Drosophila *Lyra* Mutations Are Gain-of-Function Mutations of *senseless*

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ABSTRACT

The *Lyra* mutation was first described by Jerry Coyne in 1935. *Lyra* causes recessive pupal lethality and adult heterozygous *Lyra* mutants exhibit a dominant loss of the anterior and posterior wing margins. Unlike many mutations that cause loss of wing tissue (*e.g.*, *scalloped*, *Beadex*, *cut*, and *apterous-Xasta*), *Lyra* wing discs do not exhibit increased necrotic or apoptotic cell death, nor do they show altered BrdU incorporation. However, during wing disc eversion, loss of the anterior and posterior wing margins is apparent. We have previously shown that *senseless*, a gene that is necessary and sufficient for peripheral nervous system (PNS) development, is allelic to *Lyra*. Here we show by several genetic criteria that *Lyra* alleles are neomorphic alleles of *senseless* that cause ectopic expression of SENSELESS in the wing pouch. Similarly, overexpression of SENSELESS in the wing disc causes loss of wing margin tissue, thereby mimicking the Lyra phenotype. *Lyra* mutants display aberrant expression of DELTA, VESTIGIAL, WINGLESS, and CUT. As in *Lyra* mutants, overexpression of SENSELESS in some areas of the wing pouch also leads to loss of WINGLESS and CUT. In summary, our data indicate that overexpression of SENSELESS causes a severe reduction in NOTCH signaling that in turn may lead to decreased transcription of several key genes required for wing development, leading to a failure in cell proliferation and loss of wing margin tissue.

LIKE *Lyra*, many mutations that affect wing morphogenesis have mutant phenotypes in which there are missing sectors of the wing margins. These include *vestigial*, *Notch*, *Delta*, *cut*, *apterous*, and others (Jack *et al.* 1991; Cohen *et al.* 1993; Kim *et al.* 1996; Artavanis-Tsakonas *et al.* 1999), which have been shown to play important roles during fruitfly development. It is known that many of these so-called "wing scalloping" mutations, including *cut* and *vestigial*, are caused by excessive cell death in the prospective wing margins of late larvae following a period of apparently normal development (Fristrom 1968, 1969). In *Lyra* mutants, although there is a significant reduction (10–20%) of the number of cells in the adult wing, no evidence of apoptotic or necrotic cell death was found by transmission electron microscopy, acridine orange, or trypan blue staining in third instar and pupal discs (Abbott 1986). These and other data have led to the suggestion that *Lyra* may affect more fundamental parameters of cell growth and specification. The *Lyra* mutant has therefore been of interest to those interested in wing margin development (*e.g.*, Abbott 1986; Abbott and Sprey 1990; Jack and Delotto 1992; Sturtevant and Bier 1995).

The *Lyra*[1] mutation is associated with an X-ray-induced deletion uncovering cytological bands 70A2-3;A5-6 (Coyne 1935; Zhimulev and Feldman 1982; Lindsley and Zimm 1992). It is a dominant mutation that causes a regular and predictable pattern of loss of the anterior and posterior wing margins along with a small amount of nearby wing surface tissue. The presence of a duplication of the chromosomal region carrying the wild-type *Lyra* locus in a *Lyra*[1] background does not suppress the Lyra phenotype (Abbott and Sprey 1990). Hence, the dominant phenotype is not due to haploinsufficiency. This suggested that *Lyra* is a gain-of-function mutation and is likely to be a neomorphic allele (Muller 1932) characterized by spatial and/or temporal misregulation of expression of a gene product. In addition, the results of clonal analysis with wild-type clones in *Lyra*/+ flies indicate a non-cell-autonomous function (Abbott 1986), suggesting that the *Lyra* mutation may affect processes requiring cross-talk among cells such as specification of positional information or lateral inhibition.

Properties of the Lyra[1] phenotype were studied extensively by Abbott (1986) and Abbott and Sprey (1990). Some of their key observations and conclusions were the following. First, excessive cell death in the putative wing margins of third instar and early pupal wing discs does not account for margin loss in *Lyra* adult wings. Second, although *Lyra* mutants do not form anterior and posterior wing margins, there is a normal dorsal/ventral compartment boundary as well as a zone of nonproliferating cells (ZNC) along the "de facto" *Lyra* wing

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margin. Third, a monoclonal antibody, which binds the unidentified E1C antigen expressed in the larval wing margin precursor, enabled them to demonstrate that an effect of *Lyra* on anterior and posterior wing margins is apparent early in the third larval instar. Fourth, margin rescue experiments using clonal analysis showed that wild-type *Lyra* is not required for bristle development *per se*. Fifth, further analysis of shape and position of clones indicated that the wing margin, defined as a set of several rows of cells along either side of the dorsal/ventral boundary, plays an important role in wing morphogenesis. This observation presaged the current paradigm that interactions among a number of gene products expressed in the margin region, often acting across the compartment boundary, serve to organize wing development (for review see Brook *et al.* 1996).

Here we show that *Lyra* mutations correspond to neomorphic/gain-of-function mutations of *senseless*. The *senseless* gene plays a key role in peripheral nervous system (PNS) development. Its loss causes a severe loss of external peripheral sensory organs in embryos, and its overexpression causes the formation of extra PNS organs (Nolo *et al.* 2000). Our data indicate that in *Lyra* mutants, SENSELESS is ectopically expressed in the wing pouch and that this ectopic expression goes hand in hand with localized loss of DELTA, VESTIGIAL, WINGLESS, and CUT expression. We therefore propose that the loss of wing margin in *Lyra* mutants is due to ectopic expression of SENSELESS, which also causes loss of expression of genes essential for wing margin and wing development, including but not limited to *wingless*, *vestigial*, and *cut*. We suggest that it is this loss of normal pattern of gene expression in the developing wing margin that leads to failure of differentiation of the sensory organ precursors in the wing discs and, later, to the loss of wing margin tissue seen in *Lyra* wings. However, the tissue loss because of ectopic expression of SENSELESS in the wing margin is quite different from the excess PNS organs (bristles) that arise from overexpression of SENSELESS in some areas of the fly integument (Nolo *et al.* 2000).

MATERIALS AND METHODS

Drosophila stocks: The wild-type stock was Canton-S (Bloomington Stock Center). The other stocks used in this work are as follows:

yw; P[lacZ, w$^+$]64A sens^{M256} th st cu sr e^s ca/TM6, Hu P[w$^+$, abdA-lacZ] e Tb ca (Salzberg *et al.* 1994)
yw; P[lacZ, w$^+$]64A sens^{I235} th st cu sr e^s ca/TM6, Hu P[w$^+$, abdA-lacZ] eTb ca (Salzberg *et al.* 1994)
yw; Df(3L)1228/4 P[lacZ, w$^+$]/TM3, Ser Sb (Salzberg *et al.* 1997)
Ly^1/TM3, Sb (Abbott 1986)
Lyra^{Sx67}/TM3, Sb (P. Heizler, Strasbourg, France)
P{hsneo}l(3)neo19 (Spradling *et al.* 1999)
Delta^{130}P{ry$^{[+]}$=lArB}A77.1M3/TM3, Sb ry
sens^{E1} red e/TM3, Sb e (H. Irick, Bloomington, IN)
sens^{E2} red e/TM3, Sb e (H. Irick)
sens^{E53} red e/TM3, Sb e (H. Irick)
sens^{E54} red e/TM3, Sb e (H. Irick)
sens^{E58} red e/TM3, Sb e (H. Irick)
sens^{E69} red e/TM3, Sb e (H. Irick)
vgBE-lacZ and *vgQE-lacZ* (S. Carroll, Madison, WI)

The GAL4- drivers used were *C96-GAL4* (R. Bodmer, Ann Arbor, MI), *C1003-GAL4* (J. Lopez, New York), and *dpp-GAL4/TM6B* (G. Mardon, Houston) with *UAS-lacZ* and *UAS-sens (C5, C6)* (Nolo *et al.* 2000) lines.

Immunohistochemistry and antibody staining: X-Gal staining was performed as described (Bellen *et al.* 1989). Primary antibodies used were guinea pig anti-SENSELESS (1:1000, Nolo *et al.* 2000), mouse anti-β-galactosidase (1:1000, Promega, Madison, WI), mouse anti-WINGLESS (1:10, a gift from S. Cohen), mouse anti-CUT (1:30, Bodmer *et al.* 1987), rabbit anti-SCUTE (1:100, a gift from G. Panganiban), mouse anti-DELTA (1:100, a gift from M. Muskavitch), and rabbit anti-VESTIGIAL (1:500, a gift from G. Halder). Fluorescent secondary antibodies were from Molecular Probes (Eugene, OR) or Jackson ImmunoResearch (West Grove, PA; ALEXA and Cy3, respectively). Biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA) and were used according to the manufacturer's instructions. Confocal images were captured using an MRC 1024 microscope (Bio-Rad, Richmond, CA) and all figures were processed with Adobe Photoshop software.

***In situ* hybridization:** *string* cDNA (a gift from B. Edgar) was used as a template for digoxigenin-labeled RNA probes (RNA labeling kit; Roche, Indianapolis).

RESULTS

***Lyra* alleles are gain-of-function alleles of *senseless*:** Salzberg *et al.* (1994, 1997) showed that the *senseless* gene affects the development of the PNS and reported three alleles: two ethyl methanesulfonate (EMS)-induced alleles (*sens^{M256}* and *sens^{I235}*) and one induced by *P*-element dysgenesis (*sens$^{1228/4}$*). The lethality associated with these alleles was mapped by meiotic recombination to 3-40.5, where *Lyra^1* maps (Nolo *et al.* 2000). As shown in Table 1, *senseless* mutations *M256* and *I235* failed to complement the lethality of *Lyra^1*, which is associated with deletion 70A2-3;70A5-6 (Lindsley and Zimm 1992). The *Lyra^1* deletion uncovers three essential complementation groups: *l(3)70Aa*, *l(3)70Ab*, and *l(3)70Ac* (Zhimulev and Feldman 1982). Unfortunately, these mutants no longer exist. However, Holly Irick and Peter Cherbas carried out an EMS mutagenesis to identify lethal mutations uncovered by *Df(3L)BK10* (71C3;71E5). Because the *Df(3L)BK10* chromosome was marked with *Lyra^1* we suspected that some of their lethals were in the *Lyra^1* deficiency. We tested 27 lethal mutants that failed to complement the *Lyra^1 Df(3L)BK10* chromosome and isolated 8 lethal mutations that failed to complement the *Lyra^1* deficiency.

As shown in Table 1, complementation tests for the lethal phenotype showed that six of these mutations are alleles of *senseless* referred to as complementation group *l(3)70Ad* in FlyBase. This complementation group is presumably allelic to one of the lost *l(3)70A* complementa-

TABLE 1

Complementation tests

	M256	*I235*	*E1*	*E2*	*E53*	*E54*	*E58*	*E69*	*1228/4*	*Ly*^{1}	*Ly*^{Sx67}	*delta*^{130}	*P257*
M256													
I235	−												
E1	−	−											
E2	−	−	−										
E53	−	−	−	−									
E54	−	−	−	−	−								
E58	−	−	−	−	−	−							
E69	−	−	−	−	−	−	−						
1228/4	−	−	−	−	−	−	−	−					
Ly^{1}	−	−	−	−	−	−	−	−	−				
Ly^{Sx67}	+	+	+	+	+	+	+	+	+	NA			
delta^{130}	−	−	−	−	−	−	−	−	−	−	+		
P257	+	+	+	+	+	+	+	+	−	+	+	−	

M256, *sens*^{M256}; *I235*, *sens*^{I235}; *E1*, *sens*^{E1}; *E2*, *sens*^{E2}; *E53*, *sens*^{E53}; *E54*, *sens*^{E54}; *E58*, *sens*^{E58}; *E69*, *sens*^{E69}; *1228/4*, *Df(3L)1228/4*; *delta*^{130}, an imprecise excision #130 of *P{ry*$^{[+]}$*=lArB}A77.1M3*; *P257*, *P{hsneo}l(3)neo19*; NA, not applicable.

tion groups described by Zhimulev and Feldman (1982). The *sens* alleles were designated *E1*, *E2*, *E53*, *E54*, *E58*, *E64*, *E69*, and *E87*. (H. Irick and P. Cherbas, personal communication to FlyBase). All but *E64* and *E87* are still available.

The *Lyra*^{1} deficiency *in trans* to other *senseless* alleles causes lethality but these mutant embryos do not display a severe loss of neurons as typically seen in homozygous *senseless* mutations (Nolo *et al.* 2000). Indeed, the following observations suggest that the *Lyra*^{1} mutation is not a loss-of-function allele of *senseless*. First, *Lyra*^{1}*/sens* mutant embryos display either no loss of PNS neurons or a very subtle loss, indicating that the deficiency associated with *Lyra*^{1} does not result in the lack of the *senseless* gene product. Second, none of the *senseless* alleles causes a loss of wing margin phenotype in heterozygous flies (*sens/+*), indicating that haploinsufficiency of *sens* does not cause the Lyra phenotype, in agreement with the observations of Lindsley *et al.* (1972). Third, a second, independently generated dominant allele of *Lyra*, *Lyra*^{SX67}, interacts additively with *Lyra*^{1} to produce a more severe margin loss, but complements all *senseless* alleles (Table 1). Since *Lyra* mutations are dominant and their phenotype is not caused by haploinsufficiency of *senseless*, they are presumed to be either antimorphic (dominant negative) or neomorphic (gain of function) in nature (Muller 1932). An antimorphic nature is most unlikely since duplications of the chromosomal region do not ameliorate the phenotype associated with *Lyra*^{1} (Muller 1932; Abbott and Sprey 1990). Furthermore, the *Lyra*^{1}*/sens* mutants do not display obvious defects in the PNS, as would be expected if *Lyra* was a dominant-negative allele of *senseless*. We therefore conclude that *Lyra* mutations are neomorphic mutations.

The following data support the neomorphic nature of the *Lyra* mutations, that is, that they are gain-of-function, regulatory mutations of *senseless*. First, the dominant phenotype associated with *Lyra*^{1} could not be recombined onto a *senseless* mutant chromosome, indicating that both mutations map at the same site and that the Lyra phenotype may be breakpoint dependent. Second, molecular analyses show that the distal breakpoint of *Df(3L)Ly*^{1} affects a genomic fragment that contains the 3′ end, including the 3′ untranslated region, of the *sens* gene (data not shown). Third, an X-ray-induced revertant of *Lyra*^{SX67}, *Lyra*^{SX67R12}, is homozygous lethal and fails to complement all the *senseless* alleles, showing that *Lyra*^{SX67} is associated with *senseless*. Fourth, both *Lyra* mutations cause ectopic expression of SENSELESS in wing imaginal discs (see below). These observations strongly indicate that the *Lyra* alleles are neomorphic/gain-of-function mutations of *senseless*.

The complementation data, combined with data from anti-SENSELESS-stained embryos, and the analysis of the severity of the phenotypes in which there is loss of PNS neurons in embryos support the allelic series that is shown in Table 2. We propose to keep the name *senseless*, which refers to the loss of PNS organs, and to refer to *Lyra* alleles of *senseless* as neomorphic/gain-of-function alleles that affect the wing margin.

SENSELESS is ectopically expressed in the wing pouch of *Lyra* mutants: We have previously shown that *senseless* is expressed in the sensory organ precursors (SOPs) of the embryonic and adult PNS (Nolo *et al.* 2000). In wild-type imaginal wing discs, SENSELESS is expressed in the SOPs along the presumptive wing margin (Figure 1A). To determine if the expression pattern of SENSELESS is altered in wing discs of *Lyra*^{1} and *Lyra*^{Sx67} we carried out *in situ* hybridization and immunohistochemical staining with antibodies raised against the full-length SENSELESS protein. As shown in Figure 1B, in addition to the expression in SOPs, we observe a

TABLE 2

Characteristics of different *senseless* mutations

Allele	Loss or gain of function	Strength of allele	Rescue	Messenger	Protein
$sens^{M256}$	Loss	Strong	+	++	+
$sens^{I235}$	Loss	Strong	+	++	+
$sens^{E1}$	Loss	Strong	+	+	±
$sens^{E2}$	Loss	Strong	+	++	+
$sens^{E53}$	Loss	Strong	ND	++	ND
$sens^{E54}$	Loss	Strong	+	++	+
$sens^{E58}$	Loss	Strong	+	++	+
$sens^{E69}$	Loss	Weak	+	+++	++
1228/4	Loss	Strong	ND	−	−
Ly^{1}	Gain	Weak	ND	+++	+++
Ly^{Sx67}	Gain	Very weak	NA	*	*
yw	wt	N/A	NA	+++	+++

ND, not determined; NA, not applicable; +++, wild-type levels; ++, mildly reduced; +, strongly reduced; ±, extremely reduced; *, unable to establish, as the Ly^{Sx67} carries at least one other early lethal mutation.

broad band of SENSELESS-positive cells in the anterior and posterior regions of the wing pouch of $Lyra^{1}/+$ mutant wing discs. As shown in Figure 1D, the ectopic expression of SENSELESS surrounding the anterior and posterior margin precursors in the wing pouch of $Lyra^{Sx67}/+$ is even more pronounced than in that of $Lyra^{1}/+$ wing discs. The difference in expression levels is also observed with *in situ* hybridizations (data not shown). These expression levels correlate positively with the loss of wing tissue in the anterior and posterior wing margin as the phenotype is more severe in $Lyra^{Sx67}/+$ wings (Figure 1E) than in those of $Lyra^{1}/+$ (Figure 1C). Hence, these data indicate that the anterior and posterior margins of the presumptive wing are highly sensitive to ectopic SENSELESS expression and suggest that the wing margin loss in *Lyra* mutants may well be triggered by ectopic SENSELESS.

Ectopic expression of SENSELESS causes loss of wing margin: To demonstrate that ectopic expression of SENSELESS can mimic the Lyra phenotype, we constructed flies that carried different *UAS-senseless* transgenes under the control of GAL4 drivers that express GAL4 rather specifically in the wing disc. Most and possibly all GAL4 drivers that cause widespread expression of GAL4 are lethal in the presence of *UAS-senseless*. As shown in Figure 2, A and B, ectopic expression of SENSELESS in the wing disc using the *C1003-GAL4* driver causes a phenotype that is similar to that observed in *Lyra* mutants in that the wing margins are severely affected whereas the rest of the wing is unaffected. As shown in Figure 2, C and D, expression of SENSELESS in a domain that corresponds to the wing margin using the *C96-GAL4* driver (Gustafson and Boulianne 1996) also causes a loss of wing margin. In this case the loss is not as severe as that induced by the more ubiquitous driver. These observations clearly show that ectopic expression of SENSELESS is sufficient to cause loss of wing margin tissue. Conversely, they indicate that other areas of the wing disc do not respond to ectopic SENSELESS expression with tissue loss. Instead, as reported previously (Nolo *et al.* 2000), we consistently observed that ectopic SENSELESS causes scattered supernumerary bristles on nonmargin surfaces of adult wings. Moreover, ectopic expression of SENSELESS in wing discs,

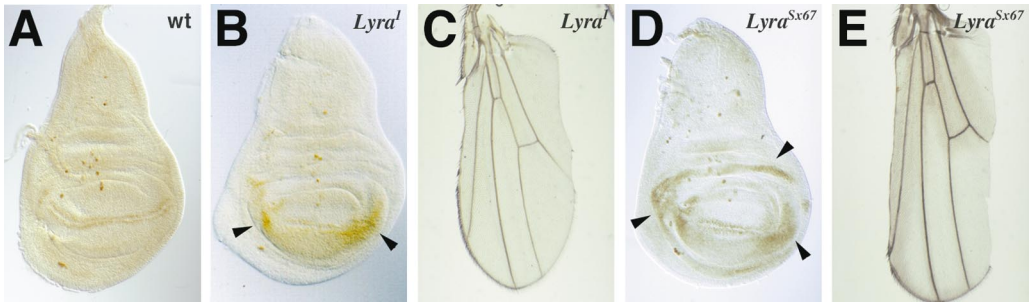

Figure 1.—Overexpression of SENSELESS in *Lyra* mutants. Third instar wing discs were stained with anti-SENSELESS antibodies (A, B, and D). (A) Canton-S wing disc. Note that the expression is confined to many single cells that correspond to SOPs (Nolo *et al.* 2000). (B) $Lyra^{1}/+$ wing disc. The arrows point to a broad domain of SENSELESS expression that is never observed in wild-type discs. (C) $Lyra^{1}/+$ wing. Wings of this genotype always show a much milder phenotype than the one shown in E. (D) $Lyra^{Sx67}/+$ wing disc. Note the ectopic expression of SENSELESS in a broad area of the wing pouch and beyond. (E) $Lyra^{Sx67}/+$ wing.

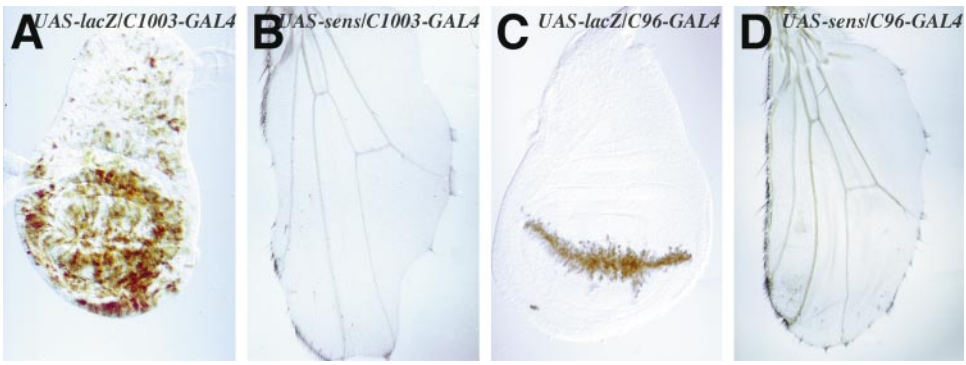

FIGURE 2.—Ectopic expression of SENSELESS affects the wing margin. Third instar wing discs (A and C). Wings (B and D). (A) *C1003-GAL4/UAS-lacZ* wing disc stained with anti-β-galactosidase. Note the scattered *lacZ* expression. (B) *C1003-GAL4/UAS-sens(C6)* wing. Although SENSELESS is expressed in many of the wing pouch cells, tissue loss is mostly observed at the wing margin. (C) *C96-GAL4/UAS-lacZ* wing disc stained with anti-β-galactosidase. *lacZ* expression is confined to the wing margin and adjacent cells. (D) *C96-GAL4/UAS-sens(C5)* wing displays some wing margin loss. The loss is more severe in the distal and posterior area of the wing.

using a *dpp-GAL4* driver, resulted in large clusters of extra bristles on the notum as well as parts of the wing surface. In leg discs, this driver caused a severe loss of all distal parts of the leg (data not shown). We conclude that ectopic expression of SENSELESS causes very different phenotypes: loss of tissue in some areas of imaginal discs and extra sensory organs in others.

***Lyra* mutations cause loss of DELTA, VESTIGIAL, WINGLESS, and CUT expression:** To determine the effects of *Lyra* mutations on the expression of key genes that have been shown to play important roles in wing development we tested four markers. The *vestigial* gene can be viewed as the wing selector gene as its lack of expression causes wing loss and its ectopic expression causes extra wing tissue (KIM *et al.* 1996). VESTIGIAL expression at the dorso-ventral boundary is essential to wing margin development. Furthermore, *vestigial* is a marker for wing identity and has an important function in wing growth (KIM *et al.* 1996; NEUMANN and COHEN 1996; KLEIN and MARTINEZ ARIAS 1999).

We tested the effect of both *Lyra* mutants on the expression pattern of *lacZ* driven by the *vestigial* boundary enhancer *vg*BE (WILLIAMS *et al.* 1994). As shown in Figure 3, A and F, *lacZ* expression of the boundary enhancer is almost entirely lost in the anterior and posterior portion of the wing pouch of $Lyra^{1}$ but is restored in the revertant, which has the same pattern of expression as the wild-type adult wing. The pattern is similar in $Lyra^{SX67}$ (data not shown), except that slightly more prospective margin is missing, in agreement with the more severe margin loss in the adult wing. *Lyra* has no effect on the *vestigial* quadrant enhancer, *vg*QE (data not shown), which controls later VESTIGIAL expression and growth of the nonmargin portion of the wing pouch (WILLIAMS *et al.* 1993, 1994; KIM *et al.* 1996). In this case VESTIGIAL is expressed throughout the wing blade but not in the prospective margin. As shown in Figure 3B, immunocytochemical staining with the anti-VESTIGIAL antibody shows a different pattern of expression in *Lyra* wing discs in the anterior and posterior area of the wing pouch when compared to wild type (Figure 3G). We do not know what underlies this altered pattern, but Figure 3A suggests that it may be due to loss of VESTIGIAL expression at the dorso-ventral boundary. Since loss-of-function clones of *vestigial* (vg^{-}/vg^{-}) do not proliferate in the wing (KIM *et al.* 1996), the loss of wing margin tissue in *Lyra* mutants could be caused by a partial loss of VESTIGIAL expression at the anterior and posterior wing boundary. This in turn may cause loss of cell proliferation during pupal wing development.

In addition to *vestigial*, *wingless* has also been shown to play an essential role in wing development (BAKER 1988). WINGLESS protein is secreted and is produced in a stripe of three to four cell rows stradling the dorso-ventral boundary (WILLIAMS *et al.* 1993; COUSO *et al.* 1994). The stripe of WINGLESS-expressing cells induces neighboring cells to differentiate into the bristles that are present at the wing margin (PHILLIPS and WHITTLE 1993; COUSO *et al.* 1994). Removing WINGLESS in second or early third instars results in the loss of tissue from the wing margin (COUSO *et al.* 1994; DIAZ-BENJUMEA and COHEN 1995; NEUMANN and COHEN 1996). The role of WINGLESS with respect to regulation of VESTIGIAL expression at the dorso-ventral boundary is still controversial (GO *et al.* 1998; KLEIN and MARTINEZ ARIAS 1999). However, it is fairly clear that NOTCH signaling is the primary inducer of *vg*BE. Hence, WINGLESS expression in *Lyra* mutants may provide an independent means to assess the effect of *Lyra* mutations on wing development. As shown in Figure 3C, WINGLESS expression is severely reduced in the anterior and posterior domain of the wing pouch of *Lyra* mutants (compare with Figure 3H). With exception of the central domain of the dorso-ventral boundary, where WINGLESS expression is apparently normal (as is the *Lyra* wing margin), its expression is confined to a narrow

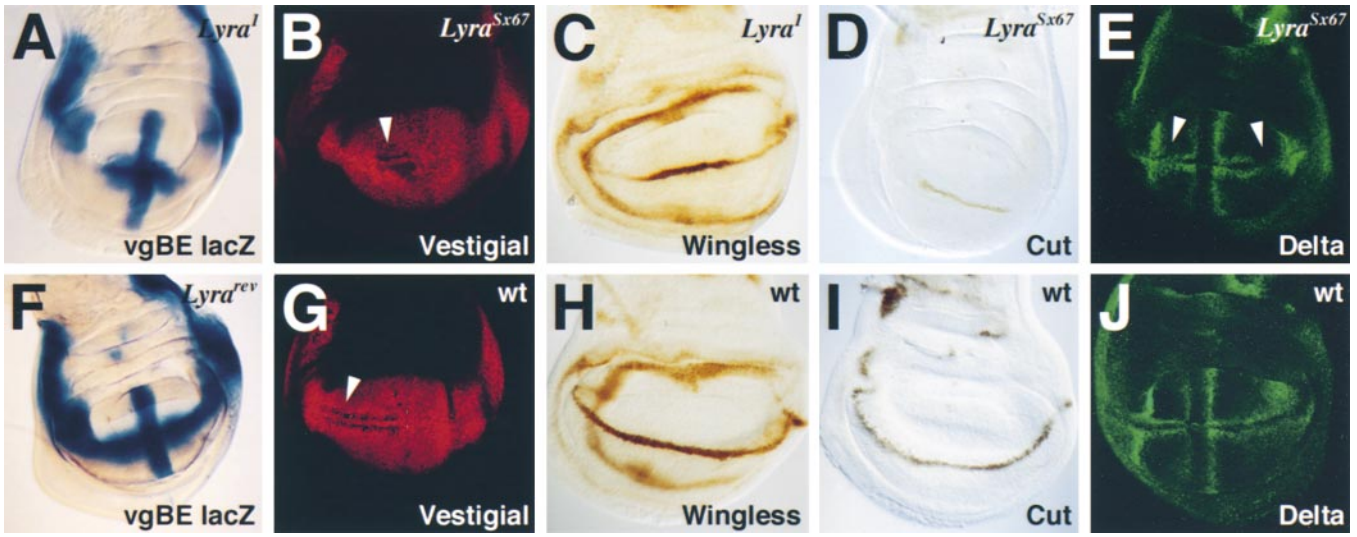

FIGURE 3.—Loss of vestigial, Wingless, Cut, and Delta in third instar wing discs of *Lyra* mutants. Third instar wing imaginal discs of *Lyra* mutants (A–E) and controls (F–J). (A and F) *Lyra*/+;*vgBE-lacZ*/+ (A) and a $Lyra^{Sx67}$ revertant (F) stained with X-gal. Note the loss of *lacZ* expression in the anterior and posterior wing margin in A. (B and G) $Lyra^{Sx67}$/+ wing disc stained with anti-VESTIGIAL antibodies shows aberrant staining in mutants when compared to wild-type disc (G). (C and H) $Lyra^{1}$/+ and wild-type discs stained with anti-WINGLESS (C). (D and I) $Lyra^{Sx67}$/+ (D) and wild-type disc (I) stained with anti-CUT. Note the loss of CUT staining in the anterior and posterior wing pouch (D). (E and J) Anti-DELTA staining of $Lyra^{Sx67}$/+ (E) and control (J) discs.

domain in which levels of Wingless protein are reduced severely. Since WINGLESS is an important secreted factor for wing margin development, this reduction in expression in *Lyra* mutants may act in an additive fashion with the loss or severe reduction of VESTIGIAL expression.

Given the similarities between the loss of wing margin tissue in some *cut* and *Lyra* mutants (JACK and DELOTTO 1992) we also investigated CUT expression in *Lyra* mutants. CUT is expressed in a row that is two to five cells wide at the dorso-ventral boundary (JACK *et al.* 1991; BLOCHLINGER *et al.* 1993). This expression is largely overlapping with that of WINGLESS and the *vestigial* boundary enhancer (WILLIAMS *et al.* 1994) but occurs in the mid-third instar, much later than either WINGLESS or *vg*BE. Loss of CUT expression on both sides of the wing boundary results in extensive notching of the margin (JACK *et al.* 1991; DORSETT 1993). *cut* has been shown to be a direct target of *Notch*, but not of *wingless* (MICCHELLI *et al.* 1997). In addition, while the initiation of WINGLESS expression is not dependent on *cut*, maintenance of WINGLESS expression is dependent on *cut* (MICCHELLI *et al.* 1997). As shown in Figure 3, D and I, CUT expression is essentially abolished in *Lyra* mutants in the anterior and posterior region of the wing pouch.

Since NOTCH signaling plays a prominent role in the regulation of the expression of *vg*BE, *wingless*, and *cut* (GO *et al.* 1998), we attempted to determine if NOTCH signaling is affected. We stained *Lyra* wing discs with anti-DELTA antibodies as anti-NOTCH antibody immunohistochemical staining of wing discs failed because of high background levels. As shown in Figure 3E, there is an obvious reduction in the expression of DELTA in the anterior and posterior wing pouch along the presumptive wing margin (compare to Figure 3J). Hence, one of the key known activators of NOTCH signaling at the dorso-ventral boundary is altered and reduced in its expression pattern. In summary, four known markers that have previously been shown to be required for the development of the wing margin and the rows of bristles along the margin are not expressed properly in *Lyra* mutants. In addition, the domains of expression that are affected in these mutants correspond to the domains that are affected in *Lyra* mutant discs and adult wings and are contained within the domains in which SENSELESS is expressed ectopically. These data suggest that ectopic expression of SENSELESS in *Lyra* mutants may be able to downregulate the expression of several genes that play a pivotal role in wing margin development, possibly by downregulating NOTCH signaling.

Ectopic expression of SENSELESS affects WINGLESS and CUT expression: To further investigate the ability of SENSELESS to downregulate the expression of specific genes, we tested the effect of ectopic expression of SENSELESS on WINGLESS and CUT expression. Ectopic expression of SENSELESS using the *C96-GAL4* wing margin driver and staining with anti-SENSELESS is shown in Figure 4A. SENSELESS overexpression causes a dramatic downregulation of WINGLESS (Figure 4B) and CUT protein levels (Figure 4C), although in both cases clusters of immunoreactive cells along the wing margin remain. Similarly, when using the *dpp-*

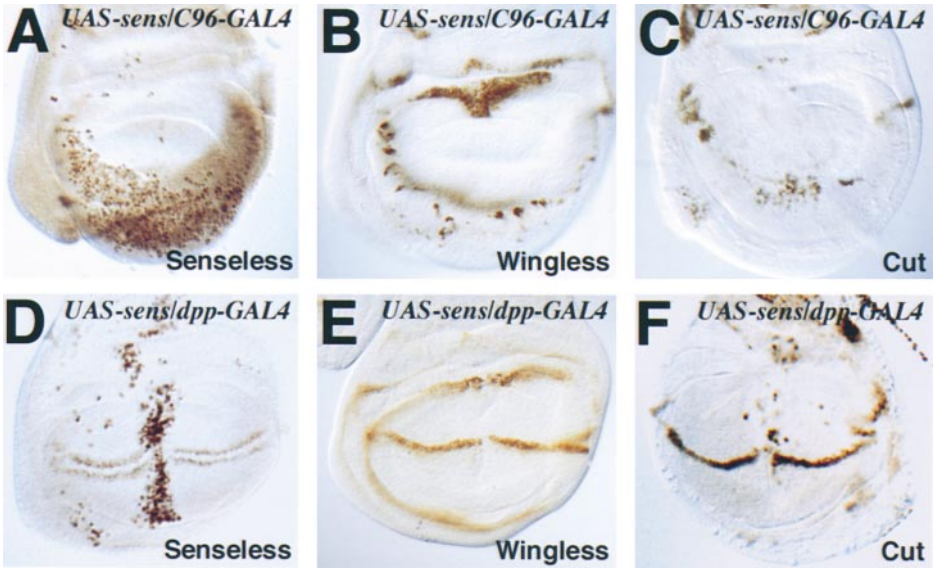

FIGURE 4.—Ectopic expression of SENSELESS causes loss of WINGLESS and CUT expression. In all panels ectopic expression of SENSELESS is achieved using the *UAS-sens(C5)* transgene (NOLO *et al.* 2000). (A–C) The driver is *C96-GAL4*, causing ectopic expression in the wing margin area. (D–F) Ectopic expression is along the anterior-posterior wing boundary using the *dpp-GAL4* driver. (A–C) Ectopic expression of SENSELESS along the wing boundary (A) causes a severe reduction of WINGLESS immunoreactivity (compare Figure 4B with 3H) and CUT staining (compare Figure 4C with 3I). (D–F) Ectopic expression of SENSELESS along the anterior-posterior wing boundary (D) causes a small gap in WINGLESS (E) and CUT (F) expression. In addition, in other areas of the disc, CUT is ectopically expressed in cells where it is not normally expressed (F). This gap in WINGLESS and CUT expression causes a loss of the distal wing margin (data not shown).

GAL4 driver to ectopically express SENSELESS along the anterior-posterior wing boundary (Figure 4D) we find a precise disruption in the continuity of WINGLESS (Figure 4E) and CUT (Figure 4F) expression where the *dpp* stripe is normally expressed. This downregulation correlates with a loss of the distal tip of the wing (data not shown). Note also that ectopic SENSELESS expression causes ectopic CUT expression in some cells of the wing pouch that normally do not express CUT, as expected from previous observations (NOLO *et al.* 2000). In summary, these data demonstrate that ectopic expression of SENSELESS in the wing margin is a potent repressor of expression of key players previously shown to function in wing margin development.

Margin loss in *Lyra* mutants: The loss of wing margin in *Lyra* mutants can be viewed as the sum of two components. The first component is an effect on margin determination in the developing wing disc. Indeed, our data are in agreement with numerous observations showing that loss of NOTCH signaling causes loss of expression in the wing margin of the patterning genes *wingless* and *vestigial* (COUSO *et al.* 1995; DIAZ-BENJUMEA and COHEN 1995; KIM *et al.* 1995, 1996; RULIFSON and BLAIR 1995; DE CELIS *et al.* 1996; DOHERTY *et al.* 1996). The second component corresponds to an effect on cell proliferation. Indeed, loss and gain of NOTCH signaling experiments have been shown to cause a severe decrease and increase in cell proliferation, respectively (GO *et al.* 1998). Our data suggest that loss of DELTA causes a loss of NOTCH signal and a loss of cell proliferation in the wing margin. The reduction in cell proliferation in *Lyra* wing development begins shortly after pupariation and continues during the first half of pupal development (ABBOTT and SPREY 1990). This is the time window in normal development when differentiation of bristles and trichomes takes place as well. To further examine how ectopic SENSELESS affects wing margin specification and differentiation during *Lyra* wing development we have studied the expression pattern of SCUTE and *string*.

scute is a proneural gene belonging to the *achaete/scute* complex and a basic Helix Loop Helix (bHLH) transcription factor required for determination of SOPs in the anterior wing margin (SKEATH and CARROLL 1991). We observe a downregulation of SCUTE expression in the anterior pouch of the wing disc (Figure 5, A and B). Indeed, in $Lyra^{Sx67}$ wings, there are few SOPs expressing SCUTE at the anterior wing margin (Figure 5B). This is in sharp contrast to ectopic expression of *senseless* in other epithelial cells of the wing disc where it causes induction of SCUTE expression (NOLO *et al.* 2000).

The failure to form SOPs in the wing discs of *Lyra* mutants predicts that the set of two cell divisions required for differentiation of margin bristles in the early pupa will not take place. The reason for the loss of the surrounding unspecialized margin cells in the adult wings of *Lyra* is not as obvious, but one hypothesis is that these cells also fail to proliferate. To test this we examined the mRNA expression pattern of *string*. *string* mRNA is normally expressed in the central cells of both the anterior and posterior wing margin during the later

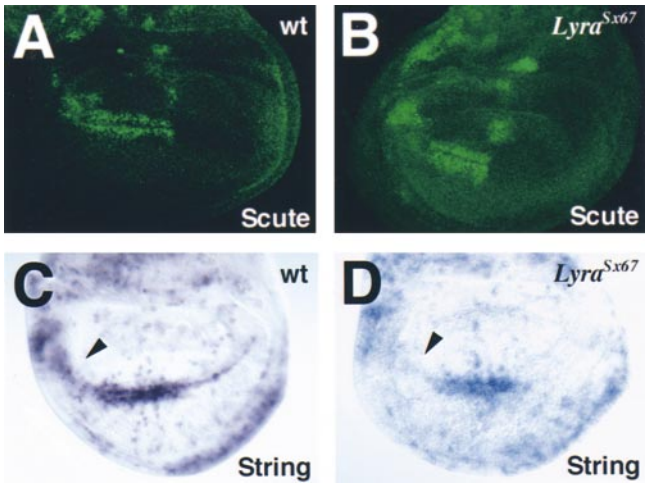

Figure 5.—Expression of SCUTE and *string* in *Lyra* wing discs. Third instar wing discs of wild-type (A and C) and $Lyra^{Sx67}/+$ larvae (B and D). (A and B) Mutant discs show decreased levels of SCUTE expression in the anterior wing margin (B). Hence, ectopic expression of SENSELESS can lead to an induction of SCUTE in some domains of the wing disc (Nolo *et al.* 2000) and loss of SCUTE in other areas. (C and D) *In situ* hybridizations with *string*. Note the loss of *string* in the anterior and posterior wing margin (D).

third instar larval stage (Johnston and Edgar 1998) even though margin cells are arrested at that time and cell proliferation does not begin until early pupariation. Our *in situ* experiments with *string* confirmed this expression pattern in wild-type discs. But in *Lyra* third instar wing discs the mitosis-inducing phosphatase STRING (Cdc25) is severely downregulated in the anterior and posterior area of the prospective wing margin as indicated by *in situ* hybridization (Figure 5D). This is consistent with an overall lack of proliferation in the anterior and posterior margin region. However, it is also possible that the non-bristle-forming cells are present in the wing margin, but that they lose their capacity to flatten and secrete margin elements (trichomes), which serve as the visible hallmark of each cell. This could be caused by their lack of exposure to the sequence of proteins required for determination of the wing margin.

DISCUSSION

The data presented in this article provide strong evidence that *Lyra* mutations are gain-of-function/neomorphic alleles of *senseless* that cause overexpression of *senseless* in third instar imaginal discs. This ectopic expression of *senseless* causes a loss of anterior and posterior wing margin tissue. The data presented in this article provide a molecular framework to understand this phenotype.

Wingless is required for differentiation of bristles late in margin development (Phillips and Whittle 1993; Blair 1994). Indeed, high levels of WINGLESS are known to be required for the proper expression of the proneural SOP determinants *acheate* and *scute*. In addition, CUT expression in third instar discs has been shown to be dependent on WINGLESS expression, while CUT is also required for the maintenance of WINGLESS expression (Neumann and Cohen 1996, 1997). Since CUT is essential for all wing margin bristles, both innervated and noninnervated (Jack *et al.* 1991), we propose that the combined reduction in WINGLESS and CUT expression in *Lyra* mutants may cause a secondary reduction in proneural gene expression in the wing margin, as revealed by SCUTE staining. This reduction in expression should lead to a loss of numerous bristles in the anterior and posterior wing margin. However, these observations do not provide a rationale for the loss of wing blade cells adjacent to the margin, which are also observed in *Lyra* mutants.

Kim *et al.* (1996) have provided compelling evidence that *vestigial* can be viewed as a "wing selector" gene, a view that is supported by the observation that its ectopic expression can rescue loss of WINGLESS (Klein and Martinez Arias 1999). Loss of VESTIGIAL in the wing disc causes also a failure of wing cells to proliferate (Kim *et al.* 1996). Go *et al.* (1998) and Klein and Martinez Arias (1999) have recently proposed a model of wing development in which the *vg*BE is induced by NOTCH signaling when and where WINGLESS is active at the developing wing margin. It has been proposed that the main function of WINGLESS is to enforce gene expression in the wing disc rather than to initiate it. Hence, the combined loss of *vestigial* expression at the boundary and the strong reduction in WINGLESS expression at the wing margin may affect cell proliferation and cell identity not only in the wing margin, but also in a few cell rows adjacent to the anterior and posterior wing margin. This model is in agreement with the observation that we find no alterations in the expression pattern of the quadrant enhancer of *vestigial* in *Lyra* mutants (data not shown) and that *Lyra* wing discs exhibit a dramatic reduction in *string* expression in the cells along the dorso-ventral boundary (Figure 5, C and D). Since *string* has been shown to induce mitosis, and since *Lyra* mutants exhibit no cell death and a loss of cells in pupal development, we propose a causal relationship between these observations. At the root of the Lyra phenotype may be the observation that the DELTA signal is impaired, which should lead to a decrease in NOTCH signaling. This decrease may explain the loss of WINGLESS, VESTIGIAL, and CUT expression, which have all previously been shown to depend on NOTCH signaling. We propose that this defect in *Lyra* mutants underlies the effect on margin determination in the developing wing disc and the reduction in cell proliferation in early pupae.

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