanisms in Wg-mediated transcription than previously anticipated. Both SWI/SNF and ISWI are able to rearrange chromatin structure, but they have distinct chromatin-remodelling activities (Racki & Narlikar, 2008). For example, it has been reported that SWI/SNF promotes the release of nucleosomes from DNA, whereas ISWI promotes their sliding (Langst & Becker, 2001b; Fan *et al*, 2003). In addition, it has been suggested that Brahma functions at a step before Pol II recruitment (Armstrong *et al*,

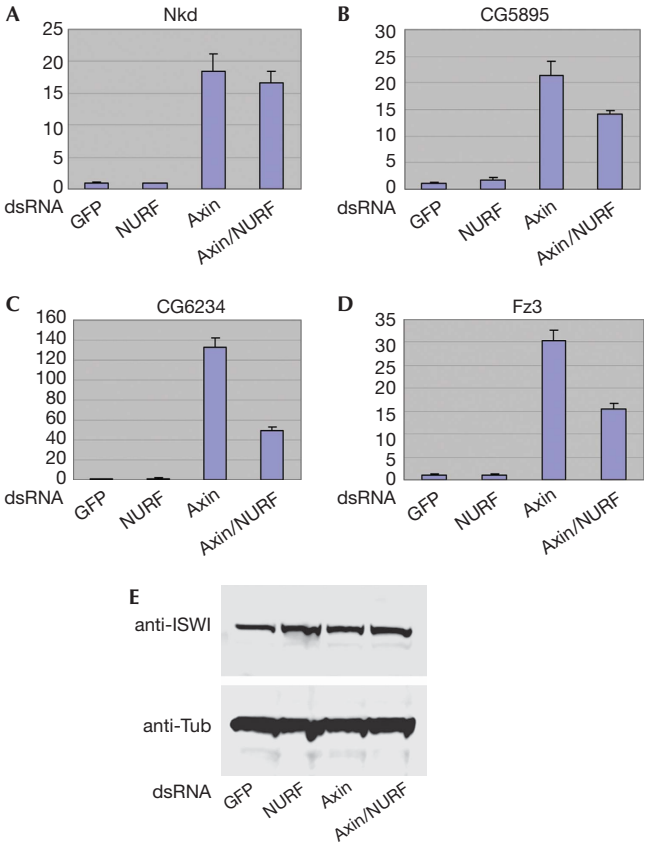

Fig 4 | The NURF complex is required for the transcriptional activation of some Wg targets in Kc cells. Cells were treated with the indicated dsRNAs for 4 days and total RNA were isolated for RT–PCR. The messenger RNA levels of actin, the ribosome protein L49 and TATA binding protein (TBP) were used as internal controls for normalization. Four Wg targets, (**A**) Nkd, (**B**) CG5895, (**C**) CG6234 and (**D**) Fz3 were measured for basal and activated transcription level. (**E**) The protein levels of ISWI and α-Tub were monitored in dsRNA-treated cells by Western blot. dsRNA, double-stranded RNA; Fz3, frizzled 3; GFP, green fluorescent protein; ISWI, imitation switch; Nkd, naked cuticle; NURF, nucleosome remodelling factor; RT–PCR, reverse transcription–PCR; Wg, wingless.

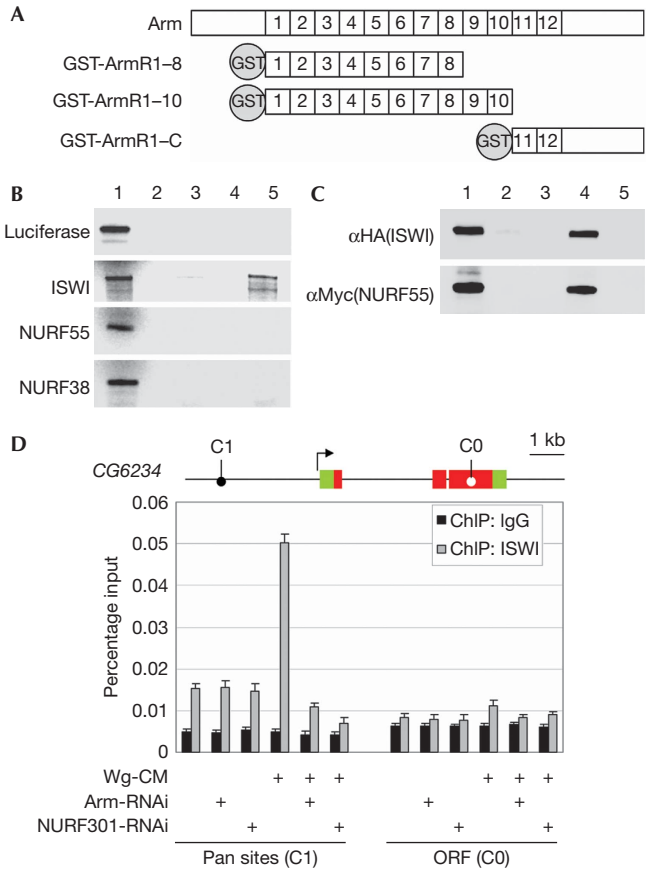

Fig 5 | Recruitment of ISWI and the NURF complex by Armadillo. (**A**) Domain structure of full-length Armadillo and truncated Armadillo fusion constructs. (**B**) *In vitro* translated samples were pulled down by Armadillo constructs and detected by autoradiography. Lane 1, 10% input. Lane 2, pulled down by GST. Lane 3, pulled down by GST-ArmR1–8. Lane 4, pulled down by GST-ArmR1–10. Lane 5, pulled down by GST-ArmR11–C. (**C**) The NURF complex was pulled down by Armadillo constructs. HA-tagged ISWI and Myc-tagged NURF55 were detected by western blot. Lane 1, 20% input. Lane 2, pulled down by GST-ArmR1–8. Lane 3, pulled down by GST-ArmR1–10. Lane 4, pulled down by GST-ArmR11–C. Lane 5, pulled down by GST. (**D**) The binding of ISWI at the *CG6234* locus. Kc cells were treated with control medium or Wg-conditioned medium (Wg-CM), combined with control RNAi, Arm RNAi or NURF301 RNAi. A locus containing Pan binding sites (C1) and a locus in the open reading frame (C0) were monitored for ISWI binding. ArmR, Armadillo repeat; ChIP, chromatin immunoprecipitation; GST, glutathione *S*-transferase; HA, haemagglutinin; ISWI, imitation switch; NURF, nucleosome remodelling factor; ORF, open reading frame; Pan, Pangolin; RNAi, RNA-mediated interference; Wg, wingless.

2002), while the preferential binding of NURF301 to H3K4me3 suggested the recruitment of the NURF complex at or near transcriptional initiation (Wysocka *et al*, 2006). We propose that Brahma and the ISWI-containing NURF complex are recruited sequentially by Armadillo. The later recruitment of the NURF complex is stabilized by binding to histone tails and functions to fine-tune Wg-mediated transcriptional activation, which is a crucial event for the expression of a subset of Wg target genes. An important step in the future will be to study the timing of the individual recruitment events and to identify the reasons that underlie the heterogeneity in the requirement of various co-factors.

METHODS

Protein interaction studies. [S^{35}]methionine-labelled proteins or the purified NURF complex was diluted in binding buffer (20 mM phosphate, pH 7.4, 200 mM NaCl, 2 mM EDTA, 5% glycerol, 0.2% Nonidet P-40, 0.5 mM dithiothreitol and protease inhibitor cocktail) and incubated with glutathione beads bearing GST or GST fusion proteins on a nutator for 3 h at 4 °C. After an extensive wash, the bound proteins were eluted and resolved by SDS–polyacrylamide gel electrophoresis and analysed by autoradiography or Western blotting.

ChIP. Kc cells were pre-treated with 5 mM dimethyl-3, 3′-dithiobispropionimidate dihydrochloride (DTBP; Sigma, St Louis,

MO, USA) on ice for 30 min and washed with 100 mM Tris (pH 8.0) and 150 mM NaCl. The cells were crosslinked with 1% formaldehyde at 25 °C, for 20 min, and ChIP assays were performed with the EZ-Magna ChIP kit (Upstate; Billerica, MA, USA). In all, 8×10^6 cells were used for each immunoprecipitation. Quantitative PCR primers for *CG6234* and *nkd* loci are described in Fang *et al* (2006) and sequence information is available upon request.

More Methods are available in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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