Research article

Splitting the Hedgehog signal: sex and patterning in *Drosophila*

Jamila I. Horabin*,†

Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA
*Present address: Room 3300-G, Department of Biomedical Sciences, 1115 West Call Street, College of Medicine, Florida State University, Tallahassee, FL 32306, USA
†Author for correspondence (e-mail: jamila.horabin@med.fsu.edu)

Summary

Sex-lethal (*Sxl*), the *Drosophila* sex-determination master switch, is on in females and controls sexual development as a splicing and translational regulator. Hedgehog (Hh) is a secreted protein that specifies cell fate during development. Previous work has demonstrated that Sxl protein is part of the Hh cytoplasmic signaling complex and that Hh promotes Sxl nuclear entry. In the wing disc anterior compartment, Patched (Ptc), the Hh receptor, acts positively in this process. Here, it is shown that the levels and rate of nuclear entry of full-length Cubitus interruptus (Ci), the Hh signaling target, are enhanced by Sxl. This effect requires the cholesterol but not palmitoyl modification on Hh, and expands the zone of full-length Ci expression. Expansion of Ci activation and its downstream targets, particularly *decapentaplegic* the *Drosophila* TGFβ homolog, suggests a mechanism for generating different body sizes in the sexes; in *Drosophila*, females are larger and this difference is controlled by *Sxl*. Consistent with this proposal, discs expressing ectopic Sxl show an increase in growth. In keeping with the idea of the involvement of a signaling system, this growth effect by Sxl is not cell autonomous. These results have implications for all organisms that are sexually dimorphic and use Hh for patterning.

Key words: Hedgehog, Sex-lethal, Decapentaplegic, Body size, *Drosophila*

Introduction

The sex determination master switch in *Drosophila*, *Sex-lethal* (*Sxl*), is activated in females early in development but remains off in males (Keyes et al., 1992). These two modes of expression are maintained through the rest of the life cycle (Sanchez and Nöthiger, 1983; Cline, 1984). Sxl promotes female differentiation by controlling the female specific splicing of *transformer* (*tra*) mRNA (Boggs et al., 1987; McKeown et al., 1987), and turns off the dose compensation system, the process that equates the gene dose imbalance of the sex chromosomes, through splicing control and translational repression of *male-specific lethal 2* (Bashaw and Baker, 1997; Kelley et al., 1997). Male development occurs by default.

Hedgehog (Hh) acts as a morphogen and specifies cell fate during development, patterning several different tissues (reviewed by Ingham and McMahon, 2001; Hooper and Scott, 2005). Its receptor Patched (Ptc), inhibits a second transmembrane protein, Smoothened (Smo). On Hh binding, the inhibition of Smo by Ptc is relieved, enabling Smo to activate the transcription factor Cubitus interruptus (Ci). Cells not exposed to Hh express the 75 kDa isoform of Ci (Aza-Blanc et al., 1997), a proteolyzed form of Ci which acts as a transcriptional repressor. Hh signaling generates full length Ci, a 155 kDa isoform, which activates transcription of target genes including *wingless* (*wg*), *decapentaplegic* (*dpp*) and *ptc*.

The regulated processing of Ci is realized through a complex of Ci with the cytoplasmic components of the Hh pathway (reviewed by Hooper and Scott, 2005). The complex is tethered to Smo (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003) and microtubules by Costal 2 (Cos2), a protein with sequence similarity to the motor domain of kinesin. On Hh signaling, the complex is released to result in full length Ci in the nucleus (Robbins et al., 1997; Sisson et al., 1997; Zhang et al., 2005).

Depending on the degree of Ci activation, different downstream Hh targets are activated. In the wing disc, graded Ci activation is accomplished by the expression of Hh in the posterior compartment; Hh diffuses into the anterior compartment and differentially activates Ci. At the highest level of activation, Ci activates *engrailed* (*en*) and *ptc* in the cells closest to the anteroposterior (AP) boundary. Slightly lower levels of Ci activation drive *dpp* expression, a few cells away from the AP boundary; still lower levels drive the expression of genes from the *Iroquois complex* further from the AP boundary.

Previously, we showed that Hh promotes the nuclear entry of Sxl in both germ cells and somatic cells (Vied and Horabin, 2001; Horabin et al., 2003). In the anterior compartment of the wing disc, Ptc appears to be a positive effector of this Hh promoted nuclear entry, while Smo has no role (Horabin et al., 2003). Here, it is shown that promotion of Sxl nuclear entry by Hh requires the cholesterol but not palmitoyl modification on Hh. The cholesterol modification also allows Sxl to enhance the levels of full-length Ci. Signaling by Hh is thus augmented, resulting in an increase in activation of Ci targets. I propose that this augmentation is how Sxl increases female size to produce sexually dimorphic animals.

Materials and methods

Fly stocks and generation of disc clones

Genes are described in Flybase (http://flybase.bio.indiana. edu). Unless otherwise stated, flies were raised at 21°C. Clones were generated by the FLP-FRT mitotic recombination system (Chou and Perrimon, 1992). For HhN expression, recombination was induced in 1st to 2nd instar larvae from a cross of *y w; act>cd2>*GAL4, *hs-flp,* UAS-*lacZ*/TM2 mated to UAS-HhN. A 20-minute heat shock at 37°C was used to induce *hs-flp* followed by recovery at room temperture for 4-6 days. Transgenes were expressed using either *y w; apterous*-GAL4/ CyO, Sp/CyO; *dpp*-GAL4/TM6 or UAS-C84SHh, *dpp*-GAL4/TM6 driver stocks mated to UAS-Sxl, UAS-C84SHh, *ptc-lacZ*; UAS-Sxl/ TM6 or *dpp-lacZ*/CyO; UAS-Sxl stocks. The Sxl isoform in UAS-*Sxl* is MS3 (Samuels et al., 1991); the same transgene was used in all experiments. Crosses with this transgene had to be maintained at 21°C to recover adequate numbers of third instar larvae of the desired genotype. The rest of the crosses were carried out at 25°C.

To generate *Sxl*-null clones, the deficiency *Sxl* allele, fP7BO, was recombined onto a FRT101 X chromosome. *hs-flp* on an MKRS balancer was introduced into the fP7BO, FRT101 stock and mated to a Ubiquitin-Green Fluorescent Protein (GFP), FRT101 stock that carried a *dpp-lacZ* transgene on the second chromosome. Clones were generated by a 55-minute heat shock at 37°C of 1st to 2nd instar larvae, followed by a recovery of 3 days at 25°C.

Immunofluorescence, LMB incubations and staining of discs

Anti-BrdU monoclonal antibodies were from Zymed and used at 1:40. Anti-Phospho Histone3 antibodies were from Upstate Biotechnology, used at 1:1000. Anti-pMad antibodies were used at 1:150. Anti-Sal were used at 1:400 and anti-Ptc at 1:5. The rest of the antibodies and staining procedures are described by Horabin et al. (Horabin et al., 2003).

Western blot analysis

These were performed as previously described (Vied and Horabin, 2001). To obtain loading matches of either Ci or β-tubulin, different amounts of adult male and female extracts were loaded on the same gel and probed with anti-Ci and anti-β-tubulin.

BrdU incorporation

Dissected and inverted larval heads from UAS-Sxl mated to *ap*-GAL4/CyO flies raised at 25°C were incubated in 20 μM BrdU (Sigma) in Schneider's Drosophila medium for 35 minutes at room temperature before fixing and staining as in Johnston and Shubiger (Johnston and Shubinger, 1996).

Results

Signaling by Ptc to Sxl requires the cholesterol but not palmitoyl moiety on Hh

Drosophila Hh is synthesized as a 45 kDa precursor that is shortened to a mature form with two lipid modifications; palmitic acid at the N terminus and cholesterol at the C terminus. Maturation involves autoproteolytic processing under the control of the C-terminal domain of Hh (Lee et al., 1994; Porter et al., 1995; Porter et al., 1996).

To test whether either of the lipid modifications plays a role in Hh promoted Sxl nuclear entry, female wing discs expressing Hh with only a single modification were examined. HhN encodes the N-terminal region of Hh that is palmitoylated but, because it does not undergo autoproteolytic processing,
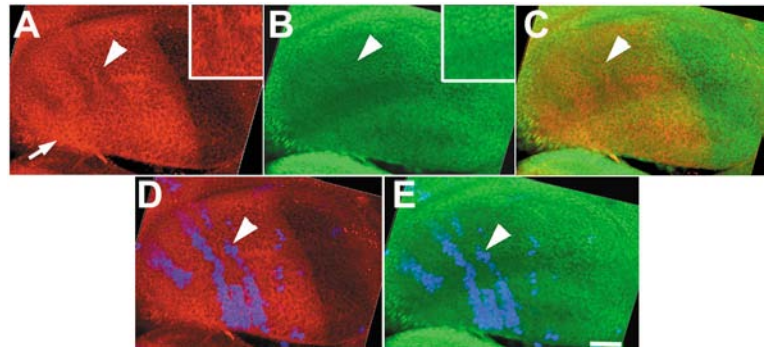

Fig. 1. HhN does not influence Sxl nuclear entry. A wing disc stained for Ci (red; A,C,D), Sxl (green; B,C,E) and β-gal (blue; D,E). β-Gal marks the actin>GAL4 clones expressing HhN (Hh with only the palmitoyl modification). The disc was treated with 100 ng/ml LMB for 3 hours. Full-length Ci is detected anterior to the AP boundary (e.g. arrow in A). In the β-gal-positive region where HhN levels are maximal (arrowheads) there is a reduction of full-length Ci, but no effect on Sxl nuclear entry (B,E; brighter, more punctate signal) (Horabin et al., 2003). Insets in A,B enlarge the areas indicated by arrowheads. (C) Overlay of Sxl and Ci; (D) overlay of Ci and β-gal; (E) overlay of Sxl and β-gal. Scale bar: 40 μm. Anterior towards the left, ventral is towards the top. Confocal images throughout.

does not contain the cholesterol moiety (Porter et al., 1996). As previously reported, this form of Hh is functional for Ci activation and full-length Ci is detected distantly anterior of the AP boundary. Where HhN levels are maximal, there is a reduction of full-length Ci (Fig. 1A,D), most likely from the activation of *en*, which inhibits Ci transcription (Schwartz et al., 1995). HhN does not increase Sxl nuclear levels, however (Fig. 1B,C,E). The normal high nuclear levels in the posterior compartment and graded nuclear localization in the anterior compartment (Horabin et al., 2003) are detected, with no change in the cells expressing HhN.

The alternative single modification [cholesterol without palmitoyl (C84S-Hh)], by contrast, was active with respect to Sxl (Fig. 2E; see Fig. S1B in the supplementary material). In Fig. 2, the *dpp*GAL4 driver was used to drive expression of C84S-Hh. Relative to endogenous Hh, about threefold less of the nuclear export inhibitor Leptomycin B (LMB) (Nishi et al., 1994) was required to detect nuclear Sxl in these discs, suggesting that the nuclear Sxl is effected primarily by the ectopic Hh.

Sxl stabilizes Ci on Hh signaling

C84S-Hh has been shown to dominantly destabilize Ci, decreasing the expression of Hh target genes. Patterning of the wing is compromised and the size of the region between veins L3 and L4 is reduced. C84S-Hh is also unable to rescue the embryonic segmentation phenotype caused by loss of Hh (Lee et al., 2001).

We also found that C84S-Hh destabilizes Ci, but only in males. Females show the opposite effect, increasing the levels of full-length Ci (compare Fig. 2A with D; see Fig. S1 in the supplementary material). The nuclear Ci detected is in a broad band reflective of the *dpp* expression zone (note that C84S-Hh is expected to diffuse from its source of expression), and requires several fold less of the nuclear export inhibitor LMB for detection. Under the same
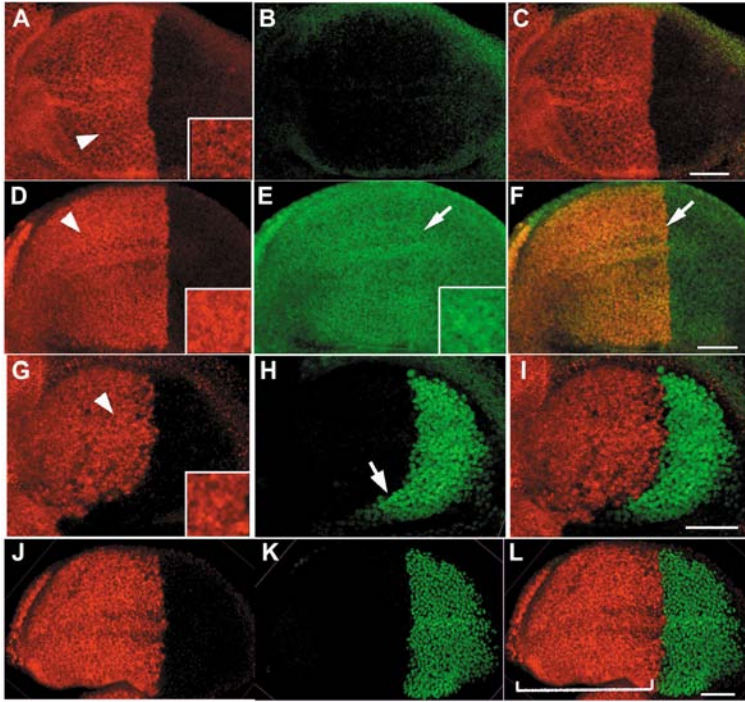
Fig. 2. Sxl allows Hh with only cholesterol modification to stabilize Ci. (A-F) Discs expressing Hh with only the cholesterol modification, C84S-Hh, with *dpp*-GAL4 driver stained for Ci (red) and Sxl (green). *dpp* is expressed in a broad band in the anterior compartment, a few cells away from the AP boundary – see *dpp-lacZ* staining in Fig. 6A. Both male and female discs were treated with 50 ng/ml LMB for 3 hours, and scanned at about the same laser intensity. (A-C) Male disc; (D-F) female disc. In males, as previously reported (Lee et al., 2001), the levels of Ci fall (arrowhead in A for example). For females, the amount of LMB is about one-third of that needed in wild-type discs to detect nuclear, full-length Ci in region around *dpp* expression zone (e.g. arrowhead in D); Sxl is also nuclear (arrows in E, F). Insets in A,C enlarge the area indicated by arrowheads; inset in E enlarges the area indicated by an arrow. (G-L) Discs expressing Sxl and C84S-Hh with *dpp*-GAL4 driver stained for Ci (red) and En (green). (G-I) Male disc; (J-L) female disc. Ci signal is punctate (e.g. G) and En-positive cells extend towards the anterior compartment (arrow in H), which was seen in all male discs (n>12) of this genotype (non-Tubby larvae). Inset in G enlarges the area indicated by an arrowhead. Male discs were treated with 50 ng/ml LMB for 3 hours; female discs were treated with half the amount of LMB. The size of the nuclei and scale bars in I,L both illustrate that the female disc is larger than the male. Punctate signal is indicative of nuclear Ci and there is a relative increase in size of the anterior compartment (bracket in L). Scale bars: in C, 20 μm for A-C; in F, 20 μm for D-F; in I, 20 μm for G-I; in L, 20 μm for J-L. For all discs, anterior is towards the left, ventral is towards the top. Confocal images throughout.

conditions, male discs have a weaker signal for Ci and the protein is not nuclear.

This sex specificity, coupled with our previous observation that Sxl is present in the Hh cytoplasmic complex, suggested that Sxl may be acting to stabilize Ci on Hh signaling. If this is the case, expressing Sxl in males should increase the levels of full-length Ci. Indeed, male discs expressing Sxl (MS3 isoform) (Samuels et al., 1991), as well as C84S-Hh under the control of *dpp*GAL4, now show higher levels of full-length Ci and the protein is more nuclear, as seen in females (Fig. 2G,I). Taken together, these results suggest that when the cholesterol moiety is present on Hh, Sxl enhances the production of full-length Ci.

Curiously, the presence of Sxl does not temper the wing patterning defect caused by the ectopic expression of C84S-Hh; the reported narrowing between wing veins L3 and L4 (Lee et al., 2001) is the same in the two sexes. The form of Ci that Sxl stabilizes through C84S-Hh must not be the form responsible for Hh patterning.

Sxl enhances the rate of Ci nuclear entry as well as its levels

The results above were obtained with variant Hh. If Sxl alters the rate of full-length Ci production and/or its nuclear entry, one might predict that the endogenous protein should show a difference between the sexes. To test this, wild-type male and female discs were treated with relatively high levels of LMB and then stained for Ci and Sxl in the same dish. Examining the discs shows that it is indeed possible to sex the discs without probing for Sxl. Male discs consistently show a more diffuse Ci signal, indicative of the protein being distributed in the cytoplasm. By contrast, female discs show a more punctate signal and Ci protein appears more distinctly nuclear (Fig. 3A-F).

If Sxl is responsible for this difference, expression of ectopic Sxl in male discs should alter the behavior of full-length Ci. So as to have an internal control, Sxl was expressed in only the dorsal compartment of the disc with the *apterous* GAL4 (*ap*-GAL4) driver, leaving the ventral half of the disc in the wild-type condition. Fig. 3G-I shows that, in the compartment where Sxl is expressed, higher levels and more nuclear full-length Ci are detected in cells near the AP boundary of male discs. Ectopic Sxl was also able to enhance full-length Ci production in females, although less dramatically (Fig. 3J-L). This is not surprising given that they already express Sxl. Under the same conditions, Tra, the immediate target of Sxl in sex differentiation, does not have an effect (data not shown) suggesting that Sxl itself, or another target regulated by Sxl, is responsible.

To further test the idea that females stabilize more Ci than males, the amount of endogenous full-length Ci in males versus females was compared using Western blot analysis. As can be seen in Fig. 4, which uses β-tubulin as a loading control, when the loading level of Ci is the same in the two sexes, there is more protein loaded in the male lane. Conversely, for the same loading level of protein, females have higher amounts of full-length Ci. These results are consistent with the tissue staining results that suggest that, in addition to increasing the rate of nuclear entry, Sxl enhances the production of full-length Ci.

Sxl boosts the Hh signal

To determine whether the effects of Sxl on full-length Ci production alters the expression of its downstream targets, the expression of *ptc*, *en* and *dpp* was analyzed in male and female wing discs expressing ectopic Sxl. In all cases, Sxl was

Fig. 3. Sxl enhances Hh signaling. Discs were treated with 150 ng/ml LMB for 3 hours and antibody incubations were carried out in the same dish (sexed using Sxl signal). (A-F) Wild-type male (A-C) and female (D-F) discs stained for Ci (red) and Sxl (green). The Ci signal in males is relatively diffuse in contrast to females where it is brighter and more discrete, indicative of nuclear localization. (G-I) Male discs expressing Sxl in dorsal compartment by *ap*-GAL4. The level of Sxl protein is low (arrowhead in H) compared with endogenous signal of females; Ci levels are increased in the dorsal half (arrowhead in G) relative to the ventral half (arrow in G) of the disc. (J-L) Female disc expressing Sxl in dorsal compartment by *ap*-GAL4. The Ci signal shows an enhancement even in females (arrowhead marks the dorsoventral boundary at the AP boundary). Bracket in I,L indicates expansion of the dorsal half of the disc. Scale bars: 20 μm. Anterior is towards the left, ventral is towards the top. Confocal images throughout.
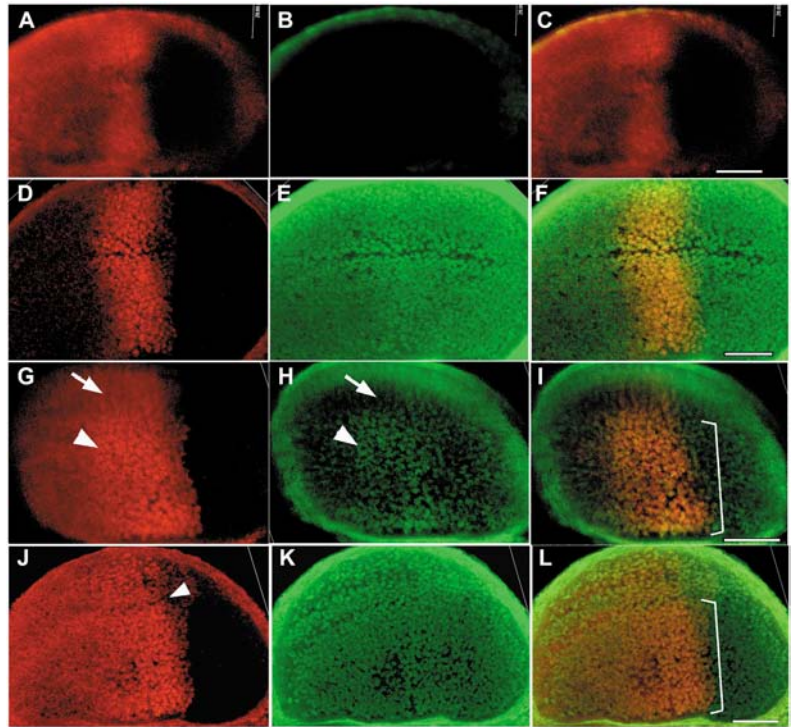
expressed in the dorsal compartment using the *ap*-GAL4 driver, so that the ventral half of the disc could be used as an internal control.

A *ptc-lacZ* transgene was used to score for *ptc* transcription. Fig. 5 shows that Sxl enhances *ptc* transcription in males; both the signal intensity increases and the stripe of β-galactosidase (β-gal) expression broadens in the dorsal half of the disc (Fig. 5B,D). Interestingly, the effect is not the same in females. Although the intensity of the β-gal signal in the dorsal half of the disc increases, the width of the β-gal-positive cells is slightly narrowed (Fig. 5C,E).

The increase in *ptc* expression in both sexes, indicates it is possible to elevate the effect of the Hh signal above endogenous levels. In females, the ensuing increase in Ptc at the membrane appears to sequester more Hh close to the AP boundary, reducing the depth of cells that turn on *ptc*. In males, it would appear that the increase in Ptc does not reach the same level, as the *ptc* expression zone is in fact slightly

broadened by the presence of Sxl. This is consistent with the idea that females normally have a slightly elevated level of Hh signaling relative to males.

A change in *en* expression in the anterior compartment was less clear cut. As only a few anterior cells at the AP boundary are normally induced to express *en* by highly activated Ci, and discs expressing Sxl in the dorsal half are frequently distorted owing to an increase in growth (see below), it was hard to
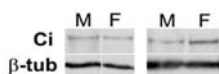

Fig. 5. *ptc* expression is enhanced by Sxl. Discs stained for β-gal which reports *ptc* transcription from a *ptc-lacZ* transgene. (A) Wild type, (B-E) discs expressing Sxl in dorsal compartment by *ap*-GAL4 driver; (B,D) male, (C,E) female. In both sexes, *ptc* expression is more intense in the dorsal compartment (arrowhead marks dorsoventral boundary at the AP boundary for all panels). Arrow in B indicates broadening of *ptc-lacZ* zone and beginning of wing pouch expansion in dorsal half; in D, the fold from overgrowth is larger and the *ptc-lacZ* forms a U (arrow). The width of *ptc-lacZ* expression narrows in females (C), more so in E (which also has a larger dorsal half). Normal width of *ptc-lacZ* in E indicated by arrows. Scale bars: 20 μm. Anterior is towards the left, ventral is towards the top. Confocal images throughout.

Fig. 4. Sxl increases Ci levels. Western blot of adult male (M) and female (F) flies stained for Ci and β-tubulin (β-tub). Left pair shows relative loading difference of protein between the sexes (females less) when the Ci signal is approximately the same; right pair has the β-tubulin levels approximately the same between the sexes, females have more Ci. Lanes taken from the same blot; for each protein, the time of exposure for male versus female lane is the same.
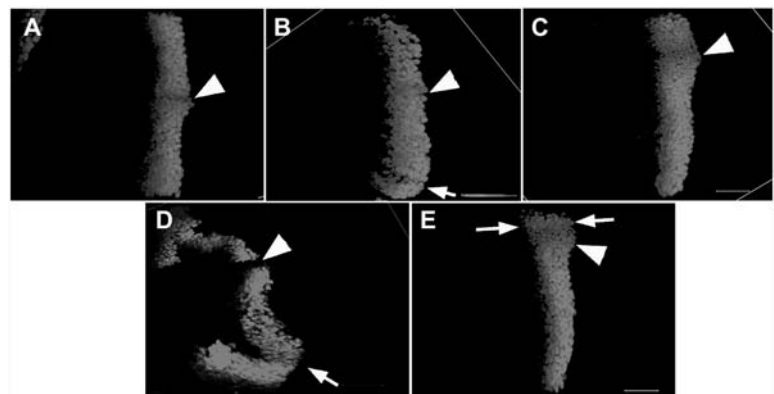
gauge whether the narrow row of cells in the anterior compartment expressing En had expanded.

dpp expression was reported by a *dpp-lacZ* transgene. Male discs showed a widening of the *dpp* expression zone and an increase in intensity of the *dpp-lacZ* stripe relative to the ventral half of the wing pouch (Fig. 6D,M). Female discs also showed an increase in intensity (Fig. 6G,J), although for some discs a widening of the *dpp-lacZ* stripe was not detected. However, in both male and female discs, the dorsal half of the wing pouch was frequently enlarged and in many cases, particularly male discs, the dorsal half had additional folds indicative of overgrowth. In some discs, the β-gal stripe was a folded U-shape instead of a stripe (see Fig. 5 for *ptc-lacZ*, Fig. 6 for *dpp-lacZ*).

To test whether the enhancement of *dpp* expression by Sxl activates the downstream targets of the Dpp signaling pathway, the levels of Spalt (Sal) and phosphorylated Mothers against Dpp (pMad) were examined. Sal is a transcription factor induced by Dpp signaling. Mad is a downstream transcriptional target of the Dpp signal that is phosphorylated on activation of the pathway (Tanimoto et al., 2000). Sxl increases the intensity of both Dpp responses (Sal in Fig. 6E,H,K,N; pMad in Fig. 7E,H) in males and females; compare the levels of the ventral half with the dorsal half of the disc in Fig. 6.

If Sxl boosts the Hh signal, removal of Sxl in female discs should reduce the relative strength of the signal. To test this prediction, *Sxl*-null clones were generated in female discs and the levels of expression of Ci, Sal and *dpp-lacZ* examined. Very few large clones were recovered in the wing pouch, but the small clones recovered near the AP border all showed a reduction in their levels of full-length Ci (Fig. 8A-C). Larger clones were found in the notum area of the wing disc and these also showed a reduction in Ci (Fig. 8D-G), as well as Sal and *dpp-lacZ* expression (Fig. 8H-K). The reduction is frequently modest. However, as males have a fully functional Hh signal, elimination of Sxl in females is only expected to reduce not eliminate the signal.

Sxl induces growth in the wing disc

Hh only patterns the region immediately anterior to the AP boundary, which gives rise to the middle of the wing – between veins L3 and L4. Patterning of the rest of the disc is accomplished by the gradient of Dpp that is elicited by Hh (Lecuit et al., 1996; Nellen et al., 1996). Dpp also regulates growth of the disc (Capdevila and Guerrero, 1994; Burke and Basler, 1996; Lecuit et al., 1996; Nellen et al., 1996), promoting the cell cycle while maintaining cell size (Martin-Castellanos and Edgar, 2002).

To test whether Sxl affects the cell division rate, male and female wing discs expressing ectopic Sxl in the dorsal compartment were analyzed for BrdU incorporation. Fig. 9A-I shows that the rate of BrdU incorporation in the dorsal half of the wing pouch is increased by ectopic Sxl. As BrdU reports DNA synthesis, this suggests that the mitotic index is elevated by Sxl. Under the same conditions, Tra did not produce this effect (data not shown). The number of cells positive for Phospho histone 3 (PH3), a modification that is also indicative
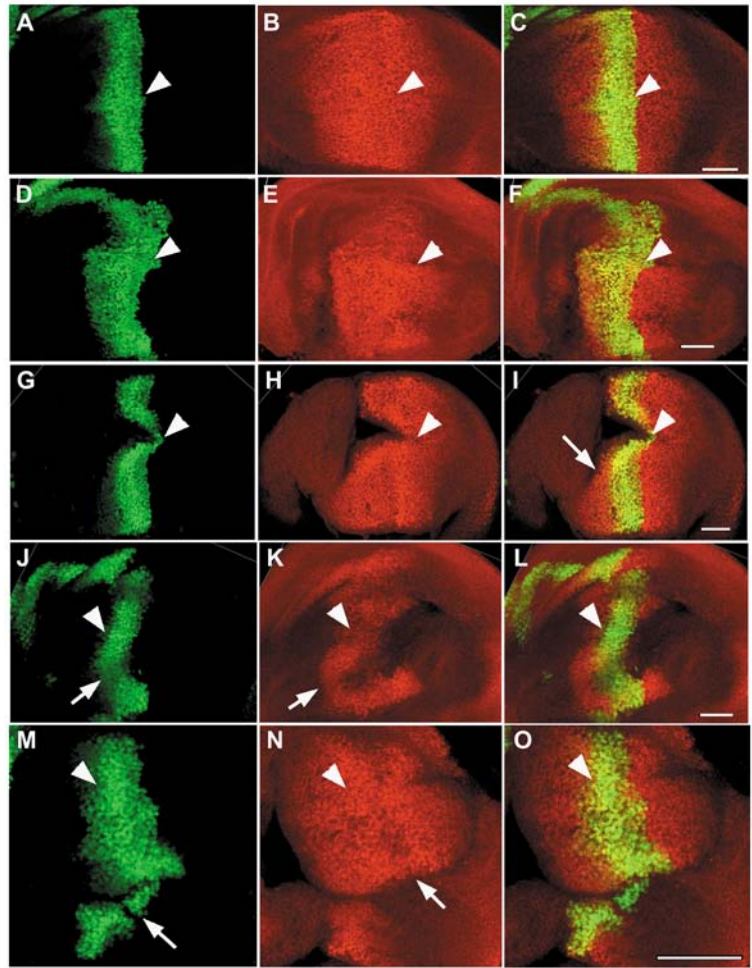

Fig. 6. Dpp signaling is enhanced by Sxl. (A-C) Wild type. (D-O) Male (D-F,M-O) and female (G-L) discs expressing Sxl in the dorsal compartment by *ap*-GAL4 driver, stained for β-gal (green; reports Dpp through a *dpp-lacZ* transgene) and Sal (red; induced by Dpp). (C,F,I,L,O) Merged images. In all panels, the arrowheads mark the dorsoventral compartment boundary. The zone of Dpp expression widens in the dorsal compartment in males (D and M), less so in females (G and J). Below the dorsoventral boundary of the wing pouch is a widened zone of Dpp expression and corresponding widening/intensity of Sal expression compared with the ventral half. Arrow in I indicates slightly distorted dorsal half; (J-O) dorsal wing pouch halves have an extra fold from the increase in size (arrows). Arrow in M indicates the wing pouch–notum boundary and arrow in N indicates the edge of fold in the distended wing pouch. Scale bars: 20 μm in A-L; 40 μm in M-O. Anterior is towards the left, ventral is towards the top. Confocal images throughout.

of mitosis, also shows an increase when Sxl is ectopically expressed (Fig. 9J-R).

If Sxl induces disc growth, as suggested by the data above, and the augmentation of Hh signaling by Sxl requires the Hh cholesterol moiety, then signaling through just C84S-Hh might be predicted to induce growth. Such an effect would have to overcome the dominant-negative effect of C84S-Hh on endogenous Hh signaling (which normally also functions to grow the disc). Despite these opposing effects, male and female wing discs expressing C84S-Hh and ectopic Sxl by the *dpp*-GAL4 driver, show a change in their normal proportions that

Fig. 7. Dpp signaling target pMad is enhanced by Sxl. (A-C) Wild type; (D-F) male and female (G-I) discs expressing Sxl in the dorsal compartment stained for β-gal (red; reports *dpp* expression though *dpp-lacZ*) and pMad (green; reports activation of Dpp signaling). (C,F,I) Merged images. Below the dorsoventral boundary of the wing pouch (arrowheads) is a widened zone of Dpp expression (D-I) and corresponding widening of pMad compared with the ventral half. Scale bars: in C, 20 μm for A-C; in F, 20 μm for D-F; in I, 20 μm for G-I. Anterior is towards the left, ventral is towards the top. Confocal images throughout.
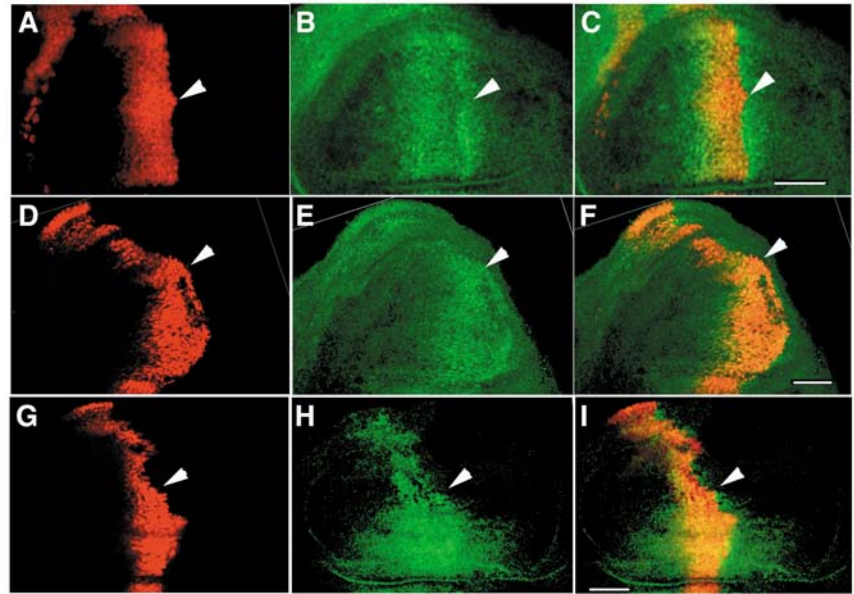
is suggestive of enlargement. All male discs show the posterior En signal with an anterior extension (Fig. 2H), with the rest of the anterior compartment relatively normal in size. In females, the anterior compartment is larger than the posterior, frequently by almost twofold (Fig. 2L).

The reason for this compartmental difference between the sexes is not clear. Co-expressing Sxl and C84S-Hh is essentially lethal to both males and females; rare female escapers had misshapen wings with bubbles and the L3 and L4 intervein region narrowed (the latter as when expressing C84S-Hh alone). Sxl and C84S-Hh are ectopically expressed in only the Dpp zone. In male discs, these cells are expected to have upsets in dose compensation and this might impair their ability to both survive and cycle through mitosis normally. Females, by contrast, normally express Sxl and are more tolerant to its overexpression. Presumably, more of their cells are able to respond to the increase in full-length Ci, which should increase the levels of Dpp and enhance cell survival (Moreno et al., 2002). In males, only the cells beyond the Sxl expression zone might benefit from the increase in full-length Ci and increase in Dpp. Either way, the data indicate Sxl is capable of

Fig. 8. Sxl-null clones in females show reduced Hh signaling. (A-C) Small Sxl-null clone near AP border (arrowhead) identified by loss of GFP signal (A), shows a reduction in Ci levels (B); (C) merged image. (D-G) Larger Sxl-null clone in notum region of wing disc (arrowhead) identified by loss of GFP (D) and Sxl signal (E), showing a reduction in Ci levels (F); (G) merge of D-F. Sxl and Ci are primarily cytoplasmic as no LMB was used. (H-K) Sxl-null clones (arrowheads), identified by loss of GFP (H), stained for β-gal (I) and Sal (J); (K) merged image. β-gal, which reflects *dpp* transcription, and Sal levels show a slight reduction. Arrow indicates slightly higher β-gal in non-clone region. The few nuclei with slightly brighter β-gal signal near bottom clone are wild type for Sxl (small arrowhead in (I)). Scale bars: in G, 20 μm for D-G; in K, 20 μm. Anterior towards the left, ventral is towards the top. Confocal images throughout.
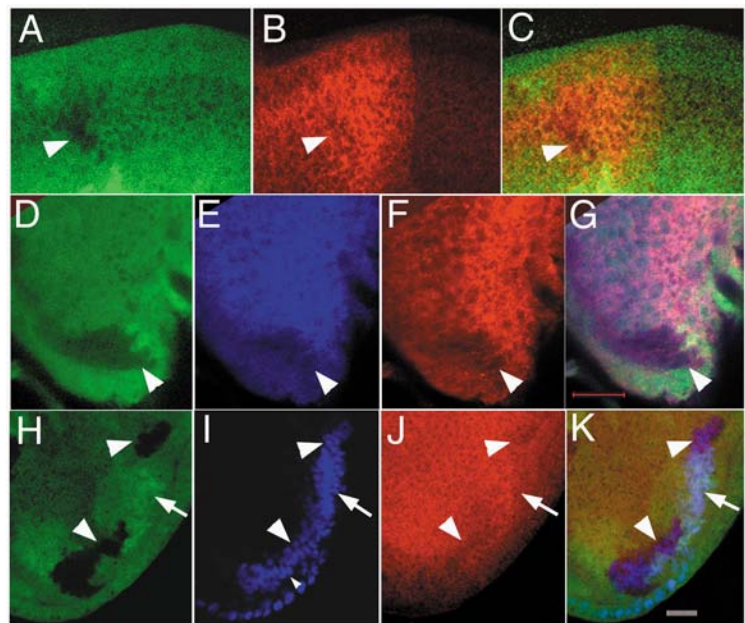
inducing growth with a form of Hh that acts negatively to the wild-type Hh ligand.

Expansion of the dorsal half of the wing pouch was also detected with wild-type Hh and the ectopic expression of Sxl. Note the increase in size of the dorsal relative to the ventral half of the wing pouch in males and females (Fig. 3I,L); in addition, cross-sections of male and female discs expressing ectopic Sxl (Fig. 10).

Growth induced by Sxl is not cell autonomous

If the growth effects by Sxl are caused through altering the Hh signal then expressing Sxl in the anterior compartment, in only the cells that respond to the Hh signal, should be sufficient to induce growth across the entire disc. To test this prediction, Sxl was expressed in the anterior compartment using the *dpp*-GAL4 driver, and effects on disc growth examined. As seen in

Fig. 9. Sxl raises mitotic rate. Wild-type (A-C); male (D-F) and female (G-I) discs expressing ectopic Sxl in the dorsal compartment by *ap*-GAL4 driver stained for BrdU incorporation (red) and Ci (green). (E-H) Expansion of Ci in the dorsal compartment identifies discs expressing ectopic Sxl (arrowhead). (D,G) The density of BrdU positive nuclei increases in the dorsal compartment in both males and females (arrow). Scale bars: in C, 20 μm for A-C; in F, 20 μm for D-F; in I, 40 μm for G-I. Wild type (J-L); male (M-O) and female (P-R) discs expressing ectopic Sxl in the dorsal compartment by *ap*-GAL4 driver stained for PH3 (green) and β-gal (red; reports *dpp* expression through *dpp-lacZ*). Arrowheads in J-R indicate dorsoventral boundary at AP boundary. (M,P) A larger number of PH3 positive cells are found below the dorsoventral boundary relative to J. (N,Q) *dpp* expression changes in the dorsal half of the disc, which is a little larger than the ventral. Scale bars: in L, 20 μm for J-L; in O, 40 μm for M-O; in R, 40 μm for P-R. Anterior is towards the left, ventral is towards the top. Confocal images throughout.

Fig. 10E,F, both males and females show overgrowth not only in the anterior compartment where it is expected, but also in the posterior compartment. These results clearly demonstrate that Sxl produces growth effects in a non-autonomous manner.

Discussion

Hh promotes the nuclear entry of Sxl in germ cells and somatic cells (Vied and Horabin, 2001; Horabin et al., 2003). In both tissues, Sxl is in a complex with all of the known cytoplasmic Hh signaling components; in somatic cells, this includes Ci. The purpose of this effect of Hh on Sxl and the function of Sxl within the complex was not clear.

The Hh signal is enhanced in females

The data presented here show that when Sxl is present, the Hh signal is augmented. This is seen as an increase in full-length Ci in whole-mount tissue (Fig. 3), and in western blots which give a more quantitative sense of protein levels (Fig. 4). In addition to elevating the levels of full-length Ci, several of the Hh downstream targets, including *ptc*, *dpp* and some of the
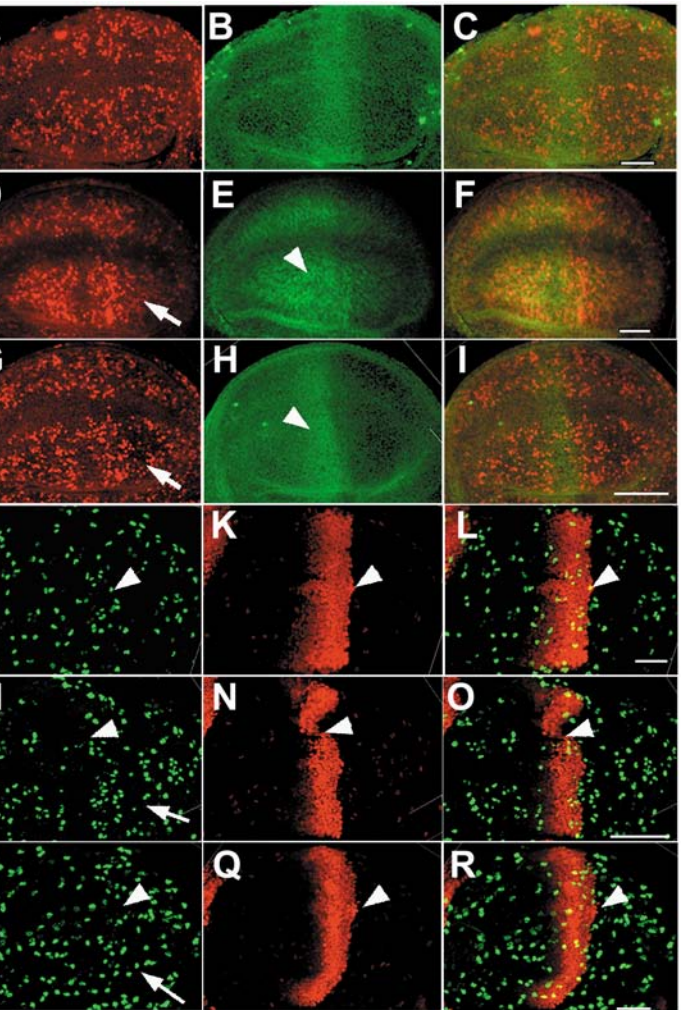
downstream targets of Dpp, show an increase in expression. Conversely, removal of Sxl in female cells shows a reduction in the strength of the Hh signal (Fig. 8).

Sxl also enhances the nuclear entry rate of Ci, with either endogenous Hh (Fig. 3) or Hh that has only the cholesterol modification (Fig. 2). In females, when Sxl is co-expressed

Fig. 10. Sxl induces disc growth which can be non autonomous. (A) Wild-type disc; (B-C) cross sections of wild-type discs (B) and male (C) and female (D) discs expressing Sxl in the dorsal compartment stained for Ci (red) and Wg (green). Broken line in A indicates the position of the cross section. Arrowheads mark the Wg stripe at the D/V boundary of the wing pouch and at the dorsal end. Arrows indicate the folds in the dorsal wing pouch caused by disc overgrowth. (C) In the male disc, the fold near the dorsoventral border is over the ventral half of the disc and an additional fold is over the dorsal half. (D) The female disc cross-section is at a slight angle to show the folds and thickening in the dorsal half. Ventral is towards the left. Confocal image stacks taken with a 40× objective (female with zoom at 0.9). (E,F) Male (E) and female (F) discs expressing Sxl in only the *dpp* zone of anterior compartment stained for Ci (red) and Wg (green). Arrows indicate more severe curve of Wg stripe caused by bulge from growth. Compare with the gentle curve of Wg stripe in the wing pouch of the wild-type disc in A, which normally is relatively flat. Arrowheads in E,F indicate folds in wing pouch in posterior compartment caused by increased growth.
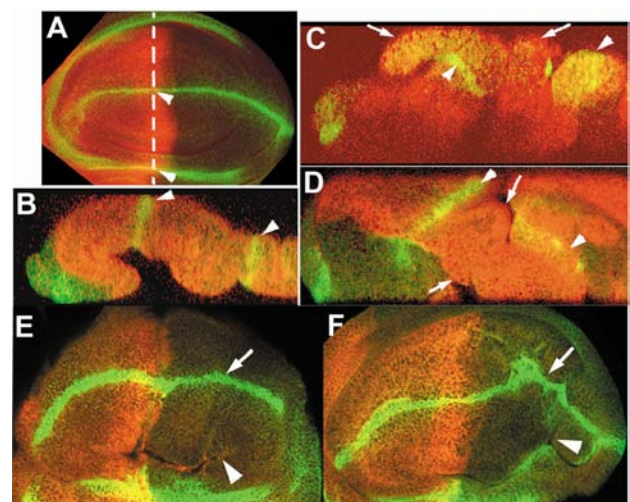
with Hh with only the cholesterol modification, the amount of LMB required to detect nuclear Ci is reduced (by almost sixfold; Fig. 2J), further supporting the idea that Sxl affects Ci nuclear entry rate on Hh signaling.

Hh enhancement of Sxl nuclear entry also depends on the cholesterol and not the palmitoyl modification. Given that Ci and Sxl are in a complex in the cytoplasm and both respond to the Hh cholesterol modification, it is tempting to speculate, although the data presented does not address this issue, that the two proteins may also enter the nucleus as a complex. This may be the method by which Sxl stabilizes Ci, diverting it from rapid proteolysis, particularly the highly activated form that is functionally detectable (Ohlmeyer and Kalderon, 1998) but has not been identified biochemically.

Stabilization of full-length Ci by Hh with only the cholesterol modification in females is in contrast to what occurs in males. We also found, as previously reported (Lee et al., 2001), that this form of Hh can destabilize Ci as well as compromise the Hh signal, but only in males (Fig. 2A). The effect of the cholesterol moiety contrasts with the palmitoyl that potentiates Hh in activating Ci for patterning. This is generally also true in vertebrates, where the cholesterol modification appears to have less of a role in patterning and a more significant role in the release and extracellular transport of the Hh ligand (Feng et al., 2004).

Hh signal is not at its maximum

In both sexes, ectopic expression of Sxl shows an increase in intensity of *ptc* expression, indicating it is possible to further elevate the Hh response. Other than *en*, which was difficult to score in our experiments, *ptc* requires the highest levels of Ci activation for its transcription.

In females, the ectopic Sxl elevates *ptc* expression in the cells near the AP boundary, but the depth of the cells showing this highest level of Ci activation is reduced. A reduction in the number of cells transcribing *ptc*, when compared with the wider but less intense width of *ptc* transcription in the control half of the disc, suggests a restriction in Hh diffusion. Elevated *ptc* transcription is expected to produce more Ptc at the membrane, which should sequester more Hh close to the AP boundary. This result shows that Sxl can both enhance the Hh response and effectively alter the Hh gradient.

In males, the increase in *ptc* transcription induced by Sxl both intensifies and widens the *ptc* expression zone. This suggests that the activation of Ci is at a lower peak in males than in females, and its enhancement by ectopic Sxl does not reach the same maximum that additional Sxl in females produces.

Ectopic expression of Sxl in the *dpp* expression zone has previously been shown to adversely affect female wing development, narrowing the region between veins L3 and L4. This defect was taken to suggest that the relative concentrations of both Ci and Sxl are important for their normal function (Horabin et al., 2003). The data presented here support that conclusion while providing an explanation for the apparent decrease in effectiveness of the Hh signal. As outlined above, when additional Sxl is expressed, the slope of the Hh gradient becomes steeper. As Hh directly patterns the L3 to L4 wing vein region, a steeper gradient of Hh will reduce the area patterned because the normal Hh patterning minimum is reached more rapidly. The L3 to L4 intervein region should

correspondingly become narrower. No adult males expressing Sxl were recovered (presumably because of upsets in dosage compensation) so their wings could not be scored.

Depending on the expression driver used, ectopic Sxl is not only lethal to males but also females. This is perhaps not altogether surprising given that Sxl can modulate the signal strength of a molecule crucial to the development of numerous tissues. The in vivo concentration of Sxl is, most likely, tightly controlled. Yanowitz et al. (Yanowitz et al., 1999) demonstrated that Sxl negatively regulates translation of its own mRNA. Combined with its positive autoregulatory splicing feedback loop, which ensures that essentially all of the *Sxl* mRNA is spliced in the productive female mode in females, this dual negative and positive autoregulation implies a homeostasis that keeps the concentration of Sxl in a predetermined fixed range. The potent effect of Sxl on the Hh signal makes the requirement for this dual regulation more readily understood.

Involvement of Hh in generating sexually dimorphic body size

Mutations in *Sxl* that produce sex transformed females generally result in animals that are small and male-like in size. Females transformed by mutations in *tra* appear as males but maintain the female size, indicating that sexual dimorphic body size is controlled by *Sxl*.

The enhanced levels of full-length Ci and the data of Figs 5-10 suggest that Sxl promotes disc growth. Indeed, when ectopic Sxl is being expressed in the dorsal half, many of the discs, both male and female, show an overgrowth phenotype with the dorsal half of the wing pouch frequently expanded and distorted. This growth effect is non autonomous (Fig. 10), indicating that it is effected by a system that signals beyond the cells expressing Sxl. This is consistent with the idea that Hh signaling is augmented to result in the overgrowth. The experiments described here do not rule out the possibility that Sxl may additionally regulate growth autonomously.

Hh with only the cholesterol modification has the greater impact on Sxl and its stabilization of full-length Ci. However, the Ci that is stabilized does not appear to accomplish Hh patterning. This raises the mechanistic question of how Sxl achieves growth of the entire disc.

Simply reducing the levels of the repressor form of Ci (which is accomplished by increasing the levels of full-length Ci) should increase the expression of the growth factor *dpp*. This is because *dpp* is affected by Ci at two levels: absence of the Ci repressor ameliorates repression to give low levels of *dpp* expression, while activated full-length Ci further elevates *dpp* transcription. Indeed, while the wing patterning defect caused by the ectopic expression of C84S-Hh narrows the region between wing veins L3 and L4 equally in the two sexes (due to its dominant-negative effect on endogenous Hh), the overall sexual dimorphic size difference is maintained. Consistent with this idea, co-expressing Sxl and Hh with only the cholesterol modification produces an overgrowth phenotype in discs (Fig. 2G-L), indicating Sxl can promote disc growth through this form of Hh.

The growth induced by Dpp has been described as 'balanced', involving both mass accumulation as well as cell cycle progression. The net effect is that cell size does not change, nor does the ploidy. This is in contrast to growth

induced by hyperactivation of Ras, Myc or Phosphoinositide 3 kinase, which increase growth but do not induce a progression through the G2/M phase of the cell cycle and, as a result, increase cell size (Martin-Castellanos and Edgar, 2002).

We propose that in the wild-type gradient of Hh with both its lipid modifications, Sxl augments the overall Hh signal to increase both full-length as well as activated full-length Ci. The two Hh targets (Ci and Sxl) respond differently to the various components of the pathway (Horabin et al., 2003). As Sxl is able to alter signal strength, the final outcome of the Hh signal must reflect the balance in activities of the components, modulated by the lipid moieties recognized, the membrane proteins used (Ptc versus Smo) and the proteins present in the Hh cytoplasmic complex. The studies reported here provide a strong rationale for why Sxl resides within the Hh cytoplasmic complex.

Sxl not only elevates expression of *dpp* and its downstream targets to induce growth, but is able to elevate *ptc* expression. Enhancing *ptc* suggests that the Hh signal is 'corrected' for the enlarged patterning field, as short-range patterning has to be controlled by Hh. By enhancing *dpp*, Sxl indirectly also enhances the long-range patterning system of the disc. Augmenting the Hh signal would thus appear an elegant solution for increasing overall size without changing the basic body plan or pattern. As Sxl is expressed in all female tissues from very early in development and this expression is maintained for the rest of the life cycle, Sxl is constantly available to upregulate the Hh signal. This augmentation must be kept within check, however, because, as argued above, too high an increase can change the overall slope of the Hh gradient, effectively changing the final patterning of the tissue.

The Hh pathway can also control body size in mammals. *ptc1* mutations in mice provide an overgrowth phenotype with large body size (Goodrich et al., 1997; Hahn et al., 1998), while increasing *ptc1* expression decreases body size (Milenkovic et al., 1999). Humans with basal cell nevus syndrome, an autosomal-dominant condition caused by the inheritance of a mutant *ptc* allele, have been reported to have multiple developmental abnormalities and, relevant to this study, larger body size (Gorlin, 1995). Whether the mechanism described here is global to sexually dimorphic organisms that use Hh for patterning remains to be seen.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/21/4801/DC1

References

Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B. (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

Bashaw, G. J. and Baker, B. S. (1997). The regulation of the Drosophila msl-2 gene reveals a function for Sex-lethal in translational control. *Cell* **89**, 789-798.

Boggs, R. T., Gregor, P., Idriss, S., Belote, J. M. and McKeown, M. (1987). Regulation of sexual differentiation in D. melanogaster via alternative splicing of RNA from the *transformer* gene. *Cell* **50**, 739-747.

Burke, R. and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122**, 2261-2269.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in Drosophila wings. *EMBO J.* **13**, 4459-4468.

Chou, T. B. and Perrimon, N. (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in Drosophila. *Genetics* **131**, 643-653.

Cline, T. (1984). Autoregulatory functioning of a *Drosophila* gene product that establishes and maintains the sexually determined state. *Genetics* **107**, 231-277.

Feng, J., White, B., Tyurina, O. V., Guner, B., Larson, T., Lee, H. Y., Karlstrom, R. O. and Kohtz, J. D. (2004). Synergistic and antagonistic roles of the Sonic hedgehog N- and C-terminal lipids. *Development* **131**, 4357-4370.

Goodrich, L. V., Milenkovic, L., Higgins, K. M. and Scott, M. P. (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* **277**, 1109-1113.

Gorlin, R. J. (1995). Nevoid basal cell carcinoma syndrome. *Dermatol. Clin.* **13**, 113-125.

Hahn, H., Wojnowski, L., Zimmer, A. M., Hall, J., Miller, G. and Zimmer, A. (1998). Rhabdomyosarcomas and radiation hypersensitivity in a mouse model of Gorlin syndrome. *Nat. Med.* **4**, 619-622.

Hooper, J. E. and Scott, M. P. (2005). Communicating with hedgehogs. *Nat. Rev. Mol. Cell. Biol.* **6**, 306-317.

Horabin, J. I., Walthall, S., Vied, C. and Moses, M. (2003). A positive role for Patched in Hedgehog signaling revealed by the intracellular trafficking of Sex-lethal, the *Drosophila* Sex Determination Master Switch. *Development* **130**, 6101-6109.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Jia, J., Tong, C. and Jiang, J. (2003). Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. *Genes Dev.* **17**, 2709-2720.

Johnston, L. A. and Shubiger, G. (1996). Ectopic expression of wingless in imaginal discs interferes with decapentaplegic expression and alters cell determination. *Development* **122**, 3519-3529.

Kelley, R. L., Wang, J., Bell, L. and Kuroda, M. I. (1997). Sex lethal controls dosage compensation in Drosophila by a non-splicing mechanism. *Nature* **387**, 195-199.

Keyes, L. N., Cline, T. W. and Schedl, P. (1992). The primary sex determination signal of Drosophila acts at the level of transcription. *Cell* **68**, 933-943.

Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387-393.

Lee, J. D., Kraus, P., Gaiano, N., Nery, S., Kohtz, J., Fishell, G., Loomis, C. A. and Treisman, J. E. (2001). An acylatable residue of Hedgehog is differentially required in Drosophila and mouse limb development. *Dev. Biol.* **233**, 122-136.

Lee, J. J., Ekker, S. C., von Kessler, D. P., Porter, J. A., Sun, B. I. and Beachy, P. A. (1994). Autoproteolysis in hedgehog protein biogenesis. *Science* **266**, 1528-1537.

Lum, L., Zhang, C., Oh, S., Mann, R. K., von Kessler, D. P., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B. and Beachy, P. A. (2003). Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. *Mol. Cell* **12**, 1261-1274.

Martin-Castellanos, C. and Edgar, B. A. (2002). A characterization of the effects of Dpp signaling on cell growth and proliferation in the Drosophila wing. *Development* **129**, 1003-1013.

McKeown, M., Belote, J. M. and Baker, B. S. (1987). A molecular analysis of *transformer*, a gene in Drosophila melanogaster that controls sexual differentiation. *Cell* **48**, 489-499.

Milenkovic, L., Goodrich, L. V., Higgins, K. M. and Scott, M. P. (1999). Mouse patched1 controls body size determination and limb patterning. *Development* **126**, 4431-4440.

Moreno, E., Basler, K. and Morata, G. (2002). Cells compete for Decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. *Nature* **416**, 755-759.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of DPP morphogen gradient. *Cell* **85**, 357-368.

Nishi, K., Yoshida, M., Fujiwara, D., Nishikawa, M., Horinouchi, S. and Beppu, T. (1994). Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. *J. Biol. Chem.* **269**, 6320-6324.

Ogden, S. K., Ascano, M., Jr, Stegman, M. A., Suber, L. M., Hooper, J. E. and Robbins, D. J. (2003). Identification of a functional interaction between the transmembrane protein Smoothened and the kinesin-related protein Costal2. *Curr. Biol.* **13**, 1998-2003.

Ohlmeyer, J. T. and Kalderon, D. (1998). Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* **396**, 749-753.

Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K. and Beachy, P. A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. *Nature* **374**, 363-366.

Porter, J. A., Young, K. E. and Beachy, P. A. (1996). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* **274**, 255-259.

Robbins, J., Nybakken, K., Kobayashi, R., Sisson, J., Bishop, M. and Therond, P. (1997). Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein *costal-2*. *Cell* **90**, 225-234.

Ruel, L., Rodriguez, R., Gallet, A., Lavenant-Staccini, L. and Therond, P. P. (2003). Stability and association of Smoothened, Costal2 and Fused with Cubitus interruptus are regulated by Hedgehog. *Nat. Cell Biol.* **5**, 907-913.

Samuels, M. E., Schedl, P. and Cline, T. W. (1991). The complex set of late transcripts from the Drosophila sex determination gene sex-lethal encodes multiple related polypeptides *Mol. Cell. Biol.* **11**, 3584-3602.

Sanchez, L. and Nöthiger, R. (1983). Sex determination and dosage compensation in Drosophila melanogaster: production of male clones in XX females. *EMBO J.* **2**, 211-214.

Schwartz, C., Locke, J., Nishida, C. and Kornberg, T. B. (1995). Analysis of cubitus interruptus regulation in Drosophila embryos and imaginal disks. *Development* **121**, 1625-1635.

Sisson, J., Ho, K., Suyama, K. and Scott, M. (1997). *Costal-2*, a novel kinesin-related protein in the *hedgehog* signaling pathway. *Cell* **90**, 235-245.

Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. *Mol. Cell.* **5**, 59-71.

Vied, C. and Horabin, J. I. (2001). The sex determination master switch, Sex-lethal, responds to Hedgehog signaling in the Drosophila germline. *Development* **128**, 2649-2660.

Yanowitz, J. L., Deshpande, G., Calhoun, G. and Schedl, P. D. (1999). An N-terminal truncation uncouples the sex-transforming and dosage compensation functions of Sex-lethal. *Mol. Cell. Biol.* **19**, 3018-3028.

Zhang, W., Zhao, Y., Tong, C., Wang, G., Wang, B., Jia, J. and Jiang, J. (2005). Hedgehog-Regulated Costal2-Kinase Complexes Control Phosphorylation and Proteolytic Processing of Cubitus Interruptus. *Dev. Cell* **8**, 267-278.