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The range of *spalt*-activating Dpp signalling is reduced in endocytosis-defective *Drosophila* wing discs

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Abstract

Pattern formation along the anterior-posterior (A/P) axis of the developing *Drosophila* wing depends on Decapentaplegic (Dpp), a member of the conserved transforming growth factor β (TGFβ) family of secreted proteins. Dpp is expressed in a stripe along the A/P compartment boundary of the wing imaginal disc and forms a long-range concentration gradient with morphogen-like properties which generates distinct cell fates along the A/P axis. We have monitored Dpp expression and Dpp signalling in endocytosis-mutant wing imaginal discs which develop severe pattern defects specifically along the A/P wing axis. The results show that the size of the Dpp expression domain is expanded in endocytosis-mutant wing discs. However, this expansion did not result in a concommittant expansion of the functional range of Dpp activity but rather its reduction as indicated by the reduced expression domain of the Dpp target gene *spalt*. The data suggest that clathrin-mediated endocytosis, a cellular process necessary for membrane recycling and vesicular trafficking, participates in Dpp action during wing development. Genetic interaction studies suggest a link between the Dpp receptors and clathrin. Impaired endocytosis does not interfere with the reception of the Dpp signal or the intracellular processing of the mediation of the signal in the responder cells, but rather affects the secretion and/or the distribution of Dpp in the developing wing cells.

Keywords: Clathrin-mediated endocytosis; α-Adaptin; Dpp signalling; pattern formation; Wing imaginal disc

1. Introduction

Anterior-posterior (A/P) pattern formation during *Drosophila* wing development is controlled by Decapentaplegic (Dpp), a secreted signalling protein of the transforming growth factor β (TGFβ) family (Padgett et al., 1987). Dpp is expressed in a narrow stripe of cells along the A/P compartment boundary (Posakony et al., 1990; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Zecca et al., 1995) and forms an activity gradient which directly instructs cells about their position along the A/P axis in a concentration-dependent manner (Lecuit et al., 1996; Nellen et al., 1996). Dpp expression pattern is controlled by Hedgehog, a conserved secreted protein expressed in the posterior wing compartment (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Like other TGFβ-like molecules such as *Xenopus* Activin (Thomsen et al., 1990), which acts as concentration-dependent morphogen (Green and Smith, 1990; Green et al., 1992; Gurdon et al., 1994, 1995), Dpp acts as a ligand for transmembrane serine/threonine kinases (reviewed in Massagué, 1998) encoded by *thick veins* (*tkv*; type I receptor) and *punt* (*put*; type II receptor) (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994; Wrana et al., 1994; Ruberte et al., 1995). Their activity results in the expression of target genes, a process mediated by the evolutionary conserved transcription factor Mad (Sekelsky et al., 1995; Hoodless et al., 1996; Newfeld et al., 1996; Wiersdorff et al., 1996).

A simple model for morphogen gradient formation involves passive diffusion of the signalling molecule through the extracellular space (Crick, 1970; Slack, 1991). The best documented case for a diffusion-generated morphogen gradient is the Activin. When Activin is provided in form of coated beads (Gurdon et al., 1996), it rapidly forms a gradient and induces different cell fates in response to different concentration thresholds (Gurdon et al., 1996; McDowell et al., 1997). This observation and *Xenopus* animal cap assays showing that relatively little of the exogenously supplied Activin is bound to its receptor suggest that Activin diffusion is basically unhindered (Dyson and Gurdon, 1998). In contrast, the Dpp gradient in the *Drosophila* wing disc forms much more slowly, over a

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period of 2 days, which argue against unhindered Dpp diffusion (Lecuit and Cohen, 1998). It has been shown previously that an impairment of endocytosis, a general cellular process for membrane internalization and vesicle trafficking (reviewed in Kirchhausen et al., 1997), interferes with signalling by Notch, Wingless and the epidermal growth factor (Haigler et al., 1979; Bejsovec and Wieschaus, 1995; Vieira et al., 1996; Seugnet et al., 1997). Here we show that impaired endocytosis interferes with A/P pattern formation in the developing *Drosophila* wing disc. We made use of α-adaptin and clathrin heavy-chain mutations of *Drosophila* to impair endocytosis (Bazinet et al., 1993; González-Gaitán and Jäckle, 1997). We found that the size of the *dpp* expression domain along the A/P boundaries was increased in the endocytosis-mutant wing discs, whereas the expression domain of the Dpp target gene *spalt* (*sal*), which served as a marker for functional Dpp activity (de Celis et al., 1996; Grimm and Pflugfelder, 1996; Lecuit et al., 1996; Nellen et al., 1996), was reduced. The results indicate that the functional range of Dpp activity in endocytosis mutant wing discs is limited to about 4 cell diameters and that endocytosis is not required for the reception of the Dpp signal or its intracellular processing. Instead, endocytosis is likely to paticipate in the propagation of the Dpp signal, a process that involves both clathrin and the Dpp receptors.

2. Results

2.1. Impaired endocytosis affects A/P wing patterning

Mutations in the *Drosophila* αadaptin gene (*DAda*) disrupt clathrin-mediated endocytosis prior to vesicle formation at the cell membrane (González-Gaitán and Jäckle, 1997). Embryos which are homozygous for a lack-of-function allele, such as $DAda^3$, develop into normal looking larvae which die in the eggshells (González-Gaitán and Jäckle, 1997). α-adaptin is also expressed at high levels at the plasma membrane of developing wing imaginal disc cells during larval stages (Fig. 1a–c). To address a possible role of α-adaptin during wing development, we generated a hypomorphic allele, $D\text{-}Ada^4$, to overcome embryonic lethality. The strongest non-lethal allelic combination, $D\text{-}Ada^3/D\text{-}Ada^4$, causes a temperature-dependent wing phenotype (Fig. 1d–i). At 18°C, the mutant wings are normal (Fig. 1d). At 25°C, wings are reduced in size and show vein pattern defects along the A/P axis (Fig. 1f). At 29°C, only wing remnants with strongly enhanced pattern defects along the A/P axis were observed (Fig. 1h). Such remnants develop diagnostic dorsoventral pattern elements, such as *sensilla campaniformia* on the hinge and the dorsal surface of the wing blade, the dorsal and ventral hairs of the wing margin triple row, and specific dorsoventral aspect of the veins. Thus, no discernible dorsoventral wing pattern defects were found. The mutant pattern formation along the A/P axis of the endocytosis-mutant wings are affected in a manner similar to hypomorphic *decapentaplegic* (*dpp*) mutants (Spencer et al., 1982; Lecuit et al., 1996).

We next asked whether wing pattern defects are also observed when clathrin-mediated endocytosis is impaired by double mutant combinations as recently shown for mutants where α-adaptin and dynamin activities are jointly reduced (González-Gaitán and Jäckle, 1997). In double heterozygous mutants for clathrin heavy-chain (*D-Chc*) (Bazinet et al., 1993) and α-adaptin (Fig. 2), wings develop a temperature-dependent phenotype. At 25°C and 29°C, the A/P pattern defects of $D\text{-}Chc/+;DAda^3/+$ mutant wings resembled those observed with *DAda* mutant wings (compare Figs. 1 and 2). Furthermore, such wings developed at 18°C a thickened posterior cross-vein (Fig. 2a, arrow) similar to mutants of the Dpp receptor *thick veins* (*tkv*) (Diaz-Benjumea et al., 1989). The *dpp*- and *tkv*-like phenotypes obtained with the endocytosis-mutant combinations are consistent with the proposal that clathrin-mediated endocytosis is necessary for proper Dpp action during wing development.

2.2. Impaired endocytosis interferes with dpp expression and signalling

The temperature-dependent wing phenotypes suggest that the effective range of Dpp signalling in the wing disc is reduced. Thus, impaired endocytosis may cause a size reduction of the *dpp* expression domain, as visualized by the expression of a *dpp-lacZ* marker gene, and/or a decrease of the level of Dpp expression. Figs. 1e and 2d show normal patterns of *dpp* expression along the A/P compartment boundary at 18°C. At 25°C, however, the *dpp* expression domain was significantly expanded, whereas the level of *dpp* expression remained comparable to wildtype (Figs. 1g and 2e). Thus, impaired endocytosis interferes with a process required for the correct spatial setting of the *dpp* expression domain such as Hedgehog signalling (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Ingham and Fietz, 1995; Zecca et al., 1995). At 29°C, this effect was even more pronounced, resulting in an altered shape of the *dpp* expression domain in addition to the broadening. Since impaired endocytosis affects the growth and/or proliferation of cells (see below), these effects on *dpp* expression may be an indirect consequence of changing the size or shape of the wing disc.

Expansion of the *dpp* expression domain should result in a mutant wing phenotype opposite to the one observed which suggests a reduction rather than an expansion of Dpp activity at the A/P compartment boundary. One possible explanation for this phenomenon is that impaired endocytosis may interfere with the Dpp signalling process by, for example, interfering with the secretion, propagation or the reception of Dpp which would limit the range of functional signalling. Since Dpp antibodies or a functional Dpp-Green Fluorescent Protein (GFP) fusion protein are not available, we could not assess the secretion process and the range of

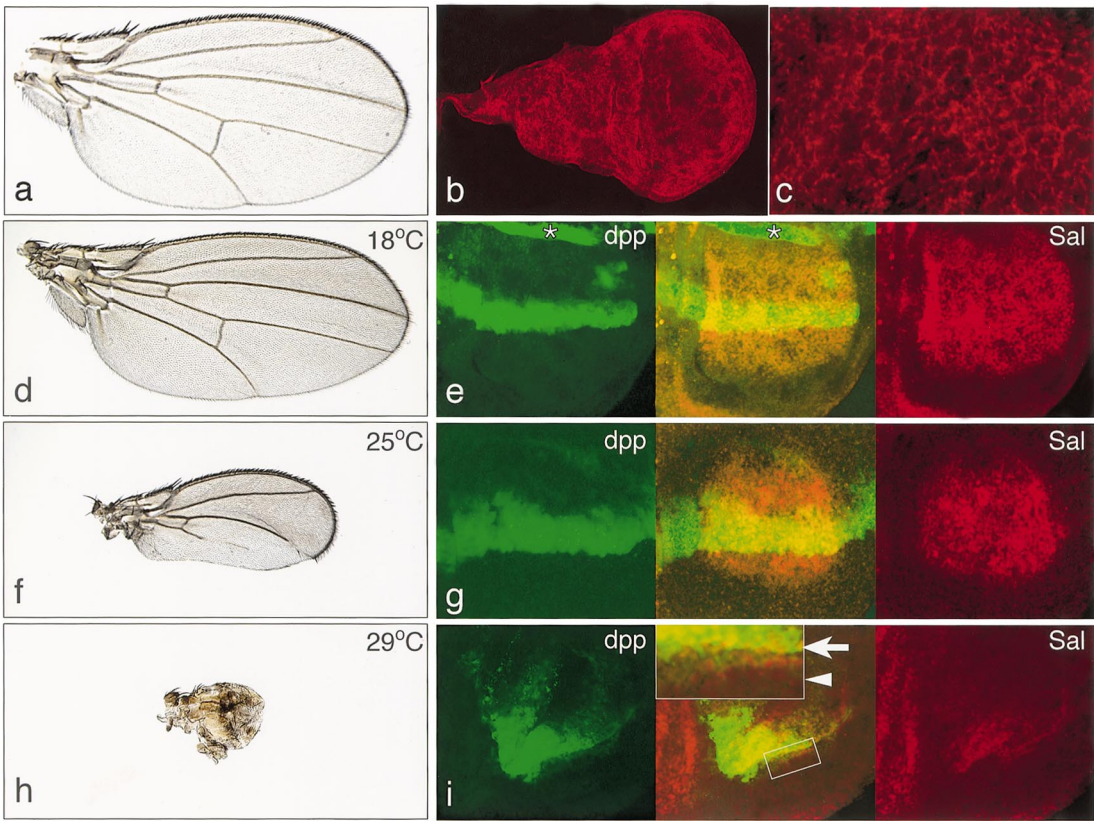

Fig. 1. (a) Wild type wing. (b,c) anti-α-Adaptin antibody staining of a third instar wing imaginal disc, (b) low magnification; (c) high magnification. Orientation of the wing and the imaginal disc is anterior up and distal to the right, and anterior up and ventral to the right, respectively. In (c) note the association of α-Adaptin to the plasma membrane. (d–i) Temperature-dependent wing phenotypes (d,f,h) and corresponding expression patterns of Dpp and *spalt* (e,g,i) in wing imaginal discs of *D-Ada*^{4}/*D-Ada*^{3} mutants at 18°C (d,e), 25°C (f,g) and 29°C (h,i). For the orientation and a comparison with a wild type wing, see (a) and (b). At 18°C (d), *D-Ada*^{4}/*D-Ada*^{3} mutant wings develop normally. At 25°C (f), wings are reduced. Note missing veins, characteristic of defects in A/P pattern formation as seen in *dpp* mutants (Spencer et al., 1982; Lecuit et al., 1996). At 29°C (h), only wing remnants similar to *dpp* extreme hypomorphic mutants develop (Lecuit et al., 1996). (e,g,i) *dpp*-lacZ reporter gene expression (''Dpp''; left panels; green), anti-Spalt antibody stainings (right panels; red) and superimposed images of both stainings (central panels) of third instar wing discs raised at 18°C (e), 25°C (g) and 29°C (i). Note the wild type-like expression of Dpp and Spalt at 18°C (e) (for comparison see Lecuit et al., 1996; Nellen et al., 1996; asterisk indicates Dpp expression in the peripodial membrane, outside the presumptive wing pouch region, in a different focal plane). At 25°C (g), the Dpp stripe is enlarged, but the Spalt expression domain is significantly reduced. At 29°C (i), the Spalt expression domain is further reduced. Note in the inset that Sal expression (arrowhead) extends beyond the posterior border of the Dpp expression domain (arrow) by four cells maximum. For details see text.

Dpp staining in the mutant discs directly. Instead we monitored Dpp activity by the size of the expression domain of the Dpp target gene *spalt* (*sal*). This target gene is activated above a distinct concentration threshold of Dpp and thus, the size of its expression domain can serve as a direct marker for the functional range of Dpp activity (de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996).

Figs. 1 and 2 show the *sal* expression domains in endocytosis-mutant wing discs at different temperatures. At 18°C, the *sal* expression domain extends in a wildtype-like manner to each side of the *dpp* expression domain (Figs. 1e and 2d). At 25°C, the *sal* expression domain is reduced, primarily within the posterior wing compartment (Figs. 1g and 2e). At 29°C, the temperature at which wing remnants develop, the *sal* expression domain is further reduced (Figs. 1i and 2f). It extends beyond the borders of the *dpp* expression domain by maximum four cell diameters instead of 10–15 cell diameters as observed in wildtype discs (González-Gaitán, unpublished observation; see inset in Fig. 1i). Thus, impaired endocytosis seems to shorten the effective range of Dpp with respect to *sal* expression.

In order to test whether the reception and intracellular processing of the Dpp signal is affected, we generated endocytosis-deficient cells in mutant mosaics and asked whether they express the target gene *sal*. Since impaired Dpp signalling affects cell growth during imaginal development (Spencer et al., 1982; Posakony et al., 1990; Lecuit and Cohen, 1998), we first tested whether endocytosis-deficient cell clones were able to proliferate and survive during wing disc development and whether they show up in adult wing blades. We generated homozygous lack-of-function *DAda*^{3} cells by X-ray-induced mitotic recombination during early larval development (Garcia-Bellido, 1972; see details in Section 4). No *DAda*^{3}/*DAda*^{3} mutant cell clones were observed, whereas the wildtype twin cells formed clones

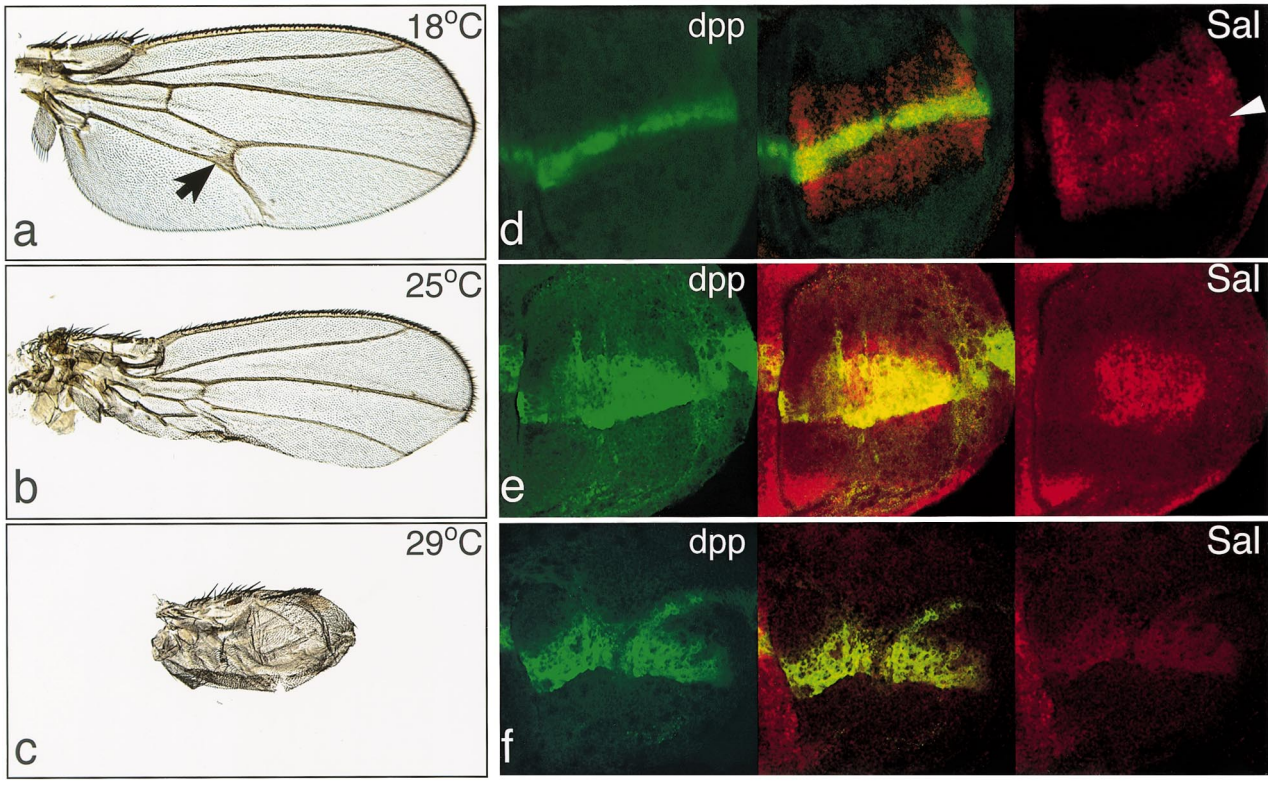

Fig. 2. Wing phenotypes (a–c) and corresponding expression of Dpp and its target gene *spalt* (d–f) in wing imaginal discs of *D-Chc/+;DAda*^{3}*/+* double heterozygous mutant at 18°C (a,d), 25°C (b,e) and 29°C (c,f). For the orientation and a comparison with a wild type wing see Fig. 1. Dpp and Spalt expression was monitored as described in the legend of Fig. 1. Note the enlargement of the Dpp expression domain and the reduction of the Sal expression domain similar to the phenotypes and effects as described for the *D-Ada*^{4}*/D-Ada*^{3} mutant in Fig. 1. Note also wildtype-like modulation of Sal level at the A/P boundary (arrowhead in d). For details see text.

of the expected size and at normal frequency (Fig. 3a,b). In contrast, endocytosis-deficient cells that were induced during late larval development developed clones which were smaller than the wildtype twins and appeared in low frequency (Fig. 3c,d). This indicates that impaired endocytosis interferes with the growth, proliferation and/or the general health of cells which, however, are able to survive and to differentiate.

To increase the frequency of endocytosis-deficient cell clones and to obtain clones of maximum size, we used the FRT/flip recombinase system (Xu and Harrison, 1994) combined with the *Minute*$^{+}$ technique (Morata and Ripoll, 1975; see details in Section 4). This way, endocytosis-deficient cell clones were obtained in higher frequency. However, they were still small, composed of a maximum of 50 cells (Fig. 3e–g). The endocytosis-deficient cells express *sal* regardless of whether the clones were located within the *dpp* expression domain or somewhere else within the limit of the wildtype *sal* expression domain (Fig. 3e–h). The results confirm that impaired endocytosis does not interfere with Dpp action over short distances (see also Fig. 1i, inset). Furthermore, the data show that the endocytosis-deficient cells receive the Dpp signal and that they can process the signal with respect to the activation of the target gene *sal*.

2.3. Genetic interactions between the Dpp receptors and clathrin

In order to hint at the level at which endocytosis affects Dpp signalling, we employed a genetic interaction assay to investigate whether A/P pattern formation and Dpp signalling are impaired in wing discs heterozygous for the *D-Chc* mutant and mutant components of the Dpp signalling pathway. *DCHC/+;put*^{10460}*/+* wings develop a normal A/P pattern at 18°C and 25°C, respectively (not shown). At 29°C, the *DChc/+;put*^{10460}*/+* individuals had wing defects similar to those observed with the *DChc/+;D-Ada*^{3}*/+* and *D-Ada*^{3}*/D-Ada*^{4} mutants (cf. Figs. 1 and 2 versus Fig. 4a,b). *DChc/+; tkv*^{strII}*/+* wings were normal at 18°C (not shown). At 25°C, both *tkv*-like mutant phenotypes (Fig. 4c) and wing remnants (not shown) were observed. At 29°C, only wing remnants developed (Fig. 4d). Correspondingly, the *sal* expression domain in *DChc/+;tkv*^{strII}*/+* mutant wing discs was normal at 18°C (not shown), whereas the size of the expression domain was reduced at 29°C (Fig. 4e). Control experiments with double heterozygous *DChc/+;dpp*^{BC86}*/+* or *DChc/+;Mad*^{B1}*/+* mutant wings resulted in normal wing patterns at 18°C, 25°C and 29°C, respectively (not shown). This suggests that endocytosis and Dpp signalling are linked at the level of the Dpp receptors.

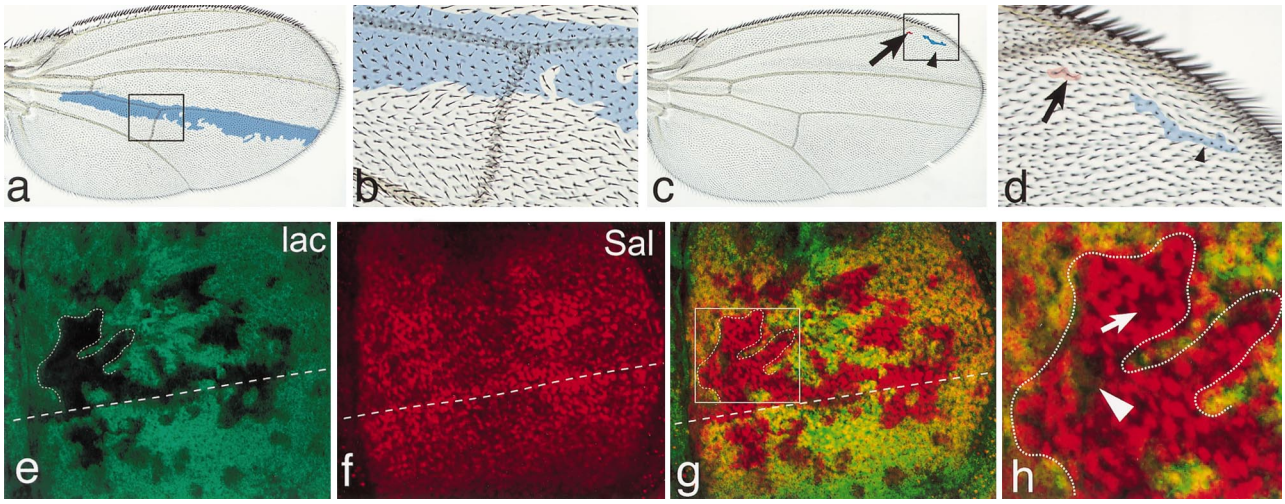

Fig. 3. *DAda*^{3} mosaic wings. (a–d) Mosaic wings bearing *DAda*^{3} homozygous mutant clones (*forked* (*f*) cell marker; red, arrow) and the corresponding wild type twins (*crinkled* (*ck*) cell marker; blue, arrowhead) that were generated in early third instar larvae (a; enlargement in b; 72–84 h after egg deposition) or in late third instar larvae (c; enlargement in d; 108–120 h after egg deposition). The absence of early induced endocytosis-deficient cell clones (60 examined wings; example in a) and the presence of late induced endocytosis-deficient cell clones (total of 35 *ck* clones and 20 *ck/f* twins in 70 wings examined; wild type clones contain on average 2.2 times the number of cells found in mutant clones; example in c,d) indicate that the lack of endocytosis interferes with cell proliferation. (e–h) Wing disc carrying multiple *DAda*^{3} homozygous mutant clones generated by FLP-FRT-dependent mitotic recombination during second and third instar larval stages. The disc was co-immunostained with anti-lacZ antibodies (e; lack of staining identifies endocytosis-deficient cell clones, example outlined by dots) and anti-Spalt antibodies (f). Superimposed image of both stainings (g) and an enlarged endocytosis-deficient clone (h, corresponds to boxed area in g). Note *spalt* expression in endocytosis-deficient cell clones close to and at a distance to the *dpp* expression domain (border shown by a dotted line in e–g) and that in some of the largest endocytosis deficient areas (arrows and filled arrowheads in h), Spalt is either reduced (arrow in h) or absent (arrowhead in h). We cannot decide whether the lack of Spalt is due to the lack of signal reception in these cells or because those cells are dying and dropping out of the epithelium.

It is important to note that the Dpp-lacZ domain of *DChc/+;tkv*^{strII}*/+*-mutant wing discs is altered at 29°C in a manner similar to the one observed in *DAda*-mutant wing discs at 29°C (compare Figs. 1i and 4e). In case of the *Chc,tkv* mutant combination, however, there is no reason to assume that Hedgehog activity is affected, nor is there any evidence that Dpp signalling regulates Dpp expression. Therefore, the enlarged *dpp* expression as visualized by the expanded *dpp-lacZ* domain may be an indirect consequence of changing the size or shape of the endocytosis-mutant wing disc. Nevertheless, the size of the resulting *sal* expression domain is reduced to a distance of only a few cell diameters beyond the *dpp* expression domain (compare Figs. 1e,g,i and 4e). This demonstrates that the range of *sal*-activating Dpp activity is significantly decreased when endocytosis is impaired.

3. Discussion

Our results show that mutant combinations affecting endocytosis reduce the functional range of Dpp signalling and cause A/P patterning defects in the developing wing disc. Endocytosis participates in many cell functions, and in several cell communication pathways including epidermal growth factor (EGF), Notch and Wingless signalling which are known to be active during wing development (Haigler et al., 1979; Bejsovec and Wieschaus, 1995; De Camilli and Takei, 1996; Vieira et al., 1996; Seugnet et al., 1997). Notch and Wingless signalling were shown to be affected in endocytosis-defective dynamin mutants (Bejsovec and Wieschaus, 1995; Seugnet et al., 1997). We have not examined Notch signalling in hypomorphic *D-Ada*^{3}*/D-Ada*^{4} mutants or in wing discs of the double mutant combination *DChc/+;D–Ada*^{3}*/+*. In both mutant combinations, however, we have noted strong pattern defects along the A/P axis of the wing, whereas pattern defects along the dorsoventral axis were not discernible. This suggests that at least Wingless signalling is not significantly affected, implying that Dpp signalling is most sensitive to impaired endocytosis as provided by the mutant combinations shown here.

We noted that *dpp* expression expands at intermediate temperatures in anterior direction of the wing disc exclusively (e.g. Figs. 1g and 2e). We take this observation as circumstantial evidence for a Hedgehog-dependent response, since its nuclear mediator, the transcription factor Cubitus interruptus, is expressed in the anterior compartment of the wing disc only (Eaton and Kornberg, 1990). A somehow more puzzling feature of the endocytosis-mutant wing discs is that the mutant defects observed are consistently more pronounced in the posterior compartment of the wing than in its anterior counterpart (e.g. Figs. 1f and 2a,b). We have no explanation for this phenomenon except that the posterior compartment is more sensitive to impaired Dpp signalling as has already been described for hypomorphic *dpp* mutants (Spencer et al., 1982)

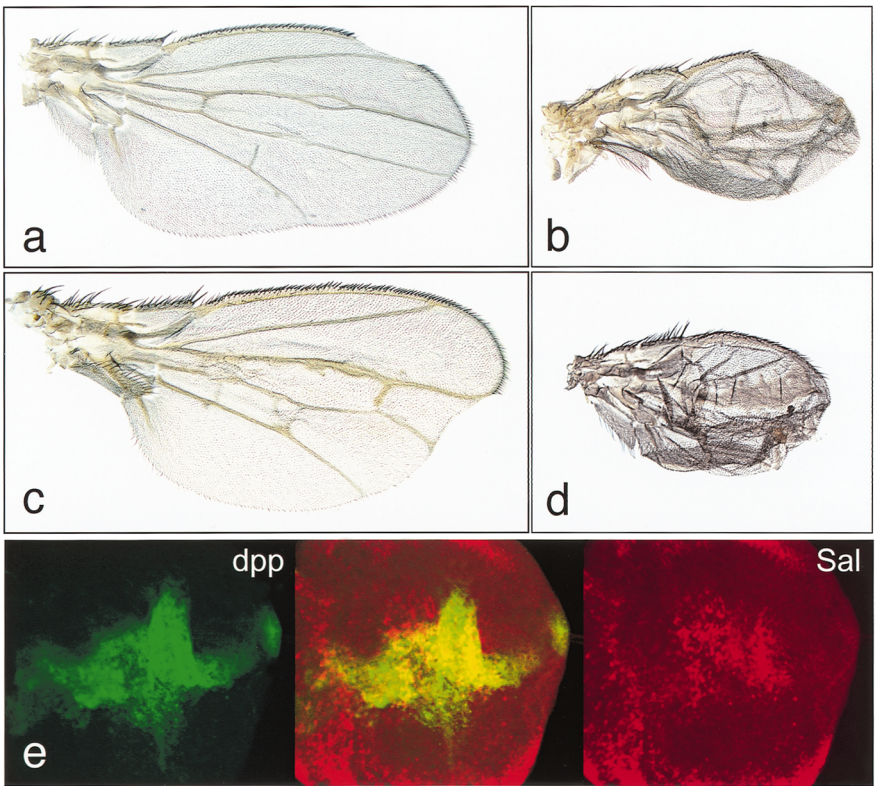

Fig. 4. (a–d) Wing phenotypes of *D-Chc/+;put*^{10460}/+ (a,b) or *D-Chc/+;tkv*^{strII}/+ (c,d) double heterozygous mutant at 25°C (c) and 29°C (a,b,d). *D-Chc/+;put*^{10460}/+ individuals raised at 29°C show either wing remnants (b; 10% penetrance) or milder wing defects (a; 60%). *D-Chc/+;tkv*^{strII}/+ individuals raised at 25°C show wing remnants (not shown; 60%) or wing defects (c; 40%). At 29°C, *D-Chc/+;tkv*^{strII}/+ individuals show wing remnants (d). Wing phenotypes were similar to the ones in endocytosis mutants. For the orientation and a comparison with a wild type wing see Fig. 1. (e) Expression of Dpp and its target gene *spalt* in a *D-Chc/+;tkv*^{strII}/+ double heterozygous mutant wing imaginal disc at 29°C. Dpp and Spalt expression were monitored as described in the legend of Fig. 1. Note that these phenotypes are similar to endocytosis mutants in Figs. 1 and 2. For details see text.

The conclusions drawn from the mutant phenotype are consistent with the finding that despite the enlarged *dpp* expression domain, the range of *sal*-activating Dpp activity is significantly reduced to 3–4 cell diameters from the source of the signal (Fig. 1i, inset). Recent results suggest that gradient formation and long-range signalling by secreted signalling proteins such as Dpp, Hedgehog and Wingless are modulated by regulatory feedback loops involving their receptors. Hedgehog activity causes elevated levels of its receptor Patched, which hinders Hedgehog diffusion and thereby limits the range of Hedgehog movement (Chen and Struhl, 1996). Wingless also regulates its receptor, but in opposite direction, i.e. the expression of the receptor is reduced in regions where Wingless levels are highest (Cadigan et al., 1998). Dpp acts in a manner like Wingless, meaning that it negatively regulates the expression of its receptor *tkv* (Lecuit and Cohen, 1998). Since endocytosis has been shown to be a prerequisite for receptor clearance at the cell membrane (Haigler et al., 1979; Kirchhausen et al., 1997), and in view of the genetic interactions between clathrin and the Dpp receptors Tkv and Put shown here, it is possible, among other explanations, that impaired endocytosis interferes with Dpp receptor levels and/or the formation of the Dpp gradient as well as with the need to recycle receptors to keep signalling working effectively.

Increased Tkv is likely to sequester free Dpp and thereby hinders its migration, resulting in an altered shape of the Dpp gradient (Lecuit and Cohen, 1998). We cannot exclude that such a mechanism also contributes to the restriction of Dpp activity in endocytosis-mutant wings. However, we noted that overexpression of Tkv by means of the UAS/GAL4 system in wing imaginal discs caused comparatively weak wing defects when compared to the phenotypes of endocytosis-mutant wings shown here (compare Figs. 1 and 2 with Fig. 3 in Lecuit and Cohen, 1998). Also, a reduction of Tkv or Put in flies which bear only one copy of the *DChc* gene causes the same defects as observed with endocytosis-mutant wings. This genetic link between the Dpp receptors and clathrin suggests that a process involving receptor-mediated endocytosis might participate in mediating Dpp action over distance, extending its functional range beyond some 4 cell diameters. However, we would like to stress that the results obtained with double mutant wing discs do not distinguish between a signalling defect, a transport defect or unrelated defects such as the need to recycle receptors to maintain effective signalling.

The mosaic analysis establishes with endocytosis-deficient wing disc cells establish that the reception of the Dpp signal is not dependent on endocytotic events. This is clearly shown by the fact that the endocytosis-deficient cells express *sal* normally, whereas cell clones of comparable size lacking the Dpp receptor Tkv, which disrupts signal reception, fail to express *sal* (Lecuit et al., 1996; Nellen et al., 1996 and references therein). Furthermore, the results establish that the intracellular processing of Dpp signal between the activated receptors and the nuclear factor(s) required to activate the target gene *sal* is not depend on clathrin-mediated endocytosis as has been reported for EGF signalling (Vieira et al., 1996). This leaves the possibility that impaired endocytosis affects the secretion or the propagation of the Dpp signal over distance, for example by transcytosis, or both processes are affected at the same time. Once Dpp antibodies or functional Dpp-GFP fusions are available to visualize the Dpp gradient and the subcellular distribution of Dpp directly, these question can be addressed in the mutant combinations described here.

4. Experimental procedures

4.1. Mutant strains

D-Ada^{3} is a lack of function allele (González-Gaitán and Jäckle, 1997). *D-Ada*^{4} was obtained in an EMS screen for thermosensitive alleles of *D-α-Adaptin* (M.G.G., unpublished result). *D-Chc*^{1} is a loss of function mutation in the *D-Clathrin heavy chain* gene (Bazinet et al., 1993). Other mutants are described in Lindsley and Zimm (1992) and flybase. *DAda* mutations were kept balanced over *T(2;3)SM6a-TM6b, Tb* (Lecuit et al., 1996) (abbreviated, *ST6*) or over *In(2LR)Gla, Bc Elp* (abbreviated, *GBE*) to identify the *D-Ada*^{3}*/D-Ada*^{4} mutants by the absence of either the *Tubby* or *Black cells* dominant markers. Double heterozygous *Chc*^{1}*/+;D-Ada*^{3}*/+* mutant larvae are specifically delayed and could thus be identified unambiguously.

4.2. Mosaic analysis

Mosaic adult wings were generated by X-ray-induced mitotic recombination (Garcia-Bellido, 1972) involving *f*^{36a}*; P(f*$^{+}$*)@30A ck pr pwn/D-Ada*^{3} *cn* males. *P(f*$^{+}$*)@30A* is a P element insertion in the cytogenetic region 30A containing a wildtype *forked* gene that rescues *f*^{36a}. Mitotic recombination proximal to *ck* results in *f*^{36a}*; D-Ada*^{3} *cn/D-Ada*^{3} *cn* cell clones associated to *ck* and D-*Ada*$^{+}$ twins. X-ray treated larvae were staged in 12 h intervals; time refers to hours after egg deposition. Wings were dissected, dehydrated in ethanol, embedded in 6:5 lactic acid:ethanol and examined in a Zeiss Axiophot microscope after embedding.

Mosaic wing imaginal discs were generated using the ''FLP-FRT'' and the ''Minute^{+}'' techniques (Morata and Ripoll, 1975; Xu and Harrison, 1994). *y HS-Flp; M(2)z arm–lacZ FRT@40A/CyO* males were crossed to *DAda*^{3} *FRT@40A/ST6* or *DAda*^{3} *FRT@40A/GBE* females. *HS-Flp*, *arm-lacZ* and *FRT@40A* are P-element insertions carrying a Flipase gene under the control of the heatshock promotor (X chromosome), lacZ as reporter gene under the control of the ubiquitous armadillo promotor (2L chromosomal arm) and a FRT site at 40A. Mitotic recombination at the FRT site of the *y HSFlp/+;DAda*^{3} *FRT@40A/ M(2)z arm–lacZ FRT@40A* F1 larvae was induced by heat-shocking two times for 2 h at 37°C during second and early third instar stage (which correspond to 72–96 h and 96–120 h after egg deposition for *M(2)z/+* individuals). Two days after the last heat-shock, *y HSFlp/+; DAda*^{3} *FRT@40A/M(2)z arm-lacZ FRT@40A* crawling female larvae were selected by screening the size of the gonads, the absence of the dominant *Tubby* or *Black cells* marker and the developmental delay characteristics of *M(2)z/+*. Their wing discs were dissected subsequently and fixed for immunostaining.

4.3. Immunostainings

Fixation, processing of the wing imaginal discs for immunofluorescence using FITC- or Cy3-coupled secondary antibody and embedding were performed as described (González-Gaitán and Jäckle, 1996). Primary antibodies were used at the following concentrations: mouse anti-β-galactosidase (Sigma), 1:200; rabbit anti-D-α-Adaptin, 1:50 (González-Gaitán and Jäckle, 1997); rabbit anti-Spalt (Kühnlein et al., 1994), 1:10. Photographs were taken with a Zeiss confocal microscope. In the case of mosaic discs, confocal Z-section series through the epithelium were projected to document staining of nuclei (Sal) and cytoplasm (lac) at different levels across the apical/basal axis.

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