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)

Accepted 23 August 2005
Development 132, 4801-4810
Published by The Company of Biologists 2005
doi:10.1242/dev.02054

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 (BasBashaw 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 temperature for 4-6 days. Transgenes were expressed using either y w; aperature-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, IP7BO, was recombined onto a FRT101 X chromosome. hs-flp on an MKRS balancer was introduced into the IP7BO, 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 Shubiger, 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, 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 influence Sxl nuclear entry (B,E; brighter, more punctate signal) (Horabin et al., 2003). Insets in A,B enlarge the area 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: 50 μm. Anterior towards the left, ventral is towards the top. Confocal images throughout.

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
Development

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 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.

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 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.

development

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
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. 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 LL 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.

**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.

**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.
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 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
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 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. 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.

**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 (J)). 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.
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...
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 response, 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 differentially 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.

I am indebted to Dr M. Yoshida for the gift of LMB and Dr D. Bopp for the UAS-Sxl line without which these experiments could not be conducted. The technical assistance of M. Moses is acknowledged. I thank S. Walthall and M. Moses for scientific discussions; Dr R. Holmgren for the anti-Ci full length monoclonal; Dr G. Marques for anti-pMad polycional antibodies; Drs A. Telemán and S. Cohen for the rat anti-Sal antibodies; Drs G. Struhl, K. Basler, J. Jiang, D. Kalderon and the Bloomington stock center for fly stocks. The anti-Engrailed antibody developed by Dr C. Goodman, the anti-Wg developed by Dr S. Cohen and the anti-β-tubulin antibodies developed by Dr M. Klymkowski were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. I also thank Albert Tousson and Shawn Williams from the UAB imaging facility, and Kim Riddle at the Biology imaging facility at FSU. This work was supported by a grant from NIH.

**Supplementary material**

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

**References**


