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SoxF is part of a novel negative-feedback loop in the *wingless* pathway that controls proliferation in the *Drosophila* wing disc

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Wnt molecules act as mitogenic signals during the development of multiple organs, and the aberrant activity of their pathway is often associated with cancer. Therefore, the production of Wnts and the activity of their signaling pathway must be tightly regulated. We have investigated the mechanisms of this regulation in the *Drosophila* hinge, a domain within the wing imaginal disc that depends on the fly Wnt1 ortholog *wingless* (*wg*) for its proliferation. Our results uncover a new feedback loop in the *wg* pathway in which the spatially restricted activation of the Sox gene *SoxF* (*Sox15*) by *wg* represses its own transcription, thus ensuring tight regulation of growth control. *rotund*, a wing proximodistal patterning gene, excludes *SoxF* from a thin rim of cells. These cells are thus allowed to express *wg* and act as the source of mitogenic signal. This novel mode of action of a Sox gene on the Wnt pathway – through transcriptional repression of a Wnt gene – might be relevant to human disease, as loss of human SoxF genes has been implicated in colon carcinoma.

KEY WORDS: *Drosophila*, SoxF, Organ growth, Wg, Wing imaginal disc

INTRODUCTION

One of the long-standing questions in biology is how organ growth is coordinated with tissue patterning. Research during recent decades has shown that a limited set of signals and signaling pathways control this coordination. Some of these signals are mitogenic, and their production at specific sites, called signaling centers, links spatial information to cell proliferation within developing organs (Freeman and Gurdon, 2002). Normal organ growth not only needs mitogens, but also mechanisms to control their production, transport, reception and/or transduction to ensure that proliferation is limited in space and time. Alterations in these control mechanisms often lead to disease.

The Wnt/β-catenin signaling pathway promotes cell proliferation during normal development and disease (Polakis, 2000). Wnts are lipid-modified glycosylated signaling molecules that can reach distant cells. Binding of Wnts to the receptor complex [composed of a Frizzled family receptor and an Arrow (LRP) co-receptor] results in the stabilization of the transcriptional co-factor β-catenin [*armadillo* (*arm*) in *Drosophila*]. Thereby, β-catenin/Arm accumulates in the nucleus, where it associates with Tcf/LEF DNA-binding transcription factors to regulate the expression of Wnt target genes (Gordon and Nusse, 2006). Research in a number of model organisms has demonstrated that the Wnt/β-catenin pathway controls cell proliferation in a variety of tissues, including the nervous system (Chenn and Walsh, 2002; Chesnutt et al., 2004; Dickinson et al., 1994) and the progenitors of the intestine and hematopoietic systems (Pinto et al., 2003; Willert et al., 2003) in mammals, and during imaginal disc development in *Drosophila* (Giraldez and Cohen, 2003; Johnston and Sanders, 2003; Neumann and Cohen, 1996). It is also known that most colorectal tumors, and a number of other tumor types, are caused by aberrant Wnt/β-catenin signaling (de Lau et al., 2007; Polakis, 2000), which underlines the necessity of tight regulation of this pathway.

The range and intensity of the signaling elicited by Wnt molecules have been shown to be regulated by many different mechanisms, including negative-feedback loops. These have been particularly well studied for the main *Drosophila* Wnt gene, *wingless* (*wg*). *wg* is required in the imaginal discs for the growth and patterning of the adult body structures (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). *wg* signaling results in the downregulation of its two receptors, *Dfz-2* (*fz2* – FlyBase) and *fz* (Cadigan et al., 1998; Muller et al., 1999) and in the upregulation of *Dfz-3* (*fz3* – FlyBase), a non-productive low-affinity receptor, and of the extracellular Wg inhibitor *Notum* (*wingful*) (Gerlitz and Basler, 2002; Giraldez et al., 2002; Sato et al., 1999; Sivasankaran et al., 2000). Intracellularly, high levels of *wg/Wnt* signaling induce the expression of two inhibitors of the pathway: *naked cuticle* (Rousset et al., 2001; Zeng et al., 2000) and *nemo* (Zeng and Verheyen, 2004). All these feedback loops result in an attenuation of the signal at the sites of maximal *wg* production and are generally implicated in all processes in which *wg* is required.

The *Drosophila* wing disc gives rise to the wing blade, the notum (body wall) and the hinge, which joins the wing blade to the body wall and articulates its movements (see Fig. 1A-D). *wg* is expressed in two concentric rings in the hinge domain (Baker, 1988) and has been shown to be required for the proliferation of hinge cells (Neumann and Cohen, 1996; Zirin and Mann, 2007). Moreover, *wg* overexpression is sufficient to drive hinge overgrowths without causing major repatterning (Neumann and Cohen, 1996; Whitworth and Russell, 2003). Therefore, the precise regulation of the *wg* pathway is crucial to control the growth of the hinge. The mitogenic effect of *wg* on hinge cells contrasts with its effect on the neighboring wing pouch cells which, upon similar *wg* overexpression, are mostly driven into sensory organ differentiation (Neumann and Cohen, 1996; Sanson et al., 1996). One prediction from these results is that the hinge-specific proliferative function of

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wg needs dedicated control mechanisms to ensure normal hinge size and shape. To identify these mechanisms, we searched genes that are differentially expressed in the hinge territory for a role in *wg*-mediated proliferation. *SoxF* (*Sox15*) belongs to the family of sequence-specific HMG Sox transcription factors and has been shown to be expressed in the prospective hinge of third larval stage (L3) wing discs (Cremazy et al., 2001). The functions of Sox genes have been extensively studied in mammals, in which they play essential roles during development (Kiefer, 2007). In addition, misregulation of Sox genes is often associated with cancer (Dong et al., 2004).

Only two of the eight Sox family genes present in the *Drosophila* genome have been studied in detail: *Dichaete* (*D*) and *SoxNeuro* (*SoxN*). They belong to the SoxB group and have prominent roles in embryonic segmentation and nervous system development (Overton et al., 2002). In addition, it has recently been shown that both genes negatively regulate the activity of the *wg/Wnt* pathway during cell fate specification in the embryonic epidermis (Chao et al., 2007; Overton et al., 2007).

Here, we report that *SoxF*, which is the sole member of this Sox group in *Drosophila*, is also required to restrain *wg* signaling, but using a novel mechanism: the transcriptional repression of *wg*. In the absence of *SoxF*, *wg* transcription spreads through the hinge causing its overproliferation. *SoxF* is itself under the control of the canonical *wg/Wnt* pathway such that *wg* and *SoxF* regulate each other's transcription through a feedback loop. Moreover, the expression of *rotund* (*rn*), which is part of the proximodistal patterning mechanism of the wing disc, allows the exclusion of *SoxF* from a thin rim of cells, allowing them to express *wg*. Thereby, this rim becomes a spatially well-defined mitogen-producing center necessary to ensure normal hinge growth. This novel mode of action of a Sox gene on the Wnt pathway – the transcriptional repression of a Wnt gene – might be relevant to human disease, as loss of human SoxF genes has been implicated in colon carcinoma.

MATERIALS AND METHODS

Generation and characterization of several mutant alleles of *SoxF*

In order to determine the role played by *SoxF* during hinge development, we first characterized one previously isolated *SoxF* allele, $Sox15^{KG09145}$ [now renamed $SoxF^{KG09145}$ (Bellen et al., 2004)]. The $SoxF^{KG09145}$ allele carries an insertion of the P[SUPor-P] transposon in an intronic region of the gene, which also harbors the *CG30071* transcript (see Fig. S2 in the supplementary material). Most homozygous $SoxF^{KG09145}$ flies die as pharate adults, and escapers are weak with held-out wings (see Fig. S2D in the supplementary material). This latter phenotype is indicative of hinge defects. In fact, these flies show abnormal proximal hinge structures: the sclerites, the alula and the costa are affected (see Fig. S2E,F in the supplementary material). Although the insertion does not affect *SoxF* coding sequence, we observed by RT-PCR (data not shown) and in situ hybridization (see Fig. S2B,C in the supplementary material) that *SoxF* expression is completely lost in the wing disc of mutant L3 larvae. As this P-element carries insulator sequences, we also checked by RT-PCR that expression of *CG30071* and of the 5′ neighboring gene, *RpS23*, was not affected by the insertion, which was indeed the case (see Fig. S2G in the supplementary material). We also generated new alleles by imprecise excision of the P transposon from the original allele. In addition to full revertants, we isolated more than ten mutant lines in which different lengths of intron sequences were deleted, without affecting the coding region, and which showed a range of phenotypic severity. These results suggest that this intronic region carries crucial elements for the regulation of *SoxF* expression. We also isolated some alleles that disrupt the coding sequence. Among them, $SoxF^{26}$ is specific to the *SoxF* gene and deletes the first exon and part of the first large intron, and is therefore likely to be a null allele (see Fig. S2A in the supplementary material). This allele has the same phenotype as the initial insertion. In addition, the phenotype and escaper rates of individuals carrying $SoxF^{KG09145}$ over a deficiency uncovering the *SoxF* locus, Df(2R)Exel7130, are the same as for homozygous $SoxF^{KG09145}$ flies. Therefore, $SoxF^{KG09145}$ behaves as a genetic null allele. Cremazy and co-workers (Cremazy et al., 2001) reported that *SoxF* is expressed in the embryonic peripheral nervous system (PNS). We obtained adult escapers of the molecular null allele $SoxF^{26}$. These animals, in addition to their abnormally folded wings, are weak and die shortly after eclosion. Other hinge mutants, such as *wg spd-fg*, are much healthier. Therefore, it is possible that the larval lethality and weakness of adult escapers is due to abnormal PNS development.

Other fly stocks and genetic manipulations

UAS-SoxF was generated by cloning the full-length *SoxF* coding region from the cDNA clone IP09065 as an *Eco*RI/*Xho*I fragment into the pUASt plasmid. Transgenic flies were generated by standard methods. To analyze, comparatively, the effects of gene overexpression using the *GAL4/UAS* system (Brand and Perrimon, 1993), the genotypes were synthesized to contain the same number of *UAS* sequences by including a 'neutral' *UAS-GFP* if needed.

GAL4 lines

wg-GAL4, Bx^{MS1096}*-GAL4* (FlyBase); $zfh2^{MS209}$*-GAL4* (Whitworth and Russell, 2003).

UAS lines

UAS GFP-Wg (Pfeiffer et al., 2002), $UAS\text{-}Arm^{S10}$ (UAS-Arm*), $UAS\text{-}dTCF^{DeltaN}$ (TCF^{DN}) and *UAS-GFP* (FlyBase); *UAS-rn* (St Pierre et al., 2002) was a gift from J. P. Couso; *UAS-dsSoxF* (number 45482, *Drosophila* Genetic Resource Center, Kyoto, Japan).

Reporter *lacZ* lines

puckered-lacZ (puc^{E69}), *wg-lacZ* (FlyBase), *SpFlag-lacZ* (Neumann and Cohen, 1996), *rn-lacZ* (St Pierre et al., 2002).

Mutant strains

$wg^{spd\text{-}fg}$ (Neumann and Cohen, 1996), *wgCX3* (Klein and Arias, 1998) w^{1118}; Df(2R)Exel7130, P+Pbac[XP5.WH5] Exel7130/CyO (FlyBase).

Clonal analysis

The allele $SoxF^{KG09145}$ was recombined onto an *FRT42D* chromosome using standard genetic procedures. Mitotic recombination $SoxF^{KG09145}$ clones were generated by the FRT/FLP method (Xu and Rubin, 1993) in L1-2 larvae from the cross between *FRT42D* $SoxF^{KG09145}$*/CyO* males to *hsFLP122;;FRT42D ubi-GFP* females. To induce the clones, 24-72 hours after egg laying (AEL) larvae were heat shocked at 37°C for 30 minutes. Mutant tissue was detected by the absence of the GFP marker.

SoxF and $dTCF^{DeltaN}$ overexpression clones were obtained by incubating *yw hs-FLP122; act>y+>Gal4, UAS-GFP/+ UAS-SoxF/+* or $UAS\text{-}dTCF^{DeltaN}$/+ larvae for 10 minutes at 35.5°C at two developmental times (48-72 hours and 48-96 hours AEL). *wg*, *rn* and *arm* overexpression clones were obtained by crossing males of their respective *UAS* lines to *yw122, act>hsCD2>GAL4* females (Basler and Struhl, 1994). Larvae from the crosses were heat shocked for 10-20 minutes at 35.5°C between 48 and 96 hours AEL. To mark the clones, CD2 was induced by subjecting late L3 (wandering) larvae to a 30-minute heat shock at 37°C, followed by a 30 minute recovery period at 25°C just prior to dissection.

Immunostaining, in situ hybridization and BrdU incorporation

The antibodies used for immunostaining were: mouse anti-β-galactosidase (Sigma, 1/1000) and rabbit anti-β-galactosidase (Cappell, 1/1000), mouse anti-GFP (Invitrogen, 1/1000), mouse anti-CD2 (Serotec, 1/400), mouse anti-Nub (Ng et al., 1995), rabbit anti-Tsh (Wu and Cohen, 2000), mouse anti-Wg (4D4, Developmental Studies Hybridoma Bank, Iowa University, 1/100), guinea pig anti-Hth (Casares and Mann, 1998), mouse anti-Arm (N27A1, Developmental Studies Hybridoma Bank, 1/50), rabbit anti-cleaved Caspase 3 (Cell Signaling, 1/500). Appropriate secondary antibodies were conjugated to Alexa 488, 568 or 647 (Invitrogen, 1/800). After dissection and fixation, larvae were incubated with primary antibodies overnight at 4°C or for 2 hours at room temperature.

Rhodamine-phalloidin staining (Invitrogen, 1/400) was performed during secondary antibody incubation or was added directly to the mounting medium.

BrdU incorporation followed standard protocols (Sullivan et al., 2000). Discs were incubated for 30 minutes in a 10 mM BrdU (Roche) solution, and BrdU was detected with a mouse anti-BrdU antibody (Roche, 1/400).

Fluorescent in situ hybridization was performed as described (Vanzo and Ephrussi, 2002) with minor modifications. *SoxF* antisense RNA probes were synthesized from a plasmid that contains the coding sequence of SoxF or the probe described by Cremazy et al. was used (Cremazy et al., 2001), with incubation at 65°C or 55°C, respectively. Probes were labeled with digoxigenin (Dig), and detected with an alkaline phosphatase-conjugated anti-Dig antibody (1/1000), both from Roche. Signal was developed using Fast Red tablets (Roche) followed by standard immunostaining (Vanzo and Ephrussi, 2002). Confocal image acquisition was performed on a SP2-AOBS confocal system (Leica). Stacks of (*x*,*y*) sections were recorded along the *z*-axis every 1 µm. Single *z*-sections ('cross-sections') were recorded as (*x*,*z*) confocal sections, with a *z*-step of 1 µm. In some cases, maximum or average projections of the *z*-series were produced in order to visualize the total signals in the samples. Confocal data processing was performed using Adobe Photoshop.

RNA extraction and RT-PCR

RNA extraction was performed using the RNeasy Kit (Qiagen). For each genotype, eight larvae were collected in lysis buffer (RLT, RNeasy, Qiagen) and ground with a pestle in an Eppendorf tube. The lysate was passed through a QIAshredder column (Qiagen) to optimize extraction and DNA digestion was performed during the process of extraction.

For the RT-PCR reactions, 5-7 µg of RNA was used for the first-strand cDNA synthesis (SuperScript First-Strand Synthesis Kit, Invitrogen). PCR was performed using 2 µl of the first-strand synthesis reaction with GoTaq polymerase (Promega). PCR conditions were: 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 42°C. Primers were: L1-SoxF (5′-TGCAACTGCAACAACATCAA-3′) and R1-SoxF (5′-GTCAGATAGCC-ACCGTGCTC-3′), which amplify a fragment specific to the *SoxF* transcript; L1RpS23 (5′-AGATCTTGGGCGTTCCTTCT-3′) and R1Rps23 (5′-TTGCAATCCAAATCACAGGA-3′) for the *RpS23* gene; L1CG30071 (5′-AGAAGCTGGAGCAGAAGCTG-3′) and R1CG30071 (5′-GCTGCTGAATTCTTGGAAGG-3′) for the *CG30071* gene; L1-8394 (5′-GCGATGGCGAGTATAGGAAC-3′) and R1-8394 (5′-CAGCGATA-CGATGAACATGC-3′) for the *CG8394* gene. For the *SoxF*, *CG8394* and *RpS23* genes, amplification was specific for the corresponding messenger RNAs, as the primers were designed against coding sequences that are separated by introns in the pre-mRNAs.

RESULTS

SoxF is specifically expressed in the hinge domain of the *Drosophila* wing disc

During the three larval stages (L1-3), the wing disc is progressively subdivided in three concentric domains: the prospective body wall, the hinge and the wing blade (Fig. 1). In L3 discs, these different domains are clearly demarcated by folds in the epithelium (Fig. 1A). The hinge is formed by two concentric bands of tissue (Fig. 1A,B) that will give rise to the distal hinge, which is contiguous with the wing blade, and the proximal hinge, which forms the axillary sclerites of the wing articulation (Bryant et al., 1978; Casares and Mann, 2000; del Alamo Rodriguez et al., 2002; Neumann and Cohen, 1996).

During L2, prospective distal hinge cells express the POU gene *nubbin* (*nub*) (Ng et al., 1995; Zirin and Mann, 2007), while proximal hinge cells express the zinc-finger gene *teashirt* (*tsh*) (Azpiazu and Morata, 2000; Fasano et al., 1991; Soanes and Bell,

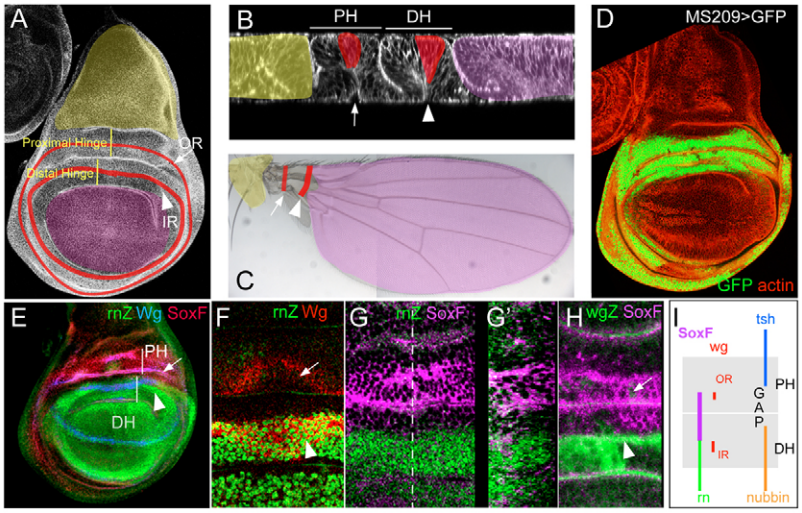

Fig. 1. *SoxF* is expressed in the hinge abutting the *wg* IR and *rn* domains. (**A**,**B**) Wild-type wing imaginal disc of a late L3 *Drosophila* larva counterstained with the actin marker rhodamine-phalloidin (B is a cross-section of the disc shown in A). The prospective hinge is formed by two concentric folds of the disc's epithelium that surround the wing pouch (pink). These two folds, called the distal (DH) and proximal (PH) hinge, give rise to the adult distal and proximal hinge structures, respectively, that articulate the wing blade (distal) (false-colored pink) with the thorax (body wall, false-colored yellow). The *wg* outer (OR) and inner (IR) rings are marked by an arrow and an arrowhead, respectively, throughout the figure, and are false-colored as red stripes in A-C. (**C**) Adult structures derived from the wing disc: wing blade (pink), notum (yellow; only a portion of this structure is shown) and hinge. The position of the two *wg* expression stripes in the hinge is marked with an arrow (IR) or arrowhead (OR). (**D**) Late L3 wing disc of *MS209-GAL4;UAS-GFP*. This line drives GFP expression specifically in hinge cells. The disc is counterstained with rhodamine-phalloidin. (**E**) *rn-lacZ* (rnZ) disc stained for β-galactosidase, Wg protein and *SoxF* transcription (fluorescent in situ hybridization). (**F**) High-magnification view of the dorsal hinge region of a *rn-lacZ* wing disc also stained for Wg antigen. The expression of *wg* in the DH (IR; arrowhead) lies at the border of the *rn-lacZ* domain. (**G**) High-magnification view of the dorsal hinge region of a *rn-lacZ* wing disc stained for β-galactosidase and *SoxF* transcription (fluorescent in situ hybridization). (**G′**) Confocal cross-section though the dashed line in G. The expression of *SoxF* abuts the *rn-lacZ* domain. (**H**) *wg-lacZ* (wgZ) disc stained for β-galactosidase and *SoxF* transcription (fluorescent in situ hybridization). *SoxF* transcription abuts the *wg* IR (arrowhead). In addition, *SoxF* overlaps the *wg* OR (arrow). (**I**) Schematic representation of the pattern of expression of *SoxF* relative to *tsh*, *nub*, *rn* and *wg*. The expression of *SoxF* straddles the gap domain delimited by Tsh and Nub. All discs (except in B) are with dorsal up and posterior to the right.

2001) (summarized in Fig. 1I). At the beginning of L3, a ring of *wg* expression appears in the prospective distal hinge, the so-called *wg* inner ring (IR). The *wg* IR expression is included within the *nub* domain in cells that also express the Kruppel-like transcription factor *rn* (del Alamo Rodriguez et al., 2002), and is driven by a specific regulatory element, the spade-flag (*spd-fg*) enhancer (Neumann and Cohen, 1996). Starting in early L3, *wg* IR drives intercalary proliferation between the *nub* and *tsh* domains generating a region that expresses neither of the two, the so-called gap domain (Zirin and Mann, 2007). By late L3, a second ring of *wg*, called the *wg* outer ring (OR), appears in the prospective proximal hinge, and abuts the distal limit of *tsh* expression (see Fig. 1I). In this paper, we focus on the regulation of the expression and function of the *wg* IR domain, as it has a major role in controlling hinge proliferation.

To identify genes that are differentially expressed in the hinge, we genetically marked hinge cells by driving GFP with the hinge-specific driver *zfh-2^{MS209}-GAL4* (Fig. 1D). This driver reproduces the pattern of the *zfh-2* (*zfh2*) gene, which is expressed in most hinge cells (Terriente et al., 2008; Whitworth and Russell, 2003). GFP^{+} (hinge) and GFP^{-} (body wall plus wing blade) cells were FACS sorted and their transcriptome profiles compared (a full account of this analysis will be published elsewhere). This experiment identified *Drosophila Sox15* (*CG8404*) as the transcript most over-represented in hinge cells. Recently, Bowles and co-workers reassigned this gene to the SoxF group of the Sox family, making it the sole *Drosophila* member of the group, which in mammals includes *Sox7*, *Sox17* and *Sox18* (Bowles et al., 2000). We adopt their nomenclature and hereafter refer to this gene as *SoxF*.

SoxF had been reported to be transcribed in the hinge of late L3 wing discs (Cremazy et al., 2001). We further mapped the *SoxF* domain relative to *wg* and *rn* reporters by in situ hybridization. The *SoxF* domain abuts *rn* and the *wg* IR on its distal border and extends into the proximal hinge overlapping the late *wg* OR (Fig. 1E-H; see Fig. S1 in the supplementary material; data not shown). Therefore, the realm of *SoxF* expression straddles the gap domain (Zirin and Mann, 2007). This adjacent, non-overlapping expression between *SoxF* and the domains of *rn* and *wg* is also observed at earlier stages (see Fig. S1 in the supplementary material).

The loss of *SoxF* function leads to hinge-specific overproliferation without loss of hinge identity

In order to determine *SoxF* function, we analyzed wing imaginal discs from larvae homozygous for the null allele *SoxF^{KG09145}* (see Materials and methods). *SoxF* mutant wing discs showed hinge overgrowths (Fig. 2) that caused misfolding of both its dorsal and ventral regions. However, the wing pouch and body wall regions seemed unaffected. To determine the origin of these overgrowths within the hinge, we mapped them relative to the expression of *nub*, *tsh* and the intervening gap domain (Fig. 2A,B). In *SoxF* mutant discs, both the prospective distal hinge, which expresses *nub*, and the gap domain were significantly enlarged (Fig. 2A,B). In addition, the overgrown hinge still expressed high levels of *homothorax* (*hth*) (not shown), which is a hinge marker (Azpiazu and Morata, 2000; Casares and Mann, 2000). Therefore, the tissue overgrowth observed in the *SoxF* mutants correlates with the *SoxF* expression domain, suggesting that *SoxF* has an autonomous effect on the control of hinge proliferation. In addition, the overgrowth cannot be explained by changes in cell fate because we still detected normal expression of hinge-specific markers.

In *SoxF* mutant discs, we detected elevated levels of incorporation of the S-phase marker BrdU specifically in the hinge, indicating that the overgrowths were in fact caused by increased cell proliferation (Fig. 2C,D). In addition, we noted an increase in apoptotic cell death,

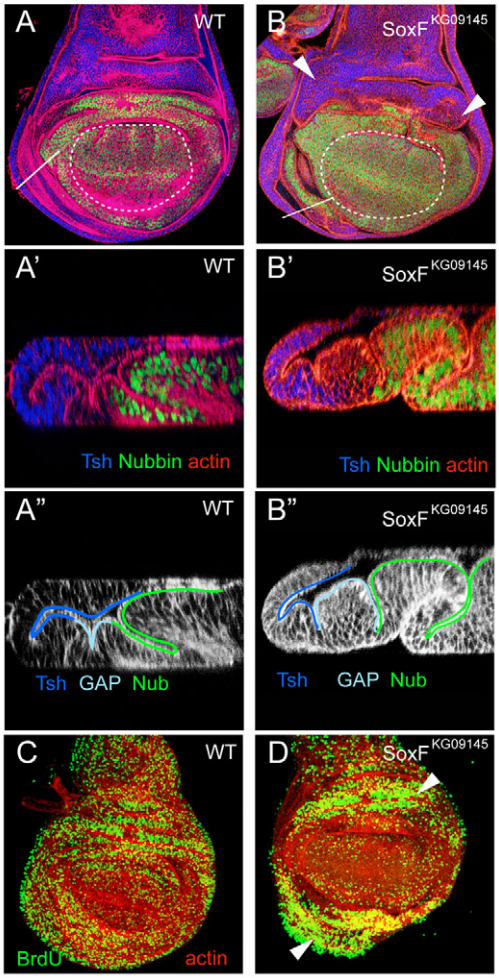

Fig. 2. Loss of *SoxF* function leads to overproliferation of hinge cells without repatterning. (**A**,**B**) Wild-type (A) and *SoxF* mutant (B) late L3 *Drosophila* wing discs stained for Nub (green), actin (rhodamine-phalloidin, red), and Tsh (blue). The wing pouch is outlined by the dashed line. The solid line in A and B marks the position of their corresponding cross-sections as shown in A′,A″ and B′, B″, respectively. In a wild-type disc (A), Nub marks the pouch and distal hinge cells and Tsh is specifically expressed in the proximal hinge and the notum. In *SoxF^{KG09145}* mutant discs (B), the prospective hinge is larger and shows extra folds both ventrally and dorsally (arrowheads). (**A′-B″**) The overgrown tissue mostly comprises the gap domain (compare B′,B″ with A′,A″). (**C**,**D**) Proliferation, as monitored by BrdU incorporation (S-phase marker), in wild-type (C) and *SoxF^{KG09145}* mutant (D) discs. A hinge-specific increase in BrdU incorporation (green) is seen in the mutant disc (arrowheads) relative to the wild type. To detect total BrdU signal, C and D are maximum projections of *z*-stacks of confocal sections. The discs were counterstained with rhodamine-phalloidin (actin, red) to visualize disc morphology.

as detected by activated Caspase 3 (Decay – FlyBase) staining (see Fig. 4D). This apoptosis is associated with activation of the Jnk pathway, as indicated by the upregulation of a transcriptional reporter of the Jnk target *puckered* (not shown).

SoxF blocks *wg* transcription in the hinge through the *spd-fg* enhancer

Since expression of *wg* at the IR is necessary for, and sufficient to induce, the proliferation of hinge cells (Neumann and Cohen, 1996; Zirin and Mann, 2007), we asked whether its expression was altered

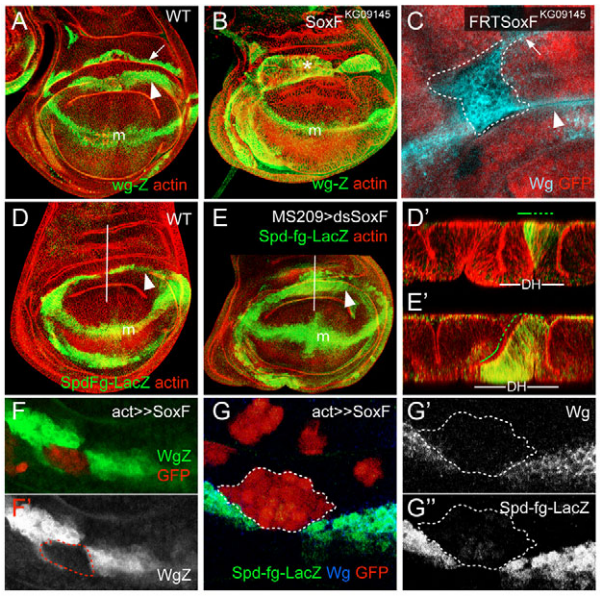

Fig. 3. *wg* is derepressed in the hinge of *SoxF* mutant discs. (**A**,**B**) *wg-lacZ* expression in wild-type (A) and $SoxF^{KG09145}$ mutant (B) L3 *Drosophila* wing discs, counterstained with rhodamine-phalloidin. The *wg-lacZ* signal is the projection of a series of confocal *z*-sections to allow visualization of the full pattern. *wg* expression spreads through the hinge region of *SoxF* mutant discs and the signal is stronger, especially in the ventral disc region. In A-E, the arrowheads, arrows and 'm' mark *wg* expression in the distal hinge, proximal hinge and wing pouch margin, respectively. In B, the expanded domain of *wg* is marked by an asterisk. (**C**) $SoxF^{KG09145}$ clone, marked by the absence of GFP (red), shows derepression of Wg (blue). (**D**,**E**) *spd-fg-lacZ* expression in L3 wing discs in a wild-type (D) or *MS209>dsSoxF* (E) background. This latter genotype knocks down *SoxF* transcription by inducing in the hinge an interference construct. Lines in D and E indicate the position of the cross-sections shown in D′ and E′. As in A and B, the *lacZ* signal is a projection and the discs are counterstained with rhodamine-phalloidin. In *MS209>dsSoxF* discs (E), the *spd-fg-lacZ* pattern in the distal hinge broadens relative to that in the wild type (D). (**D′**,**E′**) Cross-sections. The green line marks the extent of *spd-fg-lacZ* expression in the distal hinge (DH). The distal hinge fold is also indicated as a reference. (**F-G″**) Flip-out clones expressing *SoxF*, induced between 48 and 72 hours AEL, are marked positively by GFP (red). These clones cell-autonomously repress *wg-lacZ* (F), the hinge-specific *wg* reporter *spd-fg-lacZ* (G) and Wg (G). Merged (F,G) and single channels (F′,G′,G″) are shown.

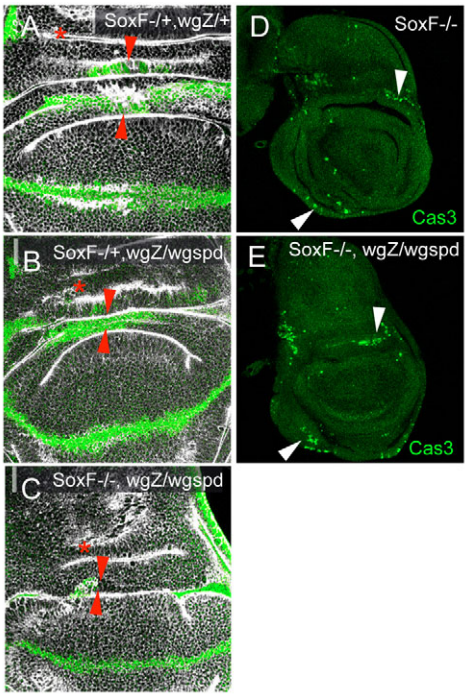

Fig. 4. *wg* drives proliferation in *SoxF* mutant hinge cells. (**A-C**) Late L3 wing discs from control (A), $wg^{spd\text{-}fg}$ (B) and $SoxF^{KG09145}$, $wg^{spd\text{-}fg}$ (C) mutant *Drosophila* larvae, stained for actin (white) and β-galactosidase (*wg-lacZ*, green). The width of the hinge is visualized as the distance between the *wg* IR and OR, which are marked by red arrowheads. A notal fold (asterisk) is marked as a reference. (**D**,**E**) Similar levels of apoptosis, as detected with anti-activated Caspase 3 (Cas3), are observed in the hinge region (arrowheads) of both $SoxF^{KG09145}$ (D) and $SoxF^{KG09145}$, $wg^{spd\text{-}fg}$ (E) mutant wing discs.

in *SoxF* mutants. We compared the expression of a *wg-lacZ* transcriptional reporter in wild-type and $SoxF^{KG09145}$ mutant wing discs. In wild-type discs, *wg-lacZ* is expressed in two distinct rings in the hinge, IR and OR, separated by a non-expressing region (Fig. 3A). However, in *SoxF* mutant discs, *wg* transcription spread throughout the hinge and no *wg*-negative territory remained (Fig. 3B). When we examined the effect of removing *SoxF* function in clones, we observed effects on *wg* expression only in clones spanning the hinge. In these clones, *wg* expression filled the domain between the IR and OR rings, which thus became connected (Fig. 3C). Sox mutant clones in the wing pouch or prospective notum had no effect on *wg* (not shown). These results indicate that *SoxF* is required cell-autonomously to repress *wg* transcription in the domain that separates the IR and OR.

The expression of *wg* in the IR is controlled by the *spd-fg* enhancer which, when linked to *lacZ*, drives reporter gene expression in the IR and wing margin expression domains of *wg* (Neumann and Cohen, 1996). In wild-type discs, *spd-fg-lacZ* expression was seen as a narrow stripe centered in the prospective distal hinge fold (Fig. 3D,D′). However, in discs in which the expression of *SoxF* had been knocked down by RNAi ($zfh\text{-}2^{MS209}$-*GAL4; UAS-dsSoxF*), the expression of this reporter was considerably wider (Fig. 3E,E′), now filling the distal hinge fold and abutting the proximal hinge fold (Fig. 3E′). Therefore, the repression of *wg* by *SoxF* is likely to occur through the *wg spd-fg* enhancer.

As *wg* expression was derepressed in *SoxF* mutant conditions, we tested whether *SoxF* was sufficient to block *wg* transcription. We observed that SoxF-expressing clones were able to repress *wg* expression in the IR at both the protein (Fig. 3G,G′) and transcriptional (Fig. 3F,F′) level. According to our previous observations, the expression of SoxF also blocks the expression of the *spd-fg-lacZ* enhancer (Fig. 3G) in a cell-autonomous manner, reinforcing the idea that the regulation of *wg* IR by *SoxF* works through the *spd-fg* enhancer. Clones overlapping the OR showed no effects on *wg* expression, in agreement with the co-expression of *SoxF* and *wg* OR found in normal discs (data not shown).

The derepression of *wg* is required for the hinge overgrowth of *SoxF* mutant discs

The correlation between *wg* derepression and hinge overgrowth, together with the known role of *wg* as an essential mitogen in the hinge, led us to test whether *wg* was itself required for the overgrowths. We recombined the $SoxF^{KG09145}$ allele into a $wg^{spd\text{-}fg}$

background. $wg^{spd\text{-}fg}$ is a regulatory mutation that deletes the enhancer that drives *wg* expression in the IR (Couso et al., 1994; Neumann and Cohen, 1996; Tiong and Nash, 1990). Accordingly, in *spd-fg* mutant discs, the prospective distal hinge underproliferates and *spd-fg* mutant adults lack distal hinge structures (Neumann and Cohen, 1996). We verified that in *spd-fg* mutant discs there is no increase in apoptosis, as monitored by anti-activated Caspase 3 staining (not shown), which confirms that reduced hinge proliferation is the major cause of the *spd-fg* adult phenotype. In $wg^{spd\text{-}fg}$, $SoxF^{KG09145}$ double mutants, the distal hinge was not overgrown (Fig. 4C). This result indicates that *wg* is required for the overproliferation observed in *SoxF* mutant discs. In fact, the reduction of the distal hinge was even stronger in the double mutant discs than in $wg^{spd\text{-}fg}$ discs, as indicated by the width of the distal hinge fold and the almost complete loss of the *wg* IR (Fig. 4B,C). This stronger phenotype can be accounted for by the apoptosis that we still detect in the hinge region of $wg^{spd\text{-}fg}$, $SoxF^{KG09145}$ discs (Fig. 4E), which is similar to that observed for *SoxF* single mutants and which does not occur in $wg^{spd\text{-}fg}$ discs.

SoxF is itself a downstream target of the *wg* pathway

The *wg* pathway activates several inducible antagonists that modulate its signaling activity, some of which, such as *Notum* and *nmo*, act in the wing (Gerlitz and Basler, 2002; Zeng and Verheyen, 2004). The fact that the domain of *SoxF* expression in the hinge coincides with the region under *wg* proliferative control prompted us to ask whether *SoxF* itself could be induced by the *wg* pathway. Indeed, clones of Arm*-expressing cells, in which the pathway is constitutively active, caused cell-autonomous activation of *SoxF* expression in regions of the notum close to the hinge (Fig. 5A). Although we have not performed a detailed study of where Arm* clones activate *SoxF* using markers for distinct domains within the notum, we noted that *SoxF* induction does not occur in the dorsal-most notal region, suggesting that factors such as *pannier* and/or the *iro-C* genes, which are expressed in this region (Calleja et al., 2000; Diez del Corral et al., 1999), might be limiting the competence to activate *SoxF* in response to Wnt signaling. In agreement with this restricted competence, *SoxF* is not found associated with two other domains of *wg* expression in the disc (not shown) that map to the prospective tegula of the wing (Casares and Mann, 2000) and the medial notum (Calleja et al., 1996). Similar to Arm*, clones expressing Wg also led to ectopic expression of SoxF, although this time the induction was, in part, non-autonomous, reflecting the diffusible nature of the Wg ligand (Fig. 5B). Conversely, clones in which the *wg* pathway is blocked by the expression of a dominant-negative form of TCF (Pangolin – FlyBase), $dTCF^{DeltaN}$, resulted in autonomous repression of *SoxF* (Fig. 5C). Similar results were observed when the expression of $dTCF^{DeltaN}$ was driven in the *dpp* domain that intersects *SoxF* expression (Fig. 5D). Therefore, these results reveal a cross-regulatory loop between *wg* and *SoxF*: *wg* induces *SoxF*, which in turn acts as a *wg* antagonist by blocking the spreading of its transcription throughout the hinge and, potentially, by attenuating its pathway. We noted that *SoxF* is expressed in late L2 wing discs, well before the rings of *wg* expression in the hinge are established (see Fig. S1 in the supplementary material). In order to determine whether *wg* is required for *SoxF* early in development, and not only in the hinge, we examined *SoxF* transcription in late L2 discs of wg^{CX3} mutants. wg^{CX3} is a regulatory mutant that lacks the earliest *wg* expression in the wing disc, and, as a consequence, wg^{CX3} discs fail to

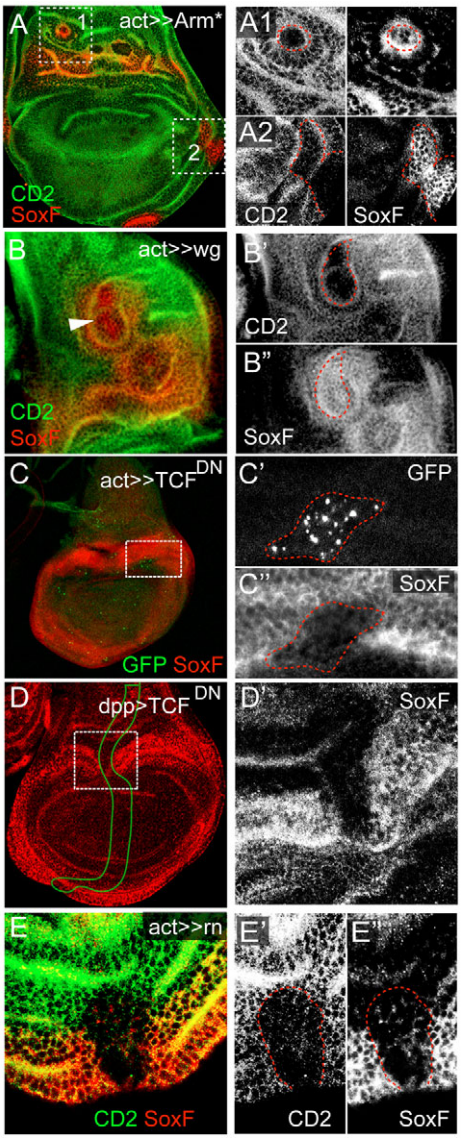

Fig. 5. *SoxF* transcription is controlled by the *wg* pathway. Fluorescent in situ hybridization detecting *SoxF* transcripts (red) in *Drosophila* wing discs containing clones overexpressing Arm* (A), Wg (B), TCF^{DN} (C) and Rn (E), or in which TCF^{DN} has been overexpressed with a *dpp-GAL4* driver (D). Merged (left) and individual signals (right) are shown. Clones are marked negatively by the absence of CD2 (A,B,E) or positively by GFP (C). In the panels on the right, dashed lines delineate the clones (A-C,E). Images in B and C are projections of confocal *z*-stacks. (**A**) In some Arm*-expressing clones, *SoxF* expression is ectopically induced in the notum (1) or pleura (2) in a cell-autonomous fashion (boxed regions are magnified in A1 and A2, respectively). (**B-B″**) In *wg*-expressing clones (arrowhead), *SoxF* expression in induced inside and outside of the clone (dashed line). (**C-C″**) Disc containing TCF^{DN}-expressing clones. (C′,C″) High-magnification view of the region boxed in C, showing a TCF^{DN} clone, detected by the punctate GFP signal (C′). In this experiment, the GFP signal is affected by the fixation and in situ hybridization protocol, allowing just the presence of clones to be detected, not their exact boundaries. In the example shown, the *SoxF* signal loss (C″) is associated with a TCF^{DN} clone (dashed line indicates inferred clone boundaries). (**D**,**D′**) *dpp*-driven expression of TCF^{DN} results in the downregulation of *SoxF* transcription where the *dpp* domain intersects the hinge (boxed area is magnified in D′). (**E-E″**) Rn ectopic expression clone represses *SoxF* cell-autonomously.

establish the wing field (Klein and Arias, 1998). These mutant discs lack *SoxF* expression (see Fig. S1A,B in the supplementary material), indicating that *wg* is required throughout development for *SoxF* transcription in the wing disc.

The activation of *SoxF* expression is spatially restricted by *rotund*

Although *wg* activates *SoxF* expression, only the cells adjacent and proximal to the *wg* IR, but not the *wg*-expressing cells themselves, express *SoxF*. This indicates that an additional regulatory mechanism operates to limit, in space, the transcription of *SoxF*.

The activation of *wg* expression in the hinge is coupled to the mechanisms that pattern the wing disc along its proximodistal (PD) axis (Azpiazu and Morata, 2000; Casares and Mann, 2000; del Alamo Rodriguez et al., 2002; Terriente Felix et al., 2007; Whitworth and Russell, 2003; Wu and Cohen, 2002). One of the genes required for *wg* IR expression is *rn*, a transcription factor that is expressed in the prospective distal hinge and wing pouch. The *wg* IR, which abuts the *SoxF* expression, appears at the edge of the *rn* domain (del Alamo Rodriguez et al., 2002). Therefore, the *rn* and *SoxF* expression domains are mutually exclusive (Fig. 1E,G; see Fig. S1 in the supplementary material). We checked whether *rn* could be repressing *SoxF* and thus polarizing its activation along the PD axis in the hinge. Ectopic clones of *rn* repressed *SoxF* expression autonomously (Fig. 5E,E′), suggesting that this is indeed the case. The reciprocal repression, of *SoxF* on *rn*, did not seem to take place, as the domain of the *rn-lacZ* reporter did not change in *SoxF* mutant discs (not shown). Therefore, *SoxF* is linked to the mechanism of PD axis formation of the disc in a way that ensures its directional activation by *wg* specifically straddling the gap domain of the hinge, the cell population whose proliferation is controlled by IR *wg*. Here, *SoxF* performs a key role in restricting the activation of the *wg* pathway and, by doing so, controls hinge growth.

DISCUSSION

Here we describe a novel negative-feedback mechanism in the *wg* pathway that is required to restrain the expression of *wg* itself, and which is essential to control organ growth.

During *Drosophila* development, the *wg* pathway often leads to the activation of genes that attenuate its signaling pathway. This is the case, for example, for *Notum* and *Dfz-3*, which are expressed in the wing disc in response to peak levels of signaling to reduce ligand availability for the Wg receptors (Sivasankaran et al., 2000), and for *nemo*, which acts intracellularly to block the signal transduction pathway (Zeng and Verheyen, 2004). In all cases described, these negative-feedback components act in all domains of *wg* expression and none regulates *wg* expression at the transcriptional level. However, in the case investigated here, the putative transcription factor *SoxF* is activated non-autonomously by *wg* in a hinge-specific manner. *SoxF* in turn represses *wg* transcription driven by the *wg spd-fg* enhancer, thus restricting the production of *wg* to the thin IR domain. Interestingly, the *SoxF* phenotype is similar to those of dominant *Dichaete* (*D*) mutations. *D* is a SoxB gene not normally expressed in the wing disc (Mukherjee et al., 2000). However, flies carrying dominant *D* mutations show reduced hinge structures. This phenotype is caused by ectopic *D* expression in the prospective hinge region of the disc (Russell, 2000). One of the salient features of *D* discs is the repression of the *wg* IR (Russell, 2000), which is reminiscent of the *wg* repression by *SoxF* we have described. Therefore, and taking into account the similarity between Sox proteins in their HMG DNA-binding domain, the ectopic *D* might be mimicking the repression of *wg* that is normally exerted by *SoxF*.

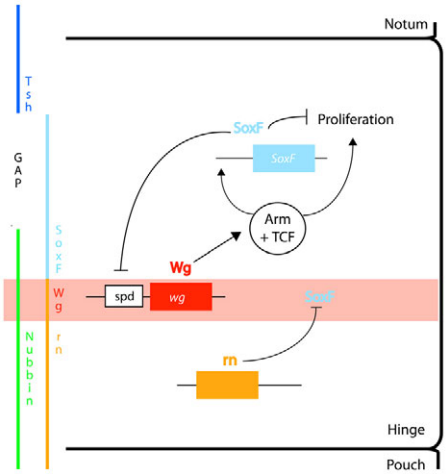

Fig. 6. Model of SoxF action in the developing wing disc hinge. The pattern of expression of Wg is tightly regulated in the hinge. *SoxF* acts to negatively regulate the *wg* pathway at two levels: (1) it blocks *wg* transcription through the *spd-fg* enhancer and (2) it antagonizes the Wg pathway parallel or downstream of Armadillo (Arm). *SoxF* is also required to maintain cell survival. More distally, Rn blocks the expression of SoxF in order to allow *wg* expression. The Wg signal produced in the IR activates the canonical pathway: Arm, together with TCF, regulates gene transcription. The activation of the pathway elicits at least three responses: (1) the establishment of a *wg* autoregulatory feedback loop, (2) the proliferation of hinge cells and (3) the activation of *SoxF* expression in the hinge. Only the expression and function of *wg* at the IR are shown.

The tight regulation of the growth of the hinge depends critically on the *wg*-induced activation of *SoxF* in the growing territory. Nevertheless, this activation is 'polarized' along the PD axis, taking place only in cells adjacent and proximal to the IR. We propose that this directionality in *SoxF* activation results from the mechanisms that pattern the wing disc along its PD axis. It has been suggested that *wg* is activated non-autonomously by a signal produced by the *vg*-expressing wing pouch cells, but excluded from them (del Alamo Rodriguez et al., 2002). This would generate a circular domain of *wg* expression surrounding the wing pouch. However, in the absence of *SoxF*, the domain of *wg* is abnormally broad and causes hinge overgrowth. This ectopic *wg* expression does not seem to result from a misregulation of hinge-specific genes: the expression of *nub*, *tsh*, *hth* and *rn* and their relative positioning in the hinge are unaffected in *SoxF* mutant discs (Figs 2 and 4; data not shown). Therefore, it seems that in the absence of *SoxF*, hinge cells cannot respond to the *wg* activating signals with enough precision to give rise to a thin ring of *wg* expression. Our results show that this precision is achieved through a double repression mechanism. First, *wg* activates its own transcriptional repressor, *SoxF*. This would lead to the extinction of *wg* expression if it were not for *rn*, which acts as a repressor of *SoxF*. Second, *rn*, by repressing *SoxF*, permits *wg* transcription. The result is that *wg* expression becomes restricted to a narrow circular stripe at the edge of the *rn* domain that provides a highly localized source of Wg. This signal activates, simultaneously and in the same cells, proliferation and the upregulation of *SoxF*, which restricts the production of the signal (Fig. 6). Therefore, *SoxF* joins *SoxN* and *SoxD* (*Sox102F* – FlyBase) (Chao et al., 2007; Overton et al., 2007) as the third *Drosophila* Sox known to antagonize the *wg* pathway. The vertebrate Sox proteins Sox9 (Mori-Akiyama et al., 2007),

XSox3 (Zorn et al., 1999) and XSox17 (Sinner et al., 2004) have also been shown to downregulate the Wnt/β-catenin pathway. Therefore, this antagonism seems evolutionarily conserved.

The relationship between SoxF genes, the *wg/Wnt* pathway and the control of tissue proliferation seems to extend to disease. The SoxF *Sox17* is normally expressed in the gut epithelium where it downregulates Wnt signaling via degradation of β-catenin and TCF. In colon carcinomas, the expression of the SoxB gene *Sox17* is often reduced, and this is associated with tissue overproliferation (Sinner et al., 2007). Moreover, inactivation of the SoxE gene *Sox9* leads to increased cell proliferation and hyperplasia in the mouse intestine (Bastide et al., 2007). The authors concluded that *Sox9* is essential for the fine-tuning of the transcriptional activity of the Wnt pathway (Bastide et al., 2007). Interestingly, the expression of *Sox9* is regulated by the Wnt pathway itself (Blache et al., 2004). Our results in *Drosophila* point to the possibility that the transcriptional regulation of Wnt expression by Sox genes might be a common feature of this proliferation-associated feedback loop.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/5/761/DC1

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