The *Drosophila* tumor suppressor gene *lethal(2)giant larvae* is required for the emission of the Decapentaplegic signal

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SUMMARY

The *Drosophila* tumor suppressor gene *lethal(2) giant larvae* (*lgl*) encodes a cytoskeletal protein required for the change in shape and polarity acquisition of epithelial cells, and also for asymmetric division of neuroblasts. We show here that *lgl* participates in the emission of Decapentaplegic (*Dpp*), a member of the transforming growth factor β (*TGFβ*) family, in various developmental processes.

During embryogenesis, *lgl* is required for the *dpp*-dependent transcriptional activation of zipper (*zip*), which encodes the non-muscle myosin heavy chain (*NMHC*), in the dorsalmost ectodermal cells – the leading edge cells. The embryonic expression of known targets of the *dpp* signaling pathway, such as *labial* or *tinman* was abolished or strongly reduced in *lgl* mutants. *lgl* mutant cuticles exhibited phenotypes resembling those observed in mutated partners of the *dpp* signaling pathway. In addition, *lgl* was required downstream of *dpp* and upstream of its receptor Thickveins (*Tkv*) for the dorsoventral patterning of the ectoderm. During larval development, the expression of *spalt*, a *dpp* target, was abolished in mutant wing discs, while it was restored by a constitutively activated form of *Tkv* (*TkvQ253D*). Taking into account that the activation of *dpp* expression was unaffected in the mutant, this suggests that *lgl* function is not required downstream of the Dpp receptor. Finally, the function of *lgl* responsible for the activation of Spalt expression appeared to be required only in the cells that produce Dpp, and *lgl* mutant somatic clones behaved non autonomously. We therefore position the activity of *lgl* in the cells that produce Dpp, and not in those that respond to the Dpp signal. These results are consistent with a same role for *lgl* in exocytosis and secretion as that proposed for its yeast ortholog *sro777* and *lgl* might function in parallel or independently of its well-documented role in the control of epithelial cell polarity.

Key words: *Drosophila*, tumor suppressor gene, decapentaplegic, signaling pathway

INTRODUCTION

Inactivation of the *Drosophila* tumor suppressor gene *lethal(2)giant larvae* (*lgl*; *l(2)gl* – FlyBase), leads to uncontrolled proliferation of the neuroblasts of the optic lobes and of the epithelial cells of the larval imaginal discs. The *lgl* mutant larvae never pupariate, and during the delayed larval stage disc cells become non-adhesive and undifferentiated, with no apparent polarity. When transplanted in a wild-type adult host, mutant tissues grow in a dispersed manner, completely invading the tissues of the host to finally kill it (Gateff, 1982; Woodhouse et al., 1998). Re-introduction of one copy of the wild-type gene into a mutant animal is sufficient to rescue the tumoral phenotype (Jacob et al., 1987), conforming to the standard definition of a tumor suppressor gene (Gateff and Schneiderman, 1969; Harris et al., 1969; Knudson, 1971).

*Lgl* belongs to a novel family of WD-40-containing proteins with homologs in many species, including humans (Strand et al., 1993; Strand et al., 1994b), mouse (Tomotsune et al., 1993), yeast (Kagami et al., 1998; Larsson et al., 1998), *C. elegans* (U51993) and the more divergent Tomosyn in rat (Fujita et al., 1998) and *Drosophila* (C617762).

The *lgl* gene encodes a protein (*P127* or *Lgl*) of the cytoskeleton mainly localized to the internal face of the plasma membrane (Strand et al., 1994a). Lgl is present in the cytoplasm as a multiprotein complex containing at least ten components, including the product of the *zipper* gene, which encodes the non-muscle myosin heavy chain (*NMHC*) (Young et al., 1993; Strand et al., 1994b), a serine-threonine kinase capable of phosphorylating Lgl (Kalnes et al., 1996) and the *Drosophila* homolog of the yeast nucleosome-associated protein 1 (*NAP1*) (Ishimi and Kikuchi, 1991; Li et al., 1999). In addition, Lgl is able to form homopolymers (Strand et al., 1994b). These different observations implicate Lgl in a cytoskeletal network (Strand et al., 1994b) whose disruption might cause the overgrowth phenotype.
These biochemical features are consistent with the recently reported function for lgl in regulation of cell-shape change and epithelial cell polarity (Manfruelli et al., 1996; Bildet al., 2000). Lgl appears to act in this latter process in cooperation with the two other neoplastic tumor suppressor genes characterized in Drosophila, discs-large (dlg; dlg1 – FlyBase) and scribble (scrib; dlg2 – FlyBase) (Bilder et al., 2000). Moreover, Lehman et al. (Lehman et al., 1999) have clearly demonstrated that the two yeast homologs of lgl, sro7 and sro77, are involved in exocytosis by interacting directly with Sec9, a component of the t-SNARE complex. sro7/77 can be functionally replaced by the human lgl homolog to rescue their mutant phenotypes (Kagami et al., 1998). A similar function has been proposed for rat Tomosyn, in that it facilitates secretory vesicles fusion to specific membrane domain in neurons (Fujita et al., 1998).

Lgl function is also essential for asymmetric cortical localization of all known basal determinants in mitotic neuroblasts and is therefore necessary for asymmetric division and creation of intrinsic differences between daughter cells (Peng et al., 2000; Ohshiro et al., 2000). Similar to its function in epithelial cell polarization, Lgl seems to cooperate withDlg in asymmetric division. All these observations point towards a function of the Lgl family proteins in some specific aspects of intracellular proteins sorting and proteins targeting to specific membrane domains.

The use of lgl temperature-sensitive alleles had led us to implicate lgl function in shape change of various epithelial cells in the embryo, including the dorsalmost ectodermal cells, the leading-edge (LE) cells (Manfruelli et al., 1996), and such a function might be related to that described above. At dorsal closure, the LE cells have to change their shape and to undergo a remodeling of their polarity. During germ band shortening, all the epithelial ectodermal cells of the trunk display a characteristic apical-basal polarity – their apical membrane facing the outside of the embryo. At the end of germ band shortening, when dorsal closure begins, the LE cells are the first ectodermal cells to change their shape (Young et al., 1993; Ring and Martinez Arias, 1993). They elongate in the dorsoventral axis and, concomitantly, they acquire a planar polarity, such that the membrane domain facing the amnioserosa has now changed from its initial basolateral character to a typical apical polarity (Martin-Blanco, 1998). For example, it no longer expresses Fasciclin III (FasIII), a basolateral marker (Young et al., 1993; Ring and Martinez Arias, 1993).

The shape change initiated in the LE cells propagates from cell to cell along the dorsoventral axis (Young et al., 1993; Ring and Martinez Arias, 1993). The onset of dorsal closure is associated with an accumulation of actin and NMHC in these LE cells. It has been suggested that the nonmuscle myosin could, as a mechanically contractile element, drive the epidermal sheet movement (Young et al., 1993). Mutations in the zipper gene (Nusslein-Volhard et al., 1984; Young et al., 1993) result in a prominent dorsal opening that resembles the dorsal holes observed in mutants of the DJNK (Drosophila Jun N-terminal Kinase) pathway (Martin-Blanco, 1997; Noselli, 1998). The amounts of actin and NMHC in the LE cells are greatly reduced in all those mutants (see, for example, Hou et al., 1997). Cell differentiation and morphogenesis during dorsal closure are mediated by the activation of the DJNK signaling pathway, which in turn allows, from the middle of germ band shortening, the expression of dpp (decapentaplegic), the Drosophila homolog of transforming growth factor β (TGFβ), in the LE cells (Martin-Blanco, 1997; Noselli, 1998).

As lgl seems to be involved in dorsal closure, our aim was to position its function in a differentiation process in which cell-shape change and epithelial polarity remodeling are triggered by a well-characterized signaling pathway. The results show that, during dorsal closure, lgl functions in the transmission of the Dpp signaling and this observation has prompted us to investigate the function of lgl in other dpp-regulated developmental processes. The analysis has revealed a new aspect of a general function for lgl in polarized membrane proteins targeting, and suggests its participation in exocytosis and secretion of the Dpp morphogen.

**MATERIALS AND METHODS**

**Drosophila strains**

Wild-type embryos were from the Oregon R strain. The lglH3 hypomorphic temperature-sensitive allele has previously been described (Manfruelli et al., 1996; DeLorenzo et al., 1999). Three amorphic mutations were used: a deficiency, Dfnet62/Cyo, which totally uncovered the lgl locus (Korochkina and Golubov ska, 1978), lglH/Cyo (Mecler et al., 1985) and lglH/Cyo (Bilder et al., 2000).

UAS-tkvQ233D, en-GAL4 and paired-GAL4 were obtained from M. Frasch, A. Gallet and L. Fasano, respectively. The UAS-dpp, dpp[bkI140C;6-GAL4 lines were provided by the Bloomington Drosophila Stock Center. The different chromosomes were recombined either with the lglH or with the lglH– mutant chromosomes. The UAS-lgl line has already been described (Manfruelli et al., 1996).

The UAS-lgl line was as described (Mollereau et al., 2000).

The w[y(+),hs-FLP], arm-lacZ,M(2);P(yr*;neo-FRT)40A/Cyo and the w[yw56v, P(yr*,hs-FLP); ctk1452,P(yr*),neo-FRT)40A/Cyo stocks were kindly provided by H. Stocker. The other stocks used to generate somatic clones were obtained from the Bloomington Drosophila Stock Center.

**In situ hybridization on whole-mount embryos and discs**

Digoxigenin (DIG)-labeled RNA probes were generated from a dpp DNA fragment (generous gift from S. Chauvet) and a wg DNA fragment (generous gift from T. Sagnier) with the T3-T7 polymerase riboprobe kit (Promega) and DIG-UTP (Boehringer). They were used for whole-mount in situ hybridization of fixed staged embryos as described (Francois et al., 1994). Embryos were treated for hybridization as described (Tautz and Pfeifle, 1989). DNA probes were DIG-labeled according to the DIG-DNA Labeling Mixture protocol (Boehringer). The zip probe was obtained by labeling a PCR-amplified genomic fragment corresponding to the ninth exon of the zipper locus. The lab probe is described in Diederich et al. (Diederich et al., 1989). The Dpp probe was DIG-labeled RNA and DNA probes were detected with the aid of a preadsorbed anti-DIG antibody coupled to alkaline phosphatase (Boehringer) and NBT/BCIP as substrate. The embryos were mounted in Geltol medium (Immunotech, France) for further observation under a Zeiss Axiosphot microscope.

For fluorescent in-situ hybridization on imaginal discs, the dpp-DIG-UTP probe was revealed as described previously (Zaffran and Frasch, 2000).

**Immunohistochemistry**

Embryos and discs were fixed and stained with antibodies according to the protocol described previously (Ashburner, 1989). The following primary antibodies were used: anti-NMHC (1/500 concentration, 1 h at room temperature) and anti-dpp (1/500 concentration, 1 h at room temperature).
RESULTS

Igl is required for the accumulation of the zipper gene product in the LE cells

In Igl(iz3) embryos, in which both maternal and zygotic functions of Igl were reduced, the dorsalmost cells of the epidermis (LE cells) do not elongate and, consequently, hamper the dorsal closure (Manfruelli et al., 1996). In a wild-type embryo, NMHC begins to specifically accumulate in LE cells at the onset of dorsal closure, before the cells have initiated their elongation (Young et al., 1993; Fig. 1A-C). By contrast, in mutant embryos issued from homozygous Igl(iz3) parents, the concentration of NMHC in the LE cells was greatly reduced (Fig. 1D). Conversely, neither the localization nor the amount of Lgl was impaired in zipper mutant embryos (not shown). In a wild-type embryo, Lgl is ubiquitously expressed and is mainly located to the basolateral membrane of the epithelial cells (Strand et al., 1994a), whereas, in Igl(iz3) mutant embryos, the Lgl mutant protein is no longer associated to the plasma membrane and is found in the cytoplasm (Manfruelli et al., 1996, DeLorenzo et al., 1999). The replacement of Ser311 by a Phe residue in the Lgl(iz3) protein (DeLorenzo et al., 1999) appears therefore to be crucial to the membrane localization of Lgl, probably by perturbing its binding to the cytoskeleton, and Lgl is required for the specific accumulation and localization of NMHC in LE cells.

In addition, the mutant LE cells did not acquire their novel polarity as shown by Phosphotyrosine (PY), Discs-large (Dlg) (Woods and Bryant, 1993) or FasIII (Martinez-Arias, 1993) stainings (Fig. 1E-J). Interestingly, in the mutant, the epidermal polarity was affected only in those LE cells but was preserved in all other ectodermal cells of the future epidermis (Fig. 1). In particular, cell junctions were correctly assembled and positioned, as probed with anti-Dlg and anti-PY (Muller and Wieschaus, 1996, Woods et al., 1997).

zip is a transcriptional target of the dpp signaling pathway in LE cells and its activation requires Igl function

The accumulation of the NMHC protein in LE cells at the onset of dorsal closure was accompanied by an activation of zip transcription in these cells (Fig. 2A). This high level of mRNA was maintained until completion of dorsal closure, when epidermal cells had acquired their final shape (not shown). This observation strongly suggests that zip transcription is activated in the LE cells in response to the activation of the DJNK-Dpp signaling pathway and is responsible, at least in part, for the observed accumulation of NMHC.

To investigate the involvement of Dpp in zip transcriptional activation, we examined zip mRNA accumulation in LE cells in mutant embryos for thickveins (tkv), which encodes a Type I receptor for Dpp. The localized accumulation of zip mRNA did not prevail any longer in this mutant (Fig. 2B), thus confirming that zip transcription requires the Dpp pathway. The occurrence in the zip promoter region of a GC-rich sequence (Mansfield et al., 1996), which well matches the consensus sequence reported for Drosophila Mad/Meda (Smad family proteins)-binding sites (Xu et al., 1998), further strengthens the possibility that zip could be a direct transcriptional target of the dpp signaling pathway.

Mutations in other components of the JNK pathway (bsk and
jun) gave rise to the same downregulation of zip in LE cells, which correlates with the lack of accumulation of NMHC protein already observed in mutants of all characterized genes of the DJNK-Dpp signaling pathway (not shown). By contrast, in puc mutants, zip expression was, overall, similar to that in a wild-type embryo, even though some perturbations were noticed (not shown; Martin-Blanco et al., 1998).

Upregulation of zip transcription was also abolished in lgl mutant embryos at restrictive temperature (Fig. 2C). Therefore, at least part of the lgl function in the dorsal closure process might be mediated by its effect on the transcriptional activation of zip. In contrast, the expression of dpp (Fig. 2), and particularly its onset, as well as the timing of its decrease in the margin cells (not shown), were not noticeably affected in lgl mutant embryos. Thus, this positions lgl function downstream transcriptional activation in LE cells.

**lgl function is necessary in various embryonic developmental processes that are dependent on dpp signaling**

The results described above have prompted us to investigate the effect of lgl mutations on the expression of some known dpp signaling targets during embryogenesis.

The mature pattern of lab (labial) expression in the midgut depends on an inductive information issued from the adhesive visceral mesoderm (Bienz, 1994) that is mediated by the secreted product of dpp expressed in parasegment 7 of this tissue. In lgl mutant embryos, the midgut expression of lab was strongly reduced or even undetectable, whereas its expression in the intercalary segment, which is not dependent on dpp (Immergluck et al., 1990), remained unaltered (Fig. 2F,G). This observation is consistent with a function for lgl in midgut morphogenesis (Manfruelli et al., 1996).

As a result of the expression of dpp in the dorsal ectoderm, tinman expression is activated in the underlying dorsal mesodermal cells, allowing their specification and their subsequent differentiation. The even skipped (eve) gene, which is expressed in a subset of pericardial cells and in precursor cells of a dorsal muscle, is also a dpp target in the dorsal mesoderm (Bodmer and Frasch, 1998). As shown in Fig. 2H-K, the expression of these two genes was downregulated in several regions of the heart in lgl mutants, and this was accompanied by an abnormal morphogenesis of the dorsal vessel (not shown).

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The function of dpp is crucial to the specification and the differentiation of the dorsal epidermis in the embryo. Cuticle preparations of lgl homozygous embryos, reared at restrictive temperature (29°C), displayed a large variety of phenotypes illustrated in Fig. 3. The various phenotypes, probably due to the fact that lgl is a hypomorphic allele, were all related to the establishment of the dorsoventral axis. The most frequent ones consisted in a lack of internal head structures and head involution (86% of the total lgl cuticles), as well as in a lack of externalization of the Filzkörpers (90%) (Fig. 3C). The cephalopharyngeal skeleton and the Filzkörpers are derived respectively from the anterior dorsal region and from the posterior dorsolateral region (Jürgens et al., 1986). A large proportion of cuticles (45%) appeared twisted (Fig. 3B) and they were similar to cuticles prepared from screw, shrew, tolloid or twisted-gastrulation mutants. As already mentioned, dorsal closure is blocked and 58% of the cuticles therefore displayed holes in their dorsal epidermis (Fig. 3A). Finally, lateral extension of the denticle belts was compared with that in a scab mutant, in which the differentiation of the dorsal epidermis was affected although no appearance of

**Fig. 1.** Distribution of NMHC and membrane markers in lgl mutant embryos at dorsal closure. Whole-mount embryos were stained at the beginning of dorsal closure as follows and observed under a confocal microscope: with anti-NMHC (A-D), with anti-Fasciclin III (FasIII), which labels the lateral membrane domain of epithelial cells (E,F), with anti-Phosphotyrosine (anti-PY), a marker of the most apical region of the lateral membrane (G,H), and with anti-Disco-large (Dlg), a marker of septate junctions (I,J).


(A-C) NMHC is strongly expressed in the LE cells, where it accumulates at the onset of dorsal closure. (D) In mutant embryos, LE cells do not change their shape and fail to accumulate NMHC (compare with C, a wild-type embryo (slightly younger than those in A,B) that has not yet changed its LE cells shape). (E) Fas III and (I) Dlg are absent from the membranes facing the amnioserosa in the LE cells during dorsal closure, while in lgl embryos, FasIII (F) and Dlg (J) are evenly distributed on the whole surface of the cell membranes. (G) In LE cells, PY is detected in the membranes facing the amnioserosa, as well as in the basolateral membranes in contrast to the situation prevailing in mutant embryos (H). In other ectodermal cells, epithelial cell polarity is maintained in lgl mutant embryos and cell junctions are correctly positioned (inserts in G-I). Arrowheads indicate the LE cells.
Fig. 2. Downregulation of the expression of dpp targets in lgl<sup>ts3</sup> mutant embryos. (A,D,F,H,J) Wild-type embryos; (C,E,G,I,K) lgl<sup>ts3</sup> homozygous embryos reared at 29°C; (B) tkv<sup>7</sup> homozygous embryo. (A-C) In situ hybridizations of whole-mount embryos probed with a DIG-labeled zipper cDNA. The views are focused on one of the two LE cells rows (arrowheads) where zip transcript accumulates in a wild-type embryo at the onset of dorsal closure (A) but fails to accumulate either in a tkv<sup>7</sup> homozygous embryo (B) or in a lgl<sup>ts3</sup> homozygous embryo at 29°C (C). as, amnioserosa; ec, lateral ectoderm; h, head. (D,E) In situ hybridizations of whole-mount embryos probed with a DIG-labeled dpp cDNA. (D) In a stage 13 wild-type embryo, dpp is expressed in the LE cells (arrowhead), as well as in subsets of other cells such as those in the visceral mesoderm. (E) A lgl<sup>ts3</sup> mutant embryo at the same stage expresses dpp in the LE cells (arrowhead), as does the wild-type embryo. Note the lower expression of dpp in the PS7 visceral mesoderm. (F,G) In situ hybridizations of whole-mount embryos probed with a DIG-labeled lab cDNA. lab expression was no longer detected in a lgl<sup>ts3</sup> mutant embryo (arrowhead). The most extreme phenotype, with almost complete absence of lab expression, is shown here. Persistence of a labeling in the intercalary segment should be noted in mutant embryos. (H,I) Tin is expressed in the cardial cells in wild-type embryos and absent from most of the cardial cells in mutant embryos (arrowheads). (J,K) The same situation prevails in the case of Eve expression. The anti-Eve antibody labels a subset of pericardial cells and of dorsal muscles precursors. For all embryos shown, anterior is leftwards and the dorsal side is in focus. The genotypes are mentioned in the lower left and the probe or the antibody used in the lower right of each panel.

Dorsalization could be detected (Nusslein-Volhard et al., 1984). On the average, an increase of 15% in the length of ventral denticle belts in lgl<sup>ts3</sup> mutant cuticles could be measured, reaching up to 60% of lateral extension in some mutant individuals (not shown).

In conclusion, these phenotypes, which were typical of a disruption in the dorsal patterning, were reminiscent of the defects encountered when the dpp signaling pathway is disrupted. In contrast, cuticles never displayed a ventralization with lack of amnioserosa and dorsal epidermis differentiation, which is however the phenotype the most sensitive to a lowering of the dpp gene dose. As a matter of fact, complete ventralization was not observed even in mutants in dpp signaling pathway genes which have a maternal expression (such as punt, for example, Letsou et al., 1995). A small number (less than 8%) of cuticles from lgl<sup>ts3</sup> embryos displayed segmental abnormalities such as fusion of dentine belts or extended regions of naked cuticles (Fig. 3C), suggesting that lgl is not crucial to the function of other secreted morphogens such as Wingless or Hedgehog.

However, gain-of-function experiments clearly demonstrate that lgl function is required downstream of dpp for the specification of the dorsal epidermis. Ubiquitous ectopic expression of dpp in the embryo causes a dorsalization of cuticles (Staehling-Hampton et al., 1994). A similar dorsalization prevailed when dpp or Tkv<sup>Q253D</sup>, a constitutively activated form of the Dpp receptor, was expressed in a wild-type embryo in the paired-expressing cells (Fig. 3E,G).

Dorsalization was significantly reduced when dpp was ectopically expressed in lgl<sup>ts3</sup> mutant embryos (compare Fig. 3E with 3F), suggesting that the dpp function requires lgl activity for the establishment of the dorsoventral polarity in embryos. By contrast, an absence of ventral denticles persisted in a lgl mutant background when Tkv<sup>Q253D</sup> was ectopically expressed (Fig. 3H). Similar results were obtained with other GAL4 drivers, en-GAL4 (Fig. 3I,J) or arm-GAL4 (not shown), even though in this latter case the dorsalization observed with UAS-dpp in lgl<sup>ts3</sup> was clearly higher than with prd-GAL4, probably because arm-GAL4 is expressed in all ectodermal cells and (or) at a higher level. Therefore, lgl appears to fulfill its function downstream of the dpp activation step, but not downstream of Tkv, after the receptor has been activated.

The function of lgl is required for dpp-dependent activation of spalt in wing imaginal discs

Growth and patterning of the wing imaginal disc rely on the localized expression of dpp, which in turn induces the transcriptional activation of its targets, including spalt (Nellen et al., 1996). Wing imaginal discs from homozygous null lgl alleles display a disorganized growth as early as the beginning of third instar larval stage and they do not form regular epithelial cells sheets, contrasting with what is encountered in a wild-type larva. However clear neoplasia in mutant wing discs become detectable only during the delayed larval life. In order to allow a clearer recognition of their shape, most of the analyses in mutants were conducted on wing discs from early.
third instar larvae, whose morphology still roughly resembled that of wild-type larvae. At this stage, the expression domain of dpp in the wing disc was larger than in late third instar larvae and the Spalt expression domain narrower.

The dpp-dependent expression of Spalt was abolished or strongly reduced in the presumptive wing blade of lgl mutant disc (Fig. 4A,B), while dpp expression itself was normally initiated and not noticeably affected (Fig. 4C,D). The polarity of the epithelial cells in mutant discs, as probed withDlg or Armadillo (Arm) was still preserved (Fig. 4F), in contrast to what is observed in discs from delayed lgl4 homozygous third instar larvae (Woods et al., 1997; Bilder et al., 2000). Furthermore, En (Fig. 4GJ,P) and Ptc (not shown) were normally expressed in mutant wing discs, strongly suggesting that the lgl function is involved in the dpp signaling pathway itself.

spalt expression in lgl4 mutant wing imaginal discs was restored by expressing TkvQ253D, but not Dpp, in the posterior compartment with the aid of an en-GAL4 driver (Fig. 4G-L). In this experiment, Spalt expression was restored only in the posterior compartment. In order to be able to recognize the imaginal disc cells that require the lgl function in dpp signaling (i.e. the cells that emit the Dpp signal or the cells that receive it), lgl has been specifically expressed in the dpp territory within a lgl4 mutant background. Such an ectopic expression led to a rescue of Spalt expression in the presumptive wing blade (Fig. 4M-O). By contrast, no rescue of Spalt expression (Fig. 4P-R) could be obtained by restoring lgl function in the posterior compartment. These results indicate that lgl function is required in the cells that produce Dpp to activate Spalt expression. Moreover, when lgl expression was restored in dpp-expressing cells, Spalt expression in the wing blade was detected in the cells that did not express dpp (arrowhead in Fig. 4N-O). This observation of a Spalt expression domain extending out of the dpp expression domain suggests that the function of lgl is not required for the reception of the Dpp signal.

Likewise, in eye imaginal discs, lgl seems to play a role related to dpp signaling pathway. Dpp function in this tissue is rather involved in the onset of the morphogenetic furrow movement than in ommatidia differentiation (see, for example, Burke and Basler, 1996b). In a wild-type eye disc, Spalt was detected in all the differentiated ommatidia, while in lgl4 mutants, its expression was restricted to only a few disorganized ommatidia (Fig. 5A,B). Furthermore, the advance

![Fig. 3. The dorsoventral patterning of the embryonic ectoderm mediated by the dpp signaling is affected in lglts3 mutant embryos. (A-D) Cuticles of lglts3 homozygous embryos reared at 29°C. (A) Absence of dorsal closure producing a large hole in the dorsal ectoderm is visible in 58% of lglts3 mutant embryos. (B) Shows the twisted phenotype and a lack of involution of the head. (C) The absence of cephalopharyngeal skeleton (arrow) and the lack of externalization (or the absence) of the Filzkörpers (arrowhead) were the most frequently encountered phenotypes. (D) Mutant cuticle showing a rare phenotype consisting in a partial lack of ventral cuticle (arrowhead). Cuticle preparations of wild-type (E,G,I) or lglts3 mutant (F,H,J) embryos in which have been expressed Dpp (E,F,I,J) or constitutively activated Tkv (G,H) under the control of prd (E-H) or en (I,J) regulatory elements. The extent of dorsalization was assessed by the reduction of the number of ventral cuticles (arrowheads). Dorsalization by ectopic expression of Dpp in wild-type embryos (E,I) is more pronounced than that induced by TkvQ253D in wild-type embryos (G; data not shown) probably because Dpp can diffuse some distance away from its source of production. As prd is a pair-ruled gene, dorsalization is observed in only one of two segments in (E,H). Cuticle in F is very similar to that in wild-type, indicating that lgl is required for dpp mediated dorsalization (compare E with F). More than 80% of mutant cuticles were phenotypically wild-type whereas in control embryos 100% of them were dorsalized. By contrast, the extent of dorsalization is roughly identical in G,H, indicating that TkvQ253D can rescue lgl function in this process. (I,J) When dpp is induced in en-expressing cells, the ventral denticles of all the segments can potentially be affected owing to the expression of en in the epidermal cells that gives rise to the anteriormost row of denticle belts. Dorsalization was also more effective in wild-type (I) than in lglts3 embryos (J). In all panels, cuticles are oriented with the anterior towards the left. Arrowheads point to wild-type (F,J) or phenotypically mutant (E,G-I) denticle belts.](image-url)
of the morphogenetic furrow was slowed down. This phenotype is reminiscent of that prevailing in the class of ‘furrow-stop mutants’, including dpp (Heberlein et al., 1993).

Fig. 4. Expression of Spalt, a dpp target, in lgl4 imaginal wing discs. In all panels (except E,F), Spalt (Sal) expression was revealed using an anti-Spalt antibody (in red). (A,B) The expression of Sal in the center of the presumptive wing blade (arrowhead) is abolished in early third instar mutant larva. The dpp-independent expression of Sal in the notum (arrow) is not affected by lgl loss of function. (C,D) dpp expression, revealed by in situ hybridization with a DIG-labeled dpp probe (in green) is normally initiated in a lgl4 mutant disc from early third instar larva. (E,F) En face optical sections of imaginal discs. Epithelial cell polarity probed with anti-Dlg (green) and anti-Arm (red) antibodies. In spite of a disorganization of the epithelial folds in lgl4 mutant discs (F), Dlg and Arm are normally located at the plasma membranes. (E,F') z-views of the epithelial layers at positions marked by an arrow in (E,F). As in the wild type, Arm is located in the mutant to the apicalmost region of the lateral membrane, above the septate junctions labeled with Dlg. (G-R) All discs are lgl4 mutants. (I,L,O,R) are merged views of the two separate views on their left. (G-I) en-Gal4 driven expression of a constitutively activated form of Tkv in the posterior compartment of a lgl4 mutant disc induces the expression of Sal in the posterior compartment, marked with anti-En antibody. The star indicates a trachea. (J-L) Under the same conditions as in (G-I), expression of dpp in en-expressing cells is unable to induce Sal expression in the posterior compartment (arrowhead). (M-O) Sal expression is restored in the presumptive wing blade (arrowhead) of an lgl4 mutant wing disc by expression of lgl under the control of dpp-Gal4. The dpp domain of expression was assessed by UAS-GFP (green). (P-R) expression of lgl in the posterior domain of a lgl4 mutant disc cannot rescue Sal expression in the presumptive wing blade (arrowhead). The arrows in M-R point to dpp-independent Sal expression.

Fig. 5. lgl function in the eye imaginal disc. (A,B,D) Anti-Sal staining, which labels a small number of photoreceptors in every differentiated ommatidia. (C) In situ hybridization with a DIG-labeled dpp probe. At late third instar larval stage, lgl mutant eye discs are smaller than in the wild type (compare the respective sizes of the eye disc with the antenna disc in A,B, for example). (D) The differentiation of ommatidia is almost completely restored when lgl is expressed in lgl mutant discs under the control of dpp-Gal4.

In the mutant discs, the expression of dpp was normal in the delayed morphogenetic furrow (Fig. 5C). Restoration of lgl function in the dpp expression domain was sufficient to almost completely rescue the mutant phenotype (compare Fig. 5A with 5D). It is not known whether Spalt expression in the eye disc is dependent on dpp signaling, but this latter result indicates that lgl function in the eye disc is required only in the cells that express or have expressed dpp, confirming the results obtained in the wing discs.
Loss-of-function lgl somatic clones behave non autonomously with respect to dpp signaling

Cell autonomy of the Dpp signal has been analyzed in lgl loss-of-function somatic clones in the wing imaginal disc. Two different null alleles were used (lgl⁴ and lgl⁴W3) that led to similar results. Spalt expression was taken as an indicator of the activity of the Dpp signaling cascade and Patched as a marker of the A/P compartment boundary (Maschat et al., 1998).

All the mutant clones scored (about 200 clones scored) that encompassed the Spalt expression domain exhibited a wild-type pattern of Spalt expression (Fig. 6). This observation, which holds even in the case of large clones produced in Minute background, indicates that lgl function, at least with respect to spalt activation, is not required in the cells that receive Dpp and respond to it. Because the expression of Spalt was abolished in mutant discs that were entirely lgl⁴, this behavior was interpreted as the result of a non autonomous function of lgl with respect to dpp-dependent Spalt expression: the mutant cells might be rescued by the surrounding wild-type cells that normally expressed and secreted Dpp.

However, we have never detected mutant cells within the anterior compartment in close apposition to the A/P compartment boundary, where Dpp is produced. In contrast, numerous mutant cells in large clones (25 mutant clones scored) were able to reach the A/P compartment boundary when they were located in the posterior compartment. It appears therefore that mutant cells appeared to be excluded from the region in which Dpp was produced at third instar larval stage. A striking example is shown in Fig. 6A in which a twin clone of wild-type cells reaches the A/P frontier and is much larger than the mutant clone that is composed of only a few cells expressing spalt, and that is located farther away from the A/P compartment boundary. lgl mutant cells seem to be eliminated from the Dpp domain; they probably cannot survive and the surrounding wild-type cells might not be able to rescue them. This clonal analysis, which has to be strengthened by other experiments, strongly suggests that the function of lgl is required only in the dpp-expressing cells of the imaginal wing disc. As a matter of fact, a clonal analysis by Posakony et al. had demonstrated a localized requirement for wild-type dpp expression along the A/P compartment boundary of the developing wing (Posakony et al., 1990). They never recovered dpp mutant clones located in this domain in adult wings.

Analysis of mitotic clones in the adult wing was consistent with these observations. Dpp plays a dual function in wing morphogenesis: it regulates growth and patterning of the wing disc during larval development and promotes the differentiation of veins during pupal development (de Celis, 1997). Under the conditions of induction that we have used, three types of situations have been encountered (around 200 clones were analyzed). Phenotypes of loss of veins were observed only when mutant clones were present on both sides of the wing blades (~2%, Fig. 7A,B), as already shown in the case of dpp mutant mosaics (de Celis, 1997). By contrast, numerous genetically mutant clones (~20%), which occupied only one surface of the wing blade and encompassing veins, did not affect vein differentiation (Fig. 7C). These results are consistent with the hypothesis that dpp expression in one surface is sufficient to provide dpp function to the other surface (de Celis, 1997). Finally, the large majority of mutant clones (~80%), which were found in intervein regions, did not display any mutant phenotypes and were as large as the wild-type twin clones.

Taken together, all these observations indicate a non autonomous function of lgl in the dpp signal transduction pathway during wing morphogenesis.
DISCUSSION

A function for lgl in Dpp secretion?

The results reported herein demonstrate that at least part of the lgl function is involved in the dpp signaling pathway. All the known dpp targets considered in this study were underexpressed or even not expressed at all in lgl mutants. In addition, whenever technically possible, we have shown that the dpp signaling pathway mutant phenotype was rescued by the expression of a constitutively active form of Tkv, thus assessing the specificity of lgl action in the process. This observation implies that the function of lgl resides in a participation either in the secretion and (or) maturation of Dpp, or in its long range diffusion or, eventually, in the correct activation of its receptor.

Several lines of evidence presented in this article suggest that the lgl function is required in cells that express Dpp rather than in cells that receive the signal. Spalt expression in mutant wing discs is recovered by restoring lgl activity in the Dpp domain of expression. In this situation, Spalt expression can be detected not only in the cells that produce dpp, but also in groups of mutant cells which do not express dpp (Fig. 4). Imaginal discs Dpp-dependent expression of spalt can be rescued in lgl mutant clone cells by surrounding wild-type cells. This observation supports the idea that the Dpp signal can diffuse and interact correctly with its receptor in the wing blade in lgl mutant cells, thus pointing towards a function for lgl in the production or the secretion of Dpp by the cells in which it is transcribed.

So far, any straightforward approach to the analysis of the processing or the secretion of Dpp in mutant cells has been hampered mainly because of the lack of efficient antibodies. We have nevertheless been able to detect a precursor form of Dpp that is synthesized in lgl null mutant disc cells and present at a level similar to that observed in wild-type cells (N. A., unpublished) by using an antibody directed against the N-terminal part of the protein (Groppe et al., 1998). This antibody however does not recognize the C-terminal moiety of the Dpp protein which constitutes its processed and secreted form.

It has been clearly demonstrated that the yeast homologs of Lgl, Sro7p and Sro77p, participate in polarized exocytosis and are associated to Sec9, a plasma membrane SNARE (Lehman et al., 1999). This protein family seems to be functionally conserved, as lgl or its human homolog (HUGL), are able to rescue the sro7/77 mutant phenotypes in yeast (Kagami et al., 1998). Moreover, a Sec9/Snap25 homolog is also present in the Drosophila genome (Lloyd et al., 2000). By taking into account these functional homologies, a function for lgl can be postulated in exocytosis.

lgl in the control of epithelial cell polarity

Secretion relies on intracellular vesicular trafficking and on the polarized exocytosis machinery (Mellman and Warren, 2000). Recent studies have demonstrated that Lgl function is essential for the establishment of the polarities of epithelial cells (Manfruelli et al., 1996; Bilder et al., 2000) and of dividing neuroblasts (Peng et al., 2000; Ohshiro et al., 2000). An important issue is therefore to understand whether the role of Lgl in Dpp secretion is direct or simply a consequence of the loss of epithelial cell polarity. Analysis of the temporal requirement for Lgl function argues in favor of Lgl being necessary for the establishment of cell polarity, rather than for its maintenance. Moreover, alteration in Dpp signaling can be observed in lgl mutants in epithelial cells that are correctly polarized and this supports a direct function for Lgl in Dpp secretion.

The epidermis is not affected in homozygous lgl null mutant larvae that no longer contain the maternal Lgl protein responsible for a normal embryonic development (Gateff and Schneiderman, 1969; Manfruelli et al., 1996). lgl larvae develop a cuticle that possesses the hallmarks of a wild-type cuticle by all the criteria used, thus indicating that the apical secretion of cuticle components has not been altered. Markers for epithelial cell polarity are localized in the correct position (Peng et al., 2000) in stage 16 embryos when Lgl is no longer detected (Strand et al., 1994a). Likewise, lgl homozygous embryos in which the Lgl protein has lost its cortical location have maintained their typical epithelial cell polarity (Fig. 1) and their capacity to secrete normal cuticle components (Fig. 3; see also Manfruelli et al., 1996; Ohshiro et al., 2000). In neuroblasts, Lgl seems to exert its action early during mitosis to recruit basal determinants to the cortex but it does not contribute to their maintenance in this latter location (Ohshiro et al., 2000). The polarity of epithelial wing disc cells is preserved until the middle of the third larval stage, long after the maternal Lgl contribution has ceased (Fig. 4; Gateff and Schneiderman, 1969).

In contrast, the need for Lgl function appears crucial whenever cell polarity has to be established. In neuroblasts, its function during mitosis is to assist Miranda and Prospero localization (Ohshiro et al., 2000), when a new asymmetric division requires basal localization of cortical determinants. Asymmetric division is equally impaired in late lgl mutant embryos in which the maternal contribution of Lgl might become too low to ensure the efficiency of the process (Peng et al., 2000). In embryos issued from germline clones, lacking both maternal
and zygotic lgl activities, the cells of the ectoderm form but they do not acquire their epithelial cell polarity (Bilder et al., 2000).

While embryogenesis proceeds, some epithelial cells have to change or remodel their polarity. This is the case, for example, for the LE cells during dorsal closure and our data show that Lgl is also involved in this process. During larval stages, imaginal disc cells actively proliferate and have to continuously reconstruct their membrane junctions and their polarity in different steps that are dependent on the exocytosis machinery. Loss of activity of lgl might be responsible for the progressive alteration in cell polarity leading to neoplasms in lgl mutant animals.

In conclusion, lgl activity appears to be required whenever targeting to the membrane of new components is crucial to the acquisition of cell polarity.

**Alteration of Dpp signaling precedes epithelial cell polarity disruption in lgl mutants**

Our data support the hypothesis that the effect of lgl on Dpp signaling does not result, as an indirect consequence, from altered cell polarity and, accordingly, we have provided some examples in which the two processes could be uncoupled. Epidermal cells in lgl mutant embryos are normally polarized and secrete cuticle but they are unable to promote cuticle dorsalization in response to an ectopic expression of Dpp while they induce this same dorsalization in response to TkvQ253D.

The cell polarity in lgl mutant embryos is not altered when the targets of the Dpp signaling have to be activated. When tin and eve expressions have to be induced within the mesoderm, the dorsal ectoderm, which produces Dpp, shows a wild-type epithelial cell polarity and can secrete dorsal cuticle. The mesodermal and endodermal midgut cells display a normal cell polarity when labial expression has to be induced by Dpp (Manfruelli et al., 1996). Similarly, zip accumulates in LE cells before they undertake their shape and polarity changes (Fig. 1). Dpp is actually expressed well ahead of the initiation of dorsal closure and its expression in LE cells rapidly decreases at the onset of this process. Finally, Spalt is not expressed in lgl mutant wing discs that express Dpp, even though their epithelial polarity is not yet disturbed (Fig. 4). The rescue in lgl mutants of Spalt expression by a constitutively activated form of the Dpp receptor, Tkv, strongly suggests that its localization to a specific domain of the cell membrane or (and) its integration within the membrane has not been impaired, although they are both likely to be dependent on cell polarity.

It seems reasonable to assume that there is a unique exocytosis pathway mediated by lgl to ensure both cell polarity control and secretion. Dlg and scrib might participate in this same pathway, as, indeed, they strongly interact genetically with lgl and share with this gene a large panel of identical mutant phenotypes (Bilder et al., 2000). Lgl, however, does not strictly colocalize with Dlg and Scrib in either epithelial cells (Bilder et al., 2000) or in neuroblasts (Peng et al., 2000). In addition, the Dlg cortical localization does not require lgl function (Peng et al., 2000; Ohshiro et al., 2000). One could therefore anticipate an lgl action, within a separate and distinct pathway, in parallel to that of Dlg and Scrib, and further experiments are needed to address this issue.

Whatever the situation, Lgl probably mediates a specific secretion pathway for Dpp that other secreted morphogens such as Wingless or Hedgehog or cuticle proteins do not share. This is consistent with the function of lgl in targeting specific cortical proteins to particular membrane domains in epithelial cells or neuroblasts.

**lgl and the control of the actomyosin cytoskeleton**

In yeast, sro7/77-mediated polarized exocytosis relies on a complex regulation and interaction with the actomyosin cytoskeleton. Sro7/77 display a strong genetic interaction with myo1 (encoding a Type II myosin homolog of NMHC) and with myo2 (encoding an unconventional Type V myosin) (Kagami et al., 1998). In addition, Myo1P can physically interact with Sro7P (Kagami et al., 1998), in a manner resembling that prevailing between Lgl and NMHC (Strand et al., 1994b). These observations are in favor of Lgl serving as a functional link between the actomyosin cytoskeleton polarity and a specific polarized exocytosis pathway, although the precise function exerted by Lgl in such a process has yet to be deciphered. In yeast, as in flies, myo1 (zipper) and sro7/77 (lgl) display a negative genetic interaction (Kagami et al., 1998; Peng et al., 2000; Ohshiro et al., 2000), as already mentioned during the fly dorsal closure (P. Manfruelli, PhD thesis, Marseille, 1996). Loss-of-function alleles of lgl suppress the dorsal closure phenotype in homozygous zip mutants. Conversely, overexpression of lgl enhances the dorsal closure phenotype.

The data reported in this article rather state a role for Lgl in the transcriptional activation of zip in LE cells, raising a puzzling question about the in vivo functional significance directly associated with the interaction demonstrated in vitro between Lgl and NMHC. As a matter of fact, even in LE cells in which there is an important accumulation of NMHC, there is no particular colocalization of the two proteins. The same situation is encountered in other cells in which NMHC also accumulates (Young et al., 1991; Edwards and Kiehart, 1996; C. De Lorenzo, PhD thesis, Marseille, 1998). Similarly, in yeast, an abnormal localization of actin and myosin becomes visible only long after the initial defect of exocytosis encountered in sro7/77 mutants (Lehman et al., 1999). These observations might indicate that the Lgl-NMHC physical interaction observed in vitro is not relevant in vivo and might not be functional in the LE cells. One could rather anticipate a general ability of Lgl to bind myosins. Accordingly, it is noteworthy that, in yeast, another myosin-encoding gene, myo2, interacts positively with sro7/77 (Kagami et al., 1998), and that, in Drosophila neuroblasts, two functionally different cytoskeletal actomyosin networks, one being repressive and the other positive, seem to control the cortical localization of basal determinants (Peng et al., 2000).

**Tumor suppression and TGFβ signaling**

It is particularly intriguing that lgl, a Drosophila tumor suppressor gene, could play a role in a signaling pathway such as the dpp pathway. Dpp is a member of the superfamily of TGFβs, which have been described as important key regulators in carcinogenesis (Padgett et al., 1998). TGFβ receptors and components of the TGFβ signal transduction pathway are mutated in a number of human tumors (for review see Riggins et al., 1997). Homozygous mutant animals for Smad3 have recently been obtained that are viable but that spontaneously display colorectal adenocarcinomas (Zhu et al., 1998).
However, in the wing disc, which are tissues that develop tumors in the absence of lgl function, dpp stimulates growth and proliferation (Burke and Basler, 1996a). This leads to a paradox, implying that a tumor suppressor gene, which is likely to encode a negative regulator of cell proliferation, could be primarily involved in a cell signaling pathway that activates proliferation.

While it is tempting to speculate that tumors arise in lgl-deficient animals as a direct consequence of the perturbation of dpp signaling, this has not been demonstrated in our study, and tumors could well be due to another dpp-independent function of lgl. In particular, the reported role of lgl in epithelial cells polarity might be implicated in tumorigenesis (Bilder et al., 2000). Current work is in progress to address this question specifically.

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