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# Modulation of the Suppressor of fused protein regulates the Hedgehog signaling pathway in *Drosophila* embryo and imaginal discs.

**François Dussillol-Godar[a,1], Jeanine Brissard-Zahraoui[a,1], Bernadette Limbourg-Bouchon[b], Dominique Boucher[c], Sylvaine Fouix[a], Claudie Lamour-Isnard[a], Anne Plessis[a] and Denise Busson[a,*]**

[a] *Laboratoire Génétique du Développement et Evolution, Institut Jacques Monod, UMR 7592-CNRS/Université Pierre et MarieCurie/Université Denis Diderot, 2, place Jussieu, 75251 Paris Cedex 05, France*
[b] *Laboratoire de Génétique et Biologie cellulaire, FRE 2445-CNRS/Université de Versailles, Bâtiment Buffon, 45 Avenue des Etats-Unis, 78035 Versailles Cedex, France*
[c] *Laboratoire de Biochimie cellulaire, UMR 7098-CNRS/Université Pierre et Marie Curie, Bâtiment C, 5ème étage, 9 Quai Saint-Bernard, 75252, Paris Cedex 05, France*

[1]: these authors contributed equally to this work

* Corresponding author. Fax : 33 1 44 27 52 65
*E-mail address*: bussond@ijm.jussieu.fr

**The Suppressor of fused (Su(fu)) protein is known to be a negative regulator of Hedgehog (Hh) signal transduction in *Drosophila* imaginal discs and embryonic development. It is antagonized by the kinase Fused (Fu) since *Su(fu)* null mutations fully suppress the lack of Fu kinase activity. In this study, we over-expressed the *Su(fu)* gene in imaginal discs and observed opposing effects depending on the position of the cells, namely a repression of Hh target genes in cells receiving Hh and their ectopic expression in cells not receiving Hh. These effects were all enhanced in a *fu* mutant context and were suppressed by *cubitus interruptus* (*ci*) over-expression. We also show that the Su(fu) protein is poly-phosphorylated during embryonic development and these phosphorylation events are altered in *fu* mutants. This study thus reveals an unexpected role for Su(fu) as an activator of Hh target gene expression in absence of Hh signal. Both negative and positive roles of Su(fu) are antagonized by Fused. Based on these results, we propose a model in which Su(fu) protein levels and isoforms are crucial for the modulation of the different Ci states that control Hh target gene expression**.

*Keywords* : *Drosophila* ; *Suppressor of fused* ; *fused* ; Hedgehog ; signal transduction ; Cubitus interruptus

## Introduction

The Hedgehog (Hh) signaling pathway plays a critical role in the patterning, differentiation and growth of a wide array of cell types during development of many organisms (Ingham and McMahon, 2001; Lum and Beachy, 2004; Nybakken and Perrimon, 2002). Hh proteins control segmental patterning in *Drosophila* embryos and specification of the antero-posterior axis in both vertebrate and insect limbs. In *Drosophila* imaginal discs, Hh is expressed within the posterior (P) compartment and acts on adjacent anterior (A) compartment cells to specify their fates in a concentration dependent manner (Ingham and McMahon, 2001). Cells interpret the level of Hh that they receive through complex events which regulate the proteolytic cleavage, nucleo-cytoplasmic trafficking and activation of the Cubitus interruptus (Ci) transcription factor. At least three different states are encountered in the anterior compartment of wing discs (i) in the cells abutting the A/P boundary which are exposed to a high concentration of Hh, Ci is found in its full-length (155 kDa), activated form (called $Ci^{155ACT}$) which upregulates the transcription of *engrailed* (*en)* and *patched* (*ptc*), (ii) in the cells located further inside the A compartment which receive less Hh, $Ci^{155}$ is activated at a lower level and induces *decapentaplegic* (*dpp*) expression (but not *ptc* nor *en*) and (iii) in more anterior cells where no Hh is available, Ci is cleaved into a 75 kDa form ($Ci^{75}$) that represses both *dpp* and *hh* while the remaining uncleaved fraction is sequestered in the cytoplasm (Vervoort, 2000). Such exquisite control of Ci activity seems to be achieved by one or several Hedgehog transducing complexes (called HTC) that include, along with Ci, the Fused (Fu) serine-threonine kinase, the kinesin related Costal-2 (Cos2) protein and the PEST-motif containing protein, Suppressor of fused (Su(fu)) (Monnier et al., 1998; Monnier et al., 2002; Robbins et al., 1997; Sisson et al., 1997; Stegman et al., 2000; Wang et al., 2000). At least two different complexes have been described: a Fu-Cos2-Ci trimeric complex devoid of

Su(fu) and associated to microtubules via Cos2 in the absence of Hh, and a Su(fu)-Fu-Cos2-Ci tetrameric complex present in cells responding to Hh. In addition, it was recently reported that Cos2 has the ability to tether both Fu and Ci to cellular membranes (Stegman et al., 2004). A model was therefore proposed in which a HTC associated to endosomes via Cos2 is required for the production of the repressor $Ci^{75}$, while a HTC bound to Smo through Cos2 promotes Ci activation (Stegman et al., 2004).

In this study, we focused our attention on the role of the Su(fu) protein. Su(fu) is known to negatively regulate the Hh pathway and to be antagonized by Fu (Alves et al., 1998; Méthot and Basler, 2000; Ohlmeyer and Kalderon, 1998; Pham et al., 1995; Préat, 1992; Préat et al., 1993; Wang et al., 2000). Indeed, *Su(fu)* null mutations fully suppress the lack of Fu kinase activity and enhance *cos2* phenotype. Nevertheless, *Su(fu)* null mutations lead only to a very mild adult mutant phenotype, suggesting that its inhibitory role is somewhat redundant in the regulation of the pathway (Préat, 1992; Préat et al., 1993). The Su(fu) protein, like the Cos2 protein, interacts directly with Fu and Ci (Méthot and Basler, 2000; Monnier et al., 1998; Monnier et al., 2002; Ohlmeyer and Kalderon, 1998; Stegman et al., 2000; Wang et al., 2000). Studies in cultured cells and clonal analysis have shown that Su(fu) does not appear to be involved in Ci proteolysis but rather in the cytoplasmic retention of full-length Ci and in the inhibition of Ci activation (Alves et al., 1998; Chen et al., 1999a; Méthot and Basler, 2000; Ohlmeyer and Kalderon, 1998; Wang et al., 2000). Recently, it has been proposed that Su(fu) is involved in the stability of Ci isoforms generating a sensitized background to the Hh signal (Ho et al., 2005). Finally, several studies have shown that Su(fu) is phosphorylated in response to Hh, depending on Fu kinase activity (Ho et al., 2005; Lum et al., 2003).

In order to gain new insight into the regulation and the function of the Su(fu) protein, we monitored its accumulation and post-translational modifications and analyzed the effects of its overexpression. First, we show that the Su(fu) protein is submitted to phosphorylation during embryonic development, at a time when the Hh signaling is fully active. These phosphorylation events are altered in *fu* mutants, suggesting that the Fu kinase is (directly or indirectly) involved in Su(fu) isoform modulation. Second, over-expression of *Su(fu)*, either ubiquitously or in specific parts of the imaginal discs, revealed complex and paradoxical effects, namely a repression of Hh target gene expression in cells receiving Hh at the A/P border and an ectopic expression of Hh targets more anteriorly in cells which do not receive Hh. This anterior effect can occur independently of Hh signaling at the A/P border. All effects of *Su(fu)* over-expression, both in cells that are receiving Hh and in those that are not, are enhanced in a *fu* mutant context, and are suppressed by Ci over-expression. Based on these results, we propose a model for Hh signal transduction in which Su(fu) protein levels are crucial for the modulation of the different Ci states in response to the Hh signal.

## Materials and methods

*Drosophila stocks*

The *fu* alleles used in this study were described previously (Busson et al., 1988; Thérond et al., 1996a). The $fu^{1}$ and $fu^{JB3}$ alleles are class I *fu* alleles which correspond to alterations in the kinase domain, the $fu^{A}$ allele belongs to class II *fu* alleles corresponding to alterations in the extra-catalytic domain. The *Su(fu)* gene is included in the common deleted region of $Df(3R)kar^{SZ11}$ and $Df(3R)kar^{SZ21}$ deficiencies (Préat, 1992). $Su(fu)^{LP}$ is an amorphic allele associated with a small deletion altering the 3' end of the *Su(fu)* transcript (Pham et al., 1995). *GAL4* lines used were *da-GAL4* (P[$w^{+}$, *da-GAL4]* on chromosome III), *dpp-GAL4* (P[$ry^{+}$, *dppblink-GAL4*] on chromosome III), provided by the Bloomington Stock Center, *vg-GAL4* (P[$w^{+}$, *2.5 kb vg intron 2-GAL4*] on chromosome II) (Delanoue et al., 2004), *C765-GAL4* (P[$w^{+}$, *GAL4*] on chromosome III) (Guillen et al., 1995). *lacZ* reporter lines used were *dpp-lacZ* which corresponds to the BS3.0 construct (Blackman et al., 1991), *ptc-lacZ* described in Lepage et al., (1995), *wg-lacZ* described in Neumann and Cohen, (1996), *hh-lacZ* described in Lee et al., (1992). The *UAS-ci* strain, which corresponds to a full-length *ci* cDNA, is described in Dominguez et al., (1996). The *UAS-lacZ* strain was obtained from the Bloomington Stock Center. Other strains used were: *hs-FLP; Sp/SM6-TM6B (Tb)*, *Act5C>CD2>GAL4, UAS-GFP* (chromosome III) (Neufeld et al., 1998).

*UAS-Su(fu) constructs and germ line transformation*

The 1.6 kb full-length *Su(fu)* cDNA (Pham et al., 1995) was cloned between the EcoRI and NotI sites in the polylinker of the pUAST vector (Brand and Perrimon, 1993). This vector was co-injected with a D2-3 helper plasmid into a $w^{1118}$ host line under standard conditions (Spradling et al., 1999). One *UAS-Su(fu)* transgenic line was established corresponding to a transposon inserted on the X chromosome (*w,UAS-Su(fu)* line). This line was used to obtain the $w,fu^{A},UAS\text{-}Su(fu)$ and $w,f,fu^{1},UAS\text{-}Su(fu)$ lines by chromosomal recombination. These latter strains were maintained with the FM3 balancer chromosome. Similar results were obtained with an *UAS-Su(fu)* line corresponding to an insertion on the third chromosome (gift from Hervé Tricoire and data not shown).

*Clonal analysis*

*GAL4*-expressing clones were induced by the FRT/Flip-out method (Struhl and Basler, 1993), by crossing *hsFLP/hsFLP; dpp-lacZ/CyO; +/+* or *hsFLP/hsFLP; ptc-lacZ/CyO; +/+* females with *w,UAS-Su(fu)/Y; +/+; Act5C>CD2>GAL4, UAS-GFP/Act5C>CD2>GAL4, UAS-GFP* males. Clones were heat-shock induced in the progeny 16-48 hours after egg deposition by 1 hour exposure at 37°C. Imaginal discs were dissected from third instar larvae ; clones overexpressing *Su(fu)* were recovered from female larvae of *hsFLP/w,UAS-Su(fu); dpp-lacZ (or ptc-lacZ)/+; Act5C>CD2>GAL4, UAS-GFP/+* genotype while clones recovered from male larvae of *hsFLP/Y; dpp-lacZ (or ptc-lacZ)/+; Act5C>CD2>GAL4, UAS-GFP/+* genotype served as control.

*Western blot analysis*

*Drosophila* embryos were collected at different times after oviposition. Two extraction procedures were used : in the first procedure (cf Figure 1), embryos were dechorionated with bleach (2.6 % active $Cl^-$), rinsed with water, and homogenized at 4°C by several passes of a Teflon Dounce homogenizer, in a buffer containing 50 mM Hepes buffer (pH 7.5), 150 mM NaCl, 1% Igepal, 5 mM EDTA, 2 mM PMSF and leupeptin 10 mg/ml ; in the second (cf Figure 2), embryos were sonicated 30 sec. X 8 times separated by 1 min., in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, 2% Igepal, 0.1% SDS and a cocktail of protease inhibitors (1X complete kit from Roche Molecular Biochemicals). Insoluble material was sedimented at 10,000 X g for 10 min. at 4°C and the supernatant was collected. The protein concentration of the soluble material was estimated according to the Bradford technique (Bio-Rad protein assay kit). For each sample, equal amounts of proteins (from 50 to 200 mg per lane) were incubated at 100°C for 5 min. in the gel-loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM b̄mercaptoethanol, 0.1% bromophenol blue). Extracts were separated by electrophoresis in SDS denaturing polyacrylamide gels, either Laemmli gels (acrylamide 8%, bis-acrylamide 0.1%) or Anderson gels (acrylamide 12.5%, bis-acrylamide 0.1%), with migration in Tris-Glycine-SDS buffer (Anderson et al., 1973; Laemmli, 1970). Proteins were then transferred to nitrocellulose (Schleicher and Schuell) for 1 h 30 at 1 $mA/cm^2$ using a semi-dry electrotransfer apparatus (C.B.S. Scientific Co). The pattern of proteins was evaluated by staining the filters with Ponceau Red S solution. The membranes were blocked by incubation for 1 h at room temperature in Tris-buffered saline (20 mM Tris pH 7.5, 135 mM NaCl) containing 5% non fat dry milk, 0.1 % Tween 20, followed by an overnight incubation at 4°C with a 1:5 000 dilution of purified polyclonal antiserum raised against Su(fu) in rabbit (Monnier et al., 1998). The membranes were washed three times with Tris-buffered saline containing 0.5 % Igepal, 0.1% Tween 20, 5% non fat dry milk, before incubation with goat anti-rabbit IgG coupled to horseradish peroxidase (Vector) for 1 h at room temperature at a 1:10 000 dilution in 1:4 diluted wash buffer. The filters were washed three times with Tris buffered saline, 0.1 % Tween 20, and were developed using an enhanced chemiluminescence substrate (Super Signal West Pico Chemiluminescent substrate from Pierce) and Amersham Hyperfilm to reveal the signals.

*Bi-dimensional gel electrophoresis*

Drosophila embryonic extracts were prepared as described above. The samples were treated in order to perform 2D/PAGE separation as previously described (Wolff et al., 1992) except that ampholines 4-6 (Amersham Biosciences) were used in the isoelectric focusing dimension. The second dimension was performed according to Anderson et al. (1973). The size of the slab gel used was 12.5 x 24 cm. Depending on migration conditions, the pHi of the major isoform ranged between 5.2 and 5.45, while more acidic and heavier isoforms were detected. The apparent molecular weight of these different isoforms were all situated in the range of 54 kDa which is the expected size of the Su(fu) protein.

*Treatment with phosphatase inhibitors*

Freshly prepared embryonic extracts were incubated at different times (0, 10, 30, 90 min.) at 37°C, with or without phosphatase inhibitors whose composition is as follows : 0.1 mM sodium ortho-vanadate, 10 mM b-glycerophosphate, 100 mM sodium fluorure.

*Imaginal disc labelings*

For b-galactosidase activity staining, imaginal discs were dissected in PBS, fixed in 0.5 % glutaraldehyde/PBS for 15 min. at room temperature and rinsed four times in PBS. The coloration was developed in 3.5 mM $K_4(FeII(CN)_6)$, 5 mM $K_3(FeIII(CN)_6)$, 1 mM $MgCl_2$ and 0.15 % X-Gal in PBS for 2 hours at 37°C. Discs were mounted and observed in glycerol. Immunostaining using the 2A1 rat monoclonal anti-Ci (Motzny and Holmgren, 1995), was performed as follows: imaginal discs from late third instar larvae were dissected in PBS, fixed in 4 % paraformaldehyde, 30 mM Pipes (pH 7.4), 160 mM KCl, 40 mM NaCl, 4 mM $Na_3EGTA$, 1 mM spermidine, 0.4 mM spermine, 0.2 % BSA and 0.1 % Triton X-100, for 20 min. at room temperature and washed in PBS, 0.3 % Triton X-100. Tissue was blocked for 20 min. in PBS, 0.3 % Triton X-100 and 1 % BSA, and incubated overnight at 4°C in a 1 :5 dilution of the primary antibody, washed, blocked again and incubated for 2 hours at room temperature

in a 1 :10 000 dilution of FITC anti-rat IgG antibody (Jackson Laboratories). b-galactosidase immunostaining was performed using the rabbit polyclonal anti-b-galactosidase antibody (from ICN/Cappel) in a 1 :1000 dilution and the secondary anti-rabbit Cy3 antibody (Jackson Laboratories) in a 1 :100 dilution. Discs were mounted in glycerol, observed and photographed under a Leica DMR fluorescence microscope. Confocal imaging was performed with a Leica SP2-AOBS microscope.

## Results

*Su(fu) is a phosphoprotein modulated during embryonic development*

Phosphorylation and other post-translational modifications are important for the regulation of biological activities of proteins. In cultured cells, most components of the Hh pathway have been shown to be phosphorylated in an Hh dependent manner (Chen et al., 1999a; Chen et al., 1999b; Denef et al., 2000; Ho et al., 2005; Lum et al., 2003; Robbins et al., 1997; Thérond et al., 1996b; Wang and Holmgren, 1999). Here, we analyzed the accumulation and post-translational modifications of the Su(fu) protein in developing embryos. Proteins from wild-type embryonic extracts were submitted to electrophoresis and immunoblotted with a purified polyclonal anti-Su(fu) antibody (Monnier et al., 1998). As shown in Fig. 1A, an immunoreactive species was present at the expected size (the predicted 54 kDa protein encoded by *Su(fu)* is 468 aa long) and was absent in lysates from embryos deleted for *Su(fu)*.

We were unable to detect any modification of the Su(fu) protein when using the electrophoretic conditions described above. We therefore turned to Anderson type gels to perform monodimensional and bidimensional electrophoresis (Materials and Methods). As shown in Fig. 1B, immunoblotting using monodimensional Anderson type gel revealed 4 isoforms, a major one with an apparent molecular weight of 54 kDa (arrow 1), two slower migrating isoforms (filled arrowheads 2 and 3) and a faster migrating isoform (empty arrowhead 4). Bidimensional electrophoresis (Fig. 1C) revealed a major form (arrow a), that probably corresponds to the major form seen in Fig. 1B and at least 4 minor isoforms (arrowheads b, c, d, e), more acidic and of higher weight than the major form. However, no spot corresponds to the lower molecular weight form observed in Fig. 1B (empty arrowhead 4). Taken together, these data show that at least 5 and most probably 6 isoforms of Su(fu) protein exist in wild-type embryonic extracts (Figs. 1B, C).

To assess whether Su(fu) isoforms were due to phosphorylation, we took advantage of the fact that the two higher forms (filled arrowheads 2 and 3 in Figs. 1B, D) greatly decreased upon incubation of the extracts at 37°C, whereas the lower form (empty arrowhead 4) accumulated (Fig. 1D). Incubation of the same extracts with phosphatase inhibitors blocked these effects (Fig. 1D). Therefore, the slower forms are probably due to phosphorylation (or hyperphosphorylation) of Su(fu), whereas the fastest form could correspond to the non- or less-phosphorylated protein. The major band (arrow) might correspond to a less-phosphorylated form that is stable during the treatment or to other types of modification.

We also monitored the Su(fu) phosphorylation during embryonic development (Fig. 1B). The higher molecular weight isoforms were scarcely detectable 0-2 hours after oviposition, then accumulated 4-8 hours after oviposition to diminish from 8 hours onwards (upper arrowheads). In contrast, the amount of the lower isoform (empty arrowhead), was maximal at 0-2 hours, and decreased 4-8 hours after oviposition.

In conclusion, Su(fu) protein is present in several different isoforms in the embryo, corresponding to the different degrees of phosphorylation. While the maternal form is hypophosphorylated, hyperphosphorylated isoforms accumulate at the time of activation of the Hh pathway.

*Modulation of phosphorylated Su(fu) isoforms depends on Fu kinase activity*

The Fu kinase behaves as an antagonist of Su(fu) and Cos2 activities. In cell cultures, it was shown that Fu activity is required for the Hedgehog-stimulated phosphorylation of Cos2 (Nybakken et al., 2002) and Su(fu) (Lum et al., 2003). Furthermore, the modulation of Su(fu) phosphorylation described above parallels that of Fu protein (Thérond et al., 1996b). Altogether this suggests that Su(fu) could also be a target of the Fu kinase. Therefore, we analysed the effect of different *fu* mutations on the accumulation of the different Su(fu) isoforms.

According to their genetic interactions with *Su(fu), fu* mutants have been classified into two different classes , class I alleles mutated exclusively in the kinase region and class II alleles altered in the regulatory domain. We performed the experiments with the two classes of *fu* mutants (Fig. 2) and in both classes, accumulation of the higher hyperphosphorylated forms of Su(fu) (bands 2 and 3) was significantly reduced. Two lower migrating forms were detected (bands 4 and 5). Their status differed depending on the class of the *fu* allele: in the class I *fu* mutants ($fu^{JB3}$) these forms were as abundant as in the wild-type, while they were markedly enhanced in the class II $fu^{A}$ mutant (especially band 4). The partial persistence of higher forms in all types of mutants suggests that other kinases are involved. The effect seen in the $fu^{A}$ mutant indicates that integrity of the regulatory domain is required for the activity of these kinases.

In conclusion, Fu kinase activity appears to participate (directly or indirectly) in the phosphorylation of the Su(fu) protein during embryonic development, but other kinases are most probably also involved.

*Su(fu) over-expression in imaginal discs leads to the inhibition of Hh target gene expression in anterior cells receiving the Hh signal*

The role of Su(fu) *in vivo* was assessed by examining the consequences of its over-expression in various tissues during development. We therefore drove the expression of an *UAS-Su(fu)* transgene with the ubiquitous *da-GAL4* driver (see Materials and Methods) and looked at fly viability, adult appendage phenotype and Hh target gene expression in imaginal discs. In all cases, comparable effects were obtained with several independent insertions of the *UAS-Su(fu)* transgene, and no effect was observed with either the driver alone or the *UAS-Su(fu)* transgene alone.

As shown in Table 1A, ubiquitous over-expression of *Su(fu)* led to a significant decrease in fly viability, with almost 100% lethality (mostly pupal) at 25°C and 29°C. This effect was much lighter at 18°C and 21°C, in accordance with the stronger activity of the GAL4 protein at high temperatures. Wings of rare escapers emerging at 25°C were analyzed. We first turned our attention to the effects induced at the A/P border in cells responding to the Hh signal. Wings of *UAS-Su(fu); da-GAL4* escapers did not show any obvious anomalies in the LV3-LV4 region which corresponds to the domain of Hh activity (Fig. 3B). We looked for Hh target gene expression in the corresponding imaginal discs at 25°C. The results presented in Fig. 4 show that *Su(fu)* over-expression actually leads to a reduction in *dpp* (Fig. 4B) and *ptc* (Fig. 4D) expression (as indicated by the width of the domain between red arrowheads). Again, this effect was stronger at 29°C than at lower temperatures (data not shown). Furthermore, we also observed a decrease in *dpp* and *ptc* expression in *Su(fu)* over-expressing clones induced at the A/P boundary (Supplementary data). The same down-regulation of *dpp* and *ptc* expression was also observed in leg (Figs. 5A-D) and eye-antennal (Figs. 5G-J) imaginal discs at 25°C.

Thus, at the A/P border in cells receiving the Hh signal, overexpression of *Su(fu)* leads to a decrease in the expression of two Hh target genes, *dpp* and *ptc*. This effect is in agreement with a negative role of Su(fu) in Hh signal transduction.

*Su(fu) over-expression in imaginal discs leads to the deregulation of Hh target gene expression in anterior cells not receiving the Hh signal*

Unexpectedly, ubiquitous overexpression of *Su(fu)* had an effect in the most anterior region of the appendages since nearly 100% escapers displayed clear anterior duplications in adult wings, legs and antennae (Fig. 3). Thus, in the antero-proximal region of the wing, the domain comprised between costa and subcosta was enlarged, with frequent more or less expanded costa duplications (Fig. 3B, arrow). In addition, costa bristles were more numerous than normal and were disorganized (data not shown). This phenotype is very similar to the *costal-2* loss of function phenotype (Grau and Simpson, 1987; Simpson and Grau, 1987; Whittle, 1976) suggesting an ectopic activation of the Hh pathway in the most anterior regions. Similar anterior duplications can also be seen in the antennae (data not shown) and in the legs. Indeed legs were highly deformed especially at 29°C, with anterior sex-comb duplications (Figs. 3E, F arrow), and enlargment and fusion of the articles along the proximo-distal axis (Fig. 3G).

In the wing discs over-expressing *Su(fu)*, ectopic *dpp* expression expanded, from a site at the antero-posterior border (presumptive proximal part of vein 3), to the outer part of the disc (presumptive costa) (Fig. 4B arrow). In leg and antenna imaginal discs, over-expression of *Su(fu)* led to anterior ectopic expression of both *dpp* (Figs. 5B arrow and 5H arrow) and *wg* (Fig. 5F). In the leg discs, ectopic *dpp* expression was especially strong (Fig. 5B, arrow), creating a second axis in the anterior compartment and was associated with an ectopic *wg* expression that extended anteriorly and dorsally (Fig. 5F arrow). Overall, these ectopic expressions were consistent with the distal duplications observed in the legs. In imaginal discs overexpressing *Su(fu)*, ectopic *ptc* expression appeared as a faint spot in the presumptive costa of the wing discs (Fig. 4D arrow) whereas it was stronger in leg, antenna and eye discs (Figs. 5D arrow and 5J arrows).

In conclusion, ubiquitous overexpression of *Su(fu)* in the imaginal discs led to apparent antagonistic effects: a decrease in Hh signaling at the A/P border, in cells receiving the Hh signal, and an ectopic activation of the pathway more anteriorly, in cells not receiving Hh.

*The ectopic anterior effects of Su(fu) over-expression are independent of its effects at the antero-posterior border*

The activation of the Hh pathway, in the most anterior regions where Hh is normally absent could be secondary to the effects seen at the A/P border. To test this hypothesis, we investigated whether the anterior effects of *Su(fu)* over-expression could occur independently of its over-expression at the A/P border. We thus drove *Su(fu)* overexpression by *vgBE-GAL4* which is strongly expressed at the dorso-ventral (D/V) border but not at the A/P border, except at the distal intersection between A/P and D/V borders of the wing pouch (Fig. 6A). The flies were viable. In agreement with the expression pattern of the *vgBE-GAL4* driver, no obvious effect could be

seen at the A/P border in either adult wing phenotype or wing discs. Conversely, we observed very frequent anterior anomalies in the costa region of the wing, similar to, but stronger than those observed with the *da-GAL4* driver, leading to large anterior duplications (Fig. 6B). Indeed, wing discs displayed anterior overgrowth with a correlative ectopic *dpp*, but not *ptc,* expression (Figs. 6C arrow, D, compare with Figs. 4B, D). This result was confirmed by analysing clones of cells overexpressing *Su(fu)* in wing and leg discs generated by the Flip-out method (Materials and Methods). In wing discs (Figs. 6E-G), anterior clones led to disc overgrowth. Only those clones located in the sensitive specific hinge region expressed *dpp* ectopically (Fig. 6F) whereas *ptc* ectopic expression was never detected, even in large anterior clones (data not shown). Ectopic *dpp* expression could also be seen outside the limits of the clones indicative of non-autonomous effects. Similar results were obtained for clones in leg imaginal discs (data not shown).

These results show that the anterior effects of *Su(fu)* over-expression on ectopic *dpp* expression can occur independently from its effects at the A/P border.

*ci over-expression has epistatic effects upon Su(fu) over-expression effects*

In wing imaginal discs, the transcription of *dpp* is both repressed by $Ci^{75}$ in the absence of Hh and activated by $Ci^{155}$ in response to Hh. Thus, the anterior ectopic *dpp* expression induced by *Su(fu)* over-expression could be due to a loss of $Ci^{75}$ activity and/or to an accumulation of $Ci^{155}$ (resulting from either an increase in its stability or an inhibition of its cleavage). To test whether $Ci^{75}$ was still present, we looked at the expression of another negative target of $Ci^{75}$, *hh*, using an *hh-lacZ* reporter. No ectopic *hh* expression in the anterior compartment could be detected (Fig. 6H), whereas endogenous *hh* expression was seen in the posterior compartment of the wing disc. This suggests that, at least some $Ci^{75}$ is still present in the anterior compartment. Next, we looked at full-length Ci, using an antibody specific of $Ci^{155}$. The accumulation of cytoplasmic $Ci^{155}$ is clearly visible in the anterior deformed part of the *vgBE-GAL4*; *UAS-Su(fu)* wing imaginal discs, (Fig. 6J arrow), whereas it remains absent or at very low levels in the corresponding region of the control disc (Fig. 6I).

Then, in order to check whether *Su(fu)* overexpression effects could be modulated by Ci, we overexpressed both *ci* and *Su(fu)* simultaneously (Fig. 7), using the *C765-GAL4* driver known to be weakly expressed in the entire wing pouch (Méthot and Basler, 1999). This led to different effects in the A and P compartments. In the posterior compartment, overexpressing *ci* alone led to an ectopic expression of *ptc* in the entire compartment and of *dpp* in two broad posterior stripes (Figs. 7D and 7C, respectively) indicating that the presence of Hh in this compartment leads to a fully activated Ci isoform (Méthot and Basler, 1999). The effects of *ci* over-expression in this compartment were not modified by simultaneous *Su(fu)* over-expression (compare Figs. 7G and 7C for *dpp* and Figs. 7H and 7D for *ptc* expression), suggesting that Su(fu) overproduction was unable to efficiently counteract $Ci^{act}$ production. In the anterior compartment, *ci* overexpression alone had no effect (Figs. 7C, D), indicating that the total Ci excess was converted in the repressive $Ci^{75}$ isoform. In these cells, *Su(fu)* overexpression induced ectopic *dpp* expression associated with $Ci^{155}$ accumulation; *ci* over-expression totally suppressed anterior *Su(fu)* over-expression effects, as shown by the lack of anterior ectopic *dpp* expression (compare Figs. 7G and 7E arrow). This suggests that Su(fu) overproduction was unable to counteract $Ci^{75}$ production.

In conclusion, in both compartments the effects of *ci* overexpression are totally epistastic over those of *Su(fu)* overexpression. This suggests that overexpression of *Su(fu)* does not have any effect on fully activated Ci ($Ci^{act}$) nor on $Ci^{75}$.

*Effects of Su(fu) over-expression are enhanced in a fu mutant background*

It is known that Su(fu) and Fu act antagonistically in the Hh pathway as negative and positive effectors, respectively, (Alves et al., 1998; Préat et al., 1993). To test whether *fu* could modulate the effects of *Su(fu)* over-expression, we overexpressed *Su(fu)* in class I and class II *fu* mutants, using the $fu^1$ and $fu^A$ alleles respectively (see Materials and Methods). We first observed that pupal lethality of *fu* flies overexpressing *Su(fu)* was greatly enhanced, even at 21°C or 18°C, when compared to that of *fu* mutants or to $fu^+$ flies overexpressing *Su(fu)* (Table 1). These effects were the same with both classes of *fu* alleles (Table 1). In escapers, the characteristic wing *fu* mutant phenotype was greatly enhanced : veins 3 and 4 were almost completely fused with a large delta at the margin (Fig. 3D). We also observed anomalies affecting more anterior regions of the wing, namely an enlargement of the domain between vein 2 and the margin, and the more or less complete disappearance of vein 2 (Fig. 3D, asterisk). Both features are reminiscent of an HhMoonrat (HhMrt) phenotype which corresponds to an ectopic *hh* expression in the anterior compartment (Felsenfeld and Kennison, 1995). Costa duplications were also induced (data not shown). Last, leg anomalies corresponding to anterior duplications, enlargement and fusion of articles were enhanced (Fig. 3H). These effects were seen with both classes of *fu* alleles.

Correlatively, *fuI* and *fuII* wing discs overexpressing *Su(fu)* displayed changes in *dpp* and

*ptc* expression. At the A/P border, *dpp* and *ptc* expressions in the wing pouch were further decreased (Figs. 4F and 4H, domains between arrows). These stronger phenotypes conserved certain characteristics of both *Su(fu)* overexpression, i.e., decrease in expression, and *fu* loss of function, i.e., an enlargement of the expression domain of *dpp* and *ptc*. In the anterior region, the ectopic expression of both *dpp* (Fig. 4F) and *ptc* (Fig. 4H) was considerably enhanced, especially for *ptc*. The effects were the same for both classes of *fu* alleles ($fu^1$ (Fig. 4), $fu^A$ (data not shown)) and are paradoxically reminiscent of previously reported data for $fu^A$ $Su(fu)^-$ flies (Alves et al., 1998).

In conclusion, the effects of *Su(fu)* overexpression are enhanced in a *fu* mutant background. This is consistent with an antagonistic role of Su(fu) and Fu both at the A/P border and in the more anterior regions of imaginal discs. These effects appear similar with both classes of *fu* alleles thus suggesting that the Fu kinase activity is involved both in cells receiving the Hh signal and in cells that do not.

## Discussion

Su(fu) plays a negative role in Hh signalization since it participates both in the cytoplasmic retention of Ci and in the inhibition of the activation of $Ci^{155}$ (Ingham and McMahon, 2001; Méthot and Basler, 2000; Nybakken and Perrimon, 2002; Ohlmeyer and Kalderon, 1998). Here, we analyzed the effects of *Su(fu)* over-expression on appendage developpment and on the expression of several Hh target genes in the corresponding discs. In parallel, we studied its accumulation and post-translational modifications during embryonic development in $fu^+$ and *fu* mutant backgrounds.

*Su(fu) over-expression in imaginal discs leads to opposite effects in cells receiving and not receiving the Hh signal*

The effects of *Su(fu)* over-expression on the Hh pathway were assessed by examining both the adult appendage development and the transcription of well characterized Hh targets (such as *dpp* and *ptc*) and accumulation of full-length Ci ($Ci^{155}$) in the corresponding discs. No effect was detected in the posterior compartment, but two apparently opposite effects were observed in the anterior compartment depending on the distance from the source of Hh.

(i) At the A/P border, there was a decrease in the response to low and high levels of Hh signaling. Indeed, *dpp* and, to a lesser extent, *ptc* gene expression was reduced. This result is in agreement with the known inhibitory role of the Su(fu) protein in cells transducing the Hh signal.

(ii) More anteriorly, in cells which do not receive the Hh signal, over-expression of *Su(fu)* led to anterior duplications in adult appendages. This was correlated with an ectopic expression of *dpp* in the wing disc or *dpp* and *wg* in the leg disc, associated with an accumulation of $Ci^{155}$. Ectopic *ptc* expression was also seen but at a much lower level. These effects phenocopy those of *cos2* loss of function mutants (Grau and Simpson, 1987; Simpson and Grau, 1987) or of ectopic *hh* expression (Felsenfeld and Kennison, 1995). They can be interpreted as a constitutive activation of the pathway. However, the fact that only low levels of ectopic *ptc* expression are induced shows that the highest levels of Ci activation are not attained.

*Anterior ectopic effects of Su(fu) over-expression can occur independently of its effects at the A/P border*

High Ptc protein levels at the boundary are known to sequester the Hh protein (Chen and Struhl, 1996). Thus, the anterior ectopic *dpp* expression observed here in discs overexpressing *Su(fu)* could be secondary to the deregulation of the Hh pathway at the A/P border: the initial decrease of Ptc at the A/P boundary would result in a further diffusion of Hh to the neighbouring cells in which Ci cleavage would be inhibited, allowing *hh* and *dpp* expression. So, step by step, a partial activation of the pathway could be propagated up to the anterior region of the wing pouch. Alternatively, the anterior effects of *Su(fu)* over-expression could occur independently of events at the A/P border. We favor this latter hypothesis for two reasons: (i) induction of *Su(fu)* over-expression in the A region, outside the A/P border (using either the *vgBE-GAL4* driver or clonal analysis), showed that the ectopic activation of *dpp* can occur independently of *Su(fu)* over-expression at the A/P border (Fig. 6), (ii) no significant ectopic *hh* expression could be detected (Fig. 6H and data not shown).

*Su(fu) over-expression modulates Ci states*

At least three Ci states have been postulated to exist, depending on the Hh signal gradient: (i) a fully active Ci ($Ci^{act}$) responsible for high *ptc* expression in a stripe 4-5 cells wide close to the A/P border, (ii) a full-length Ci ($Ci^{155}$) sufficient for *dpp* expression 10-15 cell diameters away from the A/P border, (iii) a cleaved Ci form ($Ci^{75}$) in anterior cells not receiving Hh which represses *hh* and *dpp* expression (Aza-Blanc et al., 1997; Dominguez et al., 1996; Méthot and Basler, 1999; Méthot and Basler, 2001; Ohlmeyer and Kalderon, 1998); (for review see Lum and Beachy, 2004; Nybakken and Perrimon, 2002). The balance between these forms of Ci depends on the regulation of non-exclusive processes such as cytoplasmic tethering, protein stability, nuclear shuttling and cleavage (Chen et al., 1999a; Ohlmeyer and Kalderon, 1998; Wang et al., 2000; Wang and Holmgren, 2000). At least two complexes

that contain Ci have been identified: a tetrameric Su(fu)-Ci-Fu-Cos2 complex (complex A) probably present in cells receiving a high level of Hh and a trimeric Ci-Fu-Cos2 complex (complex B) which is devoid of Su(fu) and bound to microtubules in the absence of Hh (Robbins et al., 1997; Sisson et al., 1997; Stegman et al., 2000; Wang and Jiang, 2004). At the molecular level, Su(fu) binds to N-terminal Ci and thus has the capacity to bind both $Ci^{155}$ and $Ci^{75}$ (Monnier et al., 1998; Stegman et al., 2000). Su(fu) was shown to sequester Ci in the cytoplasm thus controlling the nuclear shuttling of Ci (Méthot and Basler, 2000; Wang et al., 2000; Wang and Jiang, 2004; Wang and Holmgren, 2000). It was also shown to be involved in the stability of $Ci^{155}$ and $Ci^{75}$ (Ohlmeyer and Kalderon, 1998).

Here, we show that overexpression of Su(fu) differentially affects the expression of Hh target genes in Hh-receiving and non-receiving cells and that these effects are all reversed by overexpression of Ci. Moreover, the resulting anterior ectopic activation of *dpp* is associated with an important accumulation of $Ci^{155}$. To account for these data, we hypothesize that Su(fu) over-expression disturbs the balance between the different Ci complexes and thus between the different Ci states. We propose a model for Hh signaling in imaginal discs in which the effects of *Su(fu)* over-expression result mainly from the cytoplasmic retention of $Ci^{155}$ (Fig. 8). At the A/P boundary in Hh-receiving cells, $Ci^{155}$ is normally present in a tetrameric complex with Su(fu), Fu and Cos2 (complex A). In these cells, Hh signaling via the activation of Fu blocks Cos2 and Su(fu) negative effects in the tetrameric complex, thus preventing Ci cleavage and cytoplasmic retention and favoring the release of Ci, its activation and nuclear access (Fig. 8A [Hh]). Su(fu) over-expression could lead to the recruitment of a significant fraction of endogenous $Ci^{155}$ into complexes in which Su(fu) is no longer inhibited by Fu. A fraction of Ci is thus sequestered in the cytoplasm as an inactive full-length form (Fig. 8B [Hh]). Co-over-expression of Ci along with Su(fu) would provide enough Ci to buffer the excess of Su(fu), leading to the formation of active $Ci^{155}$. In the anterior region where Hh is absent, Ci is present in a microtubule-bound trimeric complex (complex B) containing Fu and Cos2 but not Su(fu), leading to Ci cytoplasmic tethering and favoring its cleavage in the $Ci^{75}$ repressive form. This complex would be in equilibrium with a Fu-Su(fu)-Ci complex. In this complex, Su(fu) would act as a safety lock for the cytoplasmic retention of an uncleaved fraction of $Ci^{155}$ potentially able to yield some active forms of Ci (Fig. 8A [noHh]). When Su(fu) is overexpressed, extra Su(fu) would bind $Ci^{155}$, preventing it from joining the microtubule-bound complex (Fig. 8B [noHh]). Ci would not be effectively processed, leading to the accumulation of uncleaved $Ci^{155}$. The reduction in the amount of $Ci^{75}$ would be sufficient to allow the expression of *dpp* but not that of *hh*, which has been reported to be more sensitive to $Ci^{75}$ repression than *dpp* (Méthot and Basler, 1999). There would be an enrichment in the other complex but only a few active Ci forms would be produced in agreement with the almost total absence of ectopic *ptc* expression.

*All effects of Su(fu) over-expression are modulated by Fu*

The present data show that all the effects induced by overexpresion of *Su(fu)* were enhanced in *fu* mutants, namely pupal lethality, ectopic anterior expression of *dpp* and *ptc* genes and their decrease at the antero-posterior border.

At the A/P border, Fu is normally required to antagonize the negative effect of Su(fu) in Hh receiving cells. In *fu* mutant discs overexpressing *Su(fu)*, the negative effects that Su(fu) exerts on $Ci^{155}$ cytoplasmic retention in the tetrameric complex would no longer be counteracted by Fu. The shifting of the equilibrium towards the inactive Su(fu)-Ci complex is increased. Less active Ci is available and the reduction in *dpp* and *ptc* expression is aggravated.

The anterior ectopic activation of the pathway seen in discs overexpressing *Su(fu)* was greatly enhanced in *fu* mutants. These unexpected results provide evidence for an inhibitory role of Fu on $Ci^{155}$ in the absence of the Hh signal. In the absence of Hh, Fu activity could favor the normal restrictive effect of Su(fu) on $Ci^{155}$ in the Fu-Su(fu)-Ci complex (Figure 8A [noHh]) In $fu^-$ mutants, the negative effect of Su(fu) on the trapped fraction of $Ci^{155}$ would be weakened and enough $Ci^{155}$ would be active to induce transcription of *dpp* and of *ptc*.

Strikingly, unlike *Su(fu)* loss of function mutations, *Su(fu)* over-expression failed to distinguish between the two classes of *fu* alleles. Since the regulatory domain is probably necessary for Fu kinase activity, the effects seen are probably all mostly due to a loss of Fu kinase activity which would reduce the level of phosphorylation of Su(fu). As shown here and in several recent reports, the Su(fu) protein is phosphorylated in the embryo (Ho et al., 2005; Lum et al., 2003). We detected multiple levels of phosphorylation, with hyperphosphorylated forms that accumulate at a period in embryonic development when Fu is activated by the Hh signal (Thérond et al., 1996b) and that are significantly reduced in *fu* mutants. Thus, Fu could modulate Su(fu) activity by controlling, directly or indirectly, its phosphorylation. In the absence of Hh signaling, a low level of Su(fu) phosphorylation by Fu would reinforce the negative effect of Su(fu), whereas a higher phosphorylation level would inactivate Su(fu) in Hh responding cells at the A/P border.

Nevertheless, phosphorylated isoforms were not totally abolished in *fu* mutants, suggesting that other kinase(s) can phosphorylate Su(fu). In

agreement with this point, numerous putative phosphorylation sites for kinases such as Caseine kinase II or PKC, but not PKA, are present in the Su(fu) protein. However, the biological implications of the Su(fu) isoforms and their modulation by the Hh transduction signal remain to be demonstrated.

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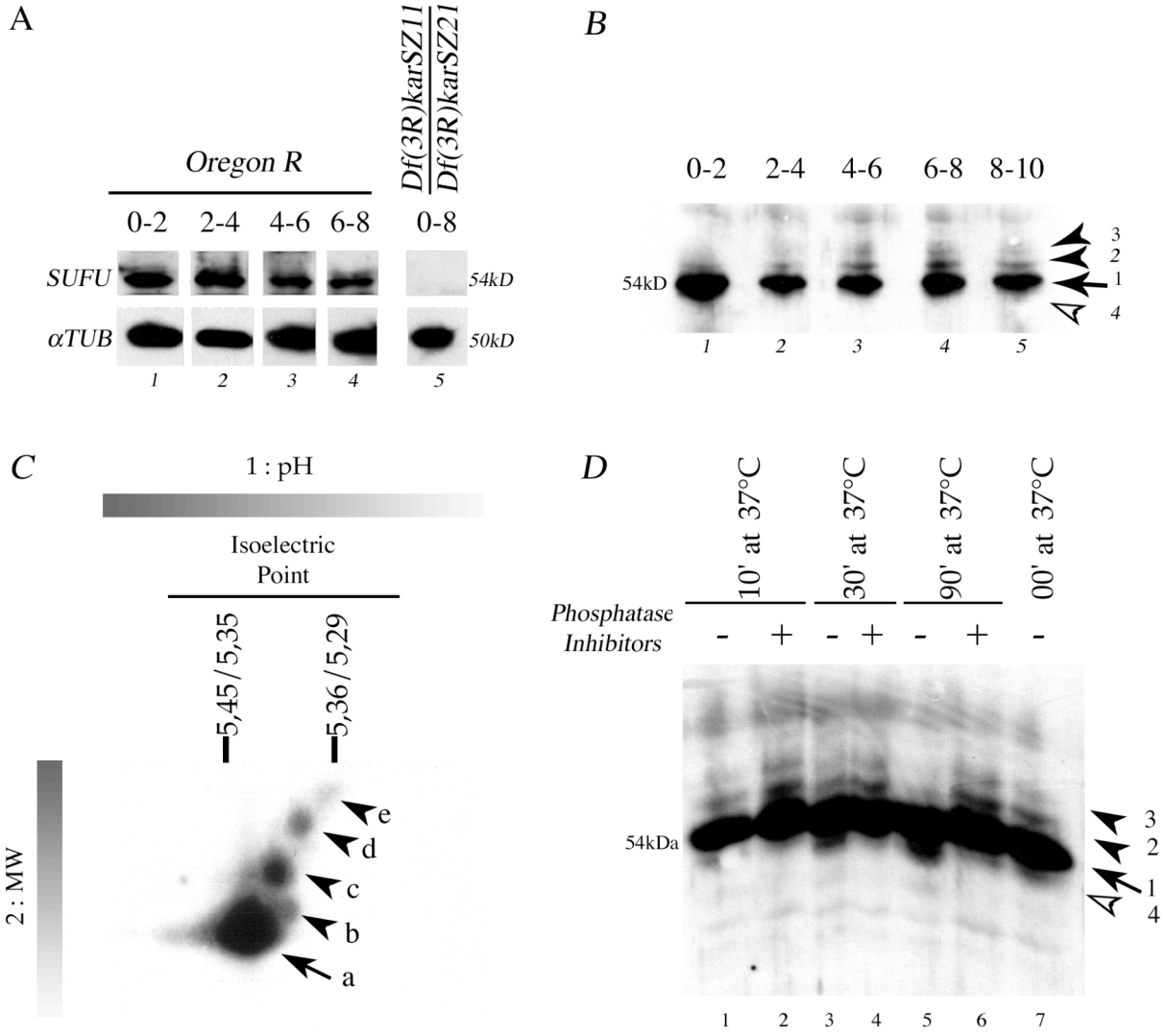


**Fig. 1:** Accumulation and post-translational modifications of the Su(fu) protein during embryonic development.
A) Immunodetection of the Su(fu) protein in embryonic extracts from 0-2 h, 2-4 h, 4-6 h, 6-8 h Oregon R embryos and from 0-8 h *Df(3R)karSZ11/Df(3R)karSZ21* embryos; electrophoresis is performed on a Laemmli type acrylamide gel; upper bands, around 54 kDa, are revealed with our anti-Su(fu) polyclonal antibody, lower bands with an anti-□tubulin antibody after stripping of the membrane; note the total absence of immuno-reactive material in embryos deleted for *Su(fu)*; Su(fu) is maternally present in 0-2 h embryos; its level increases in 2-4 h embryos to diminish in 4-6 h and 6-8 h embryos.
B) Su(fu) protein isoforms during Oregon R embryonic development; electrophoresis on an Anderson type gel reveals at least 4 isoforms, a major isoform (1, arrow) of 54kDa, two slower migrating isoforms (2 and 3, arrowheads) and a faster migrating one (4, empty arrowhead); the major isoform 1 does not vary significantly according to the developmental stage; the slower isoforms 2 and 3 appear progressively from 0-2 h (one isoform) to 6-8 h (two isoforms) to decrease from 8 h onward; a reciprocal modulation is seen for the faster isoform 4.
C) Bidimensional electrophoresis according to pHi and PM of extracts from 0-16h Oregon R embryos; at least, five isoforms are revealed, one major isoform (a, arrow) and four minor slower migrating acidic isoforms (b, c, d, e, arrowheads).
D) Su(fu) protein phosphorylation; extracts from 0-16 h embryos are incubated, 10, 30, 90 min. at 37°C, without (lanes 1, 3, 5) or with a mix of phosphatase inhibitors (lanes 2, 4, 6) and fractionated on an Anderson type gel; the control corresponds to embryonic extracts not incubated at 37°C (lane 7). In absence of phophatase inhibitors, a progressive disappearance of the higher acidic isoforms (2 and 3, arrowheads) is seen and correlated with an increase of the lower form (4, empty arrowhead). This effect is totally inhibited in the presence of phosphatase inhibitors. No modulation of the 54 kDa major isoform (1, arrow) is observed.

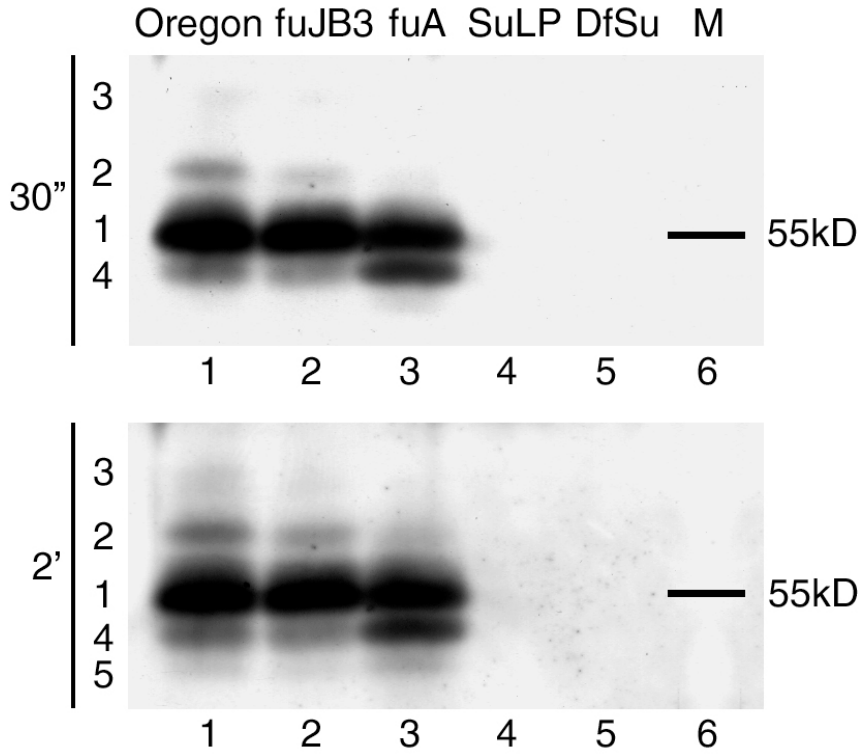


**Fig. 2:** Modulation of Su(fu) isoforms in a *fu* mutant background.
Proteic extracts from 0-24 h embryos, wild-type (lane 1) and *fu* mutants, $fu^{JB3}$ (lane 2), $fu^{A}$ (lane 3), were migrated on Anderson type gel and revealed with anti-Su(fu) antibody; extracts from $Su(fu)^{LP}$ (lane 4) and *Df(Su(fu)*) (lane 5) embryos are shown as controls. Two exposure times are given. As compared to wild-type, the relative amounts of slower migrating isoforms 2 and 3 are reduced in both class I ($fu^{JB3}$) and class II ($fu^{A}$) *fu* embryonic extracts. Unlike class I $fu^{JB3}$, class II $fu^{A}$ extracts display a strong increase in faster migrating isoforms 4 and 5.

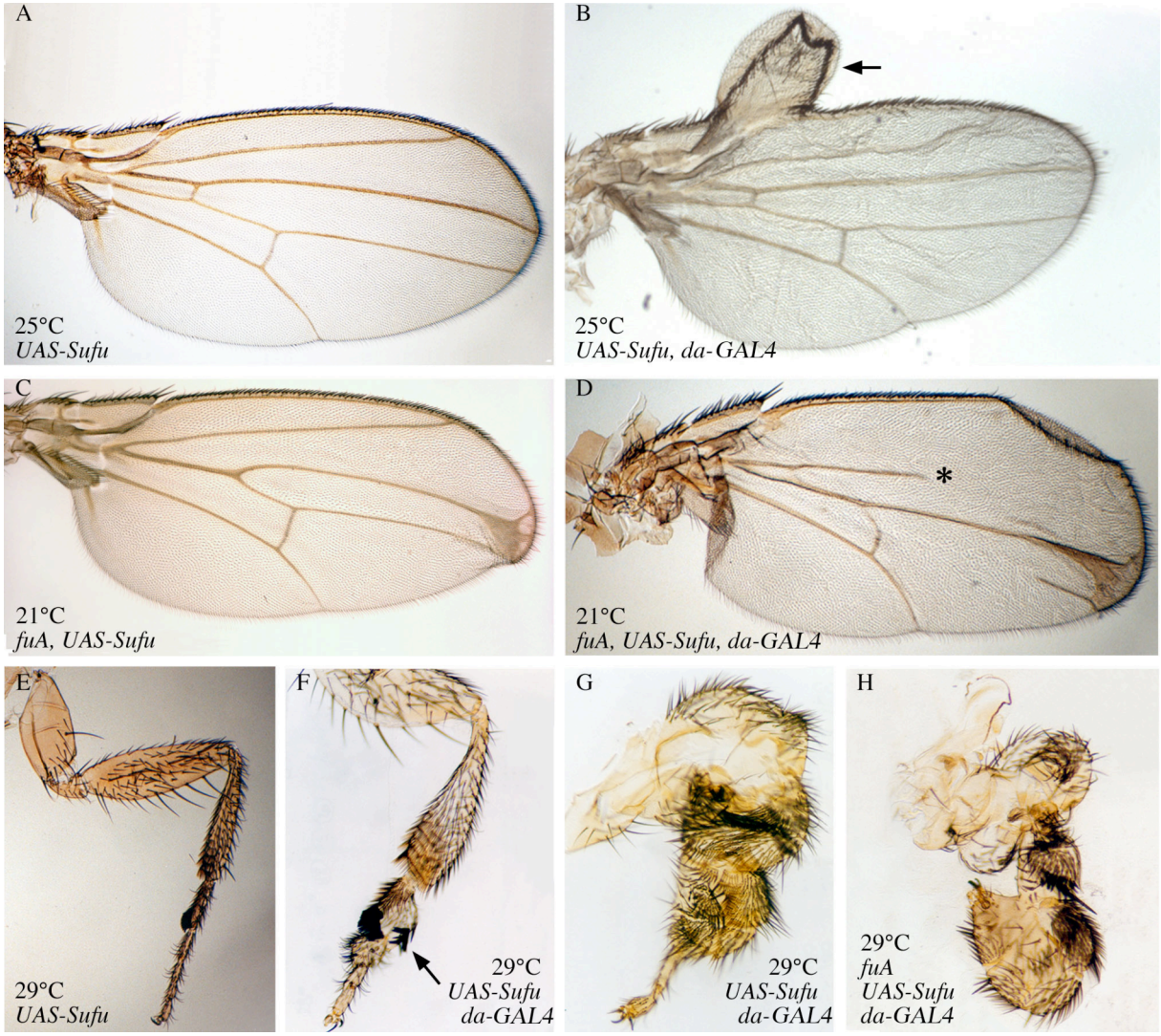


**Fig. 3**: Effects of *Su(fu)* overexpression on wing and leg phenotypes in $fu^{+}$ and *fu* mutant backgrounds.
(A, B) Wings of *UAS-Su(fu)* flies (A) and *UAS-Su(fu)*; *da-GAL4* flies (B) raised at 25°C. In wings overexpressing *Su(fu)*, the region between veins 3 and 4 is not altered but an anterior duplication on the wing margin is observed (arrow in B). (C, D) Wings of $wfu^{A}$, *UAS-Su(fu)* flies (C) and $wfu^{A}$, *UAS-Su(fu); da-GAL4* flies (D) raised at 21°C. In $fu^{A}$ wings overexpressing *Su(fu)*, the region between veins 3 and 4 completely disappeared; the vein 2 is truncated (* in D) and the domain between vein 2 and the margin is enlarged. Wings in (B) and (D) are observed from rare escapers (see Table 1). Anterior duplications can also be seen (data not shown). (E-G) Legs of *UAS-Su(fu)* flies (E) and *UAS-Su(fu); da-GAL4* flies (F, G) raised at 29°C. In the first pair of legs overexpressing *Su(fu)*, articles are shorter and thicker, and legs present a clear anterior sex comb duplication (arrow in F). Legs of the third pair are extremely deformed, with enlarged and fused articles (G). (H) Legs of *UAS-Su(fu),* $wfu^{A}$*; da-GAL4* flies raised at 29°C show anterior duplications, enlargment and fusion of articles. $wfu^{A}$, *UAS-Su(fu)* legs are similar to *UAS-Su(fu)* legs (data not shown).

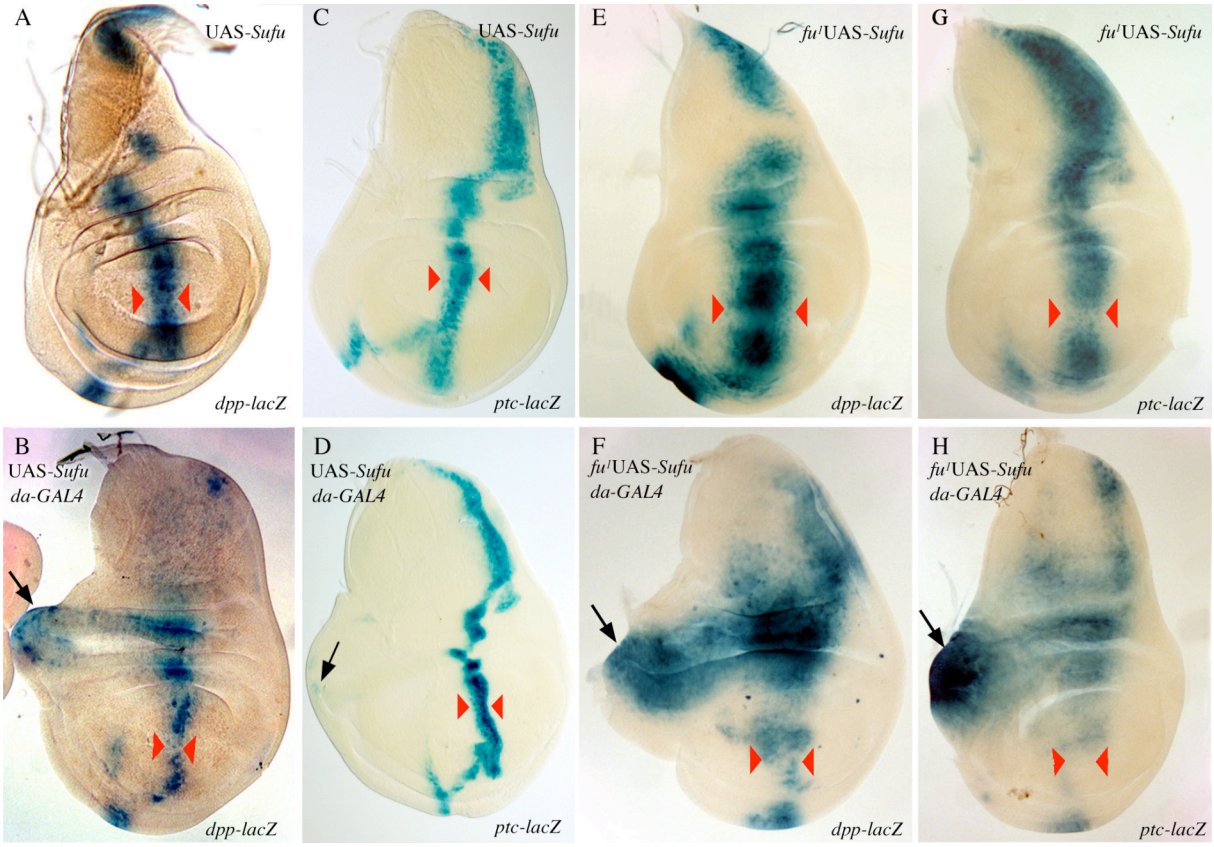


**Fig. 4:** Effects of ubiquitous *Su(fu)* overexpression on *dpp* and *ptc* expression in wing imaginal disc.
(A-D) Expression of *dpp-lacZ* (A, B) and *ptc-lacZ* (C, D) in *UAS-Su(fu)* wing discs (A, C) and *UAS-Su(fu); da-GAL4* discs (B, D) raised at 25°C. When overexpressing *Su(fu)*, the domain of expression of *dpp-lacZ* and *ptc-lacZ* along the antero-posterior boundary is reduced (compare B with A, and D with C respectively, width of the domain between red arrowheads ; note that *dpp* expression is nearly lost at the intersection between A/P and D/V borders). At the same time, *dpp-lacZ* (arrow in B) and *ptc-lacZ* (arrow in D) are ectopically expressed in the anterior compartment. (E-H) Expression of *dpp-lacZ* (E, F) and *ptc-lacZ* (G, H) in $wfu^1$, *UAS-Su(fu)* wing discs (E, G) and $wfu^1$, *UAS-Su(fu); da-GAL4* discs (F, H) raised at 25°C. In a *fu* mutant background, *dpp-lacZ* and *ptc-lacZ* expressions are nearly lost in the wing pouch of discs overexpressing *Su(fu)* (compare F with E, and H with G respectively, width of the domain between red arrowheads). The overexpression of *Su(fu)* generates also an anterior ectopic expression of *dpp-lacZ* (arrow in F) and *ptc-lacZ* (arrow in H). Note that the anterior ectopic expression of *dpp-lacZ* and *ptc-lacZ* in a *fu* mutant background is much stronger than in wild type background (compare F with B and H with D). Overexpression of *Su(fu)* in a $fu^A$ background gives the same kind of phenotype than in $fu^1$ background (data not shown).

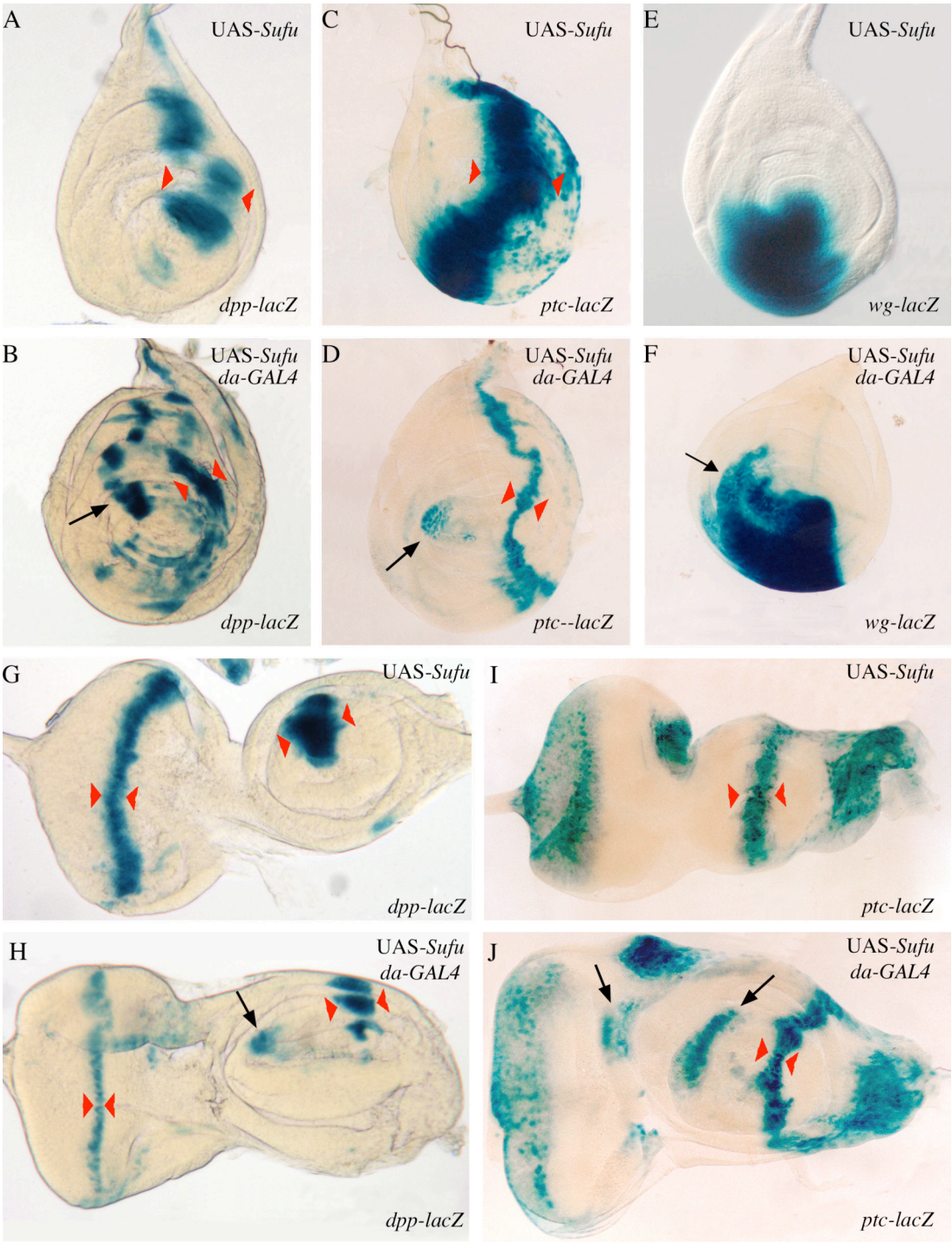


**Fig. 5:** Effects of ubiquitous *Su(fu)* overexpression on *dpp*, *wg* and *ptc* expression in leg, antenna and eye imaginal discs.
(A-F) Expression of *dpp-lacZ* (A, B), *ptc-lacZ* (C, D) and *wg-lacZ* (E, F) in *UAS-Su(fu)* leg discs (A, C, E) and *UAS-Su(fu); da-GAL4* leg discs (B, D, F) raised at 25°C. In imaginal discs overexpressing *Su(fu)*, the expression of *dpp-lacZ* and *ptc-lacZ* is reduced along the antero-posterior boundary (compare B with A and D with C respectively, width of the domain between red arrowheads), while *dpp-lacZ* and *ptc-lacZ* are ectopically expressed in the anterior compartment (arrows respectively in B and D). The expression of *wg-lacZ* is anteriorly and dorsally extended when overexpressing *Su(fu)* (compare F with E). (G-J) Expression of *dpp-lacZ* (G, H) and *ptc-lacZ* (I, J) in *UAS-Su(fu)* antenna and eye discs (G, I) and *UAS-Su(fu); da-GAL4* discs (H, J) raised at 25°C. Eye-antenna discs overexpressing *Su(fu)* are highly deformed. In those discs, *dpp-lacZ* (arrow in H) and *ptc-lacZ* (arrows in J) are ectopically and anteriorly expressed; in the eye part of the disc, the expression of *dpp-lacZ* in the furrow is reduced (compare H with G, width of the domain between red arrowheads). In H and J, X-Gal staining has been prolonged to clearly see ectopic expressions, so the expression of *dpp-lacZ* and *ptc-lacZ* along the antero-posterior boundary in the antennal part of the discs seems nearly as strong as in normal discs.

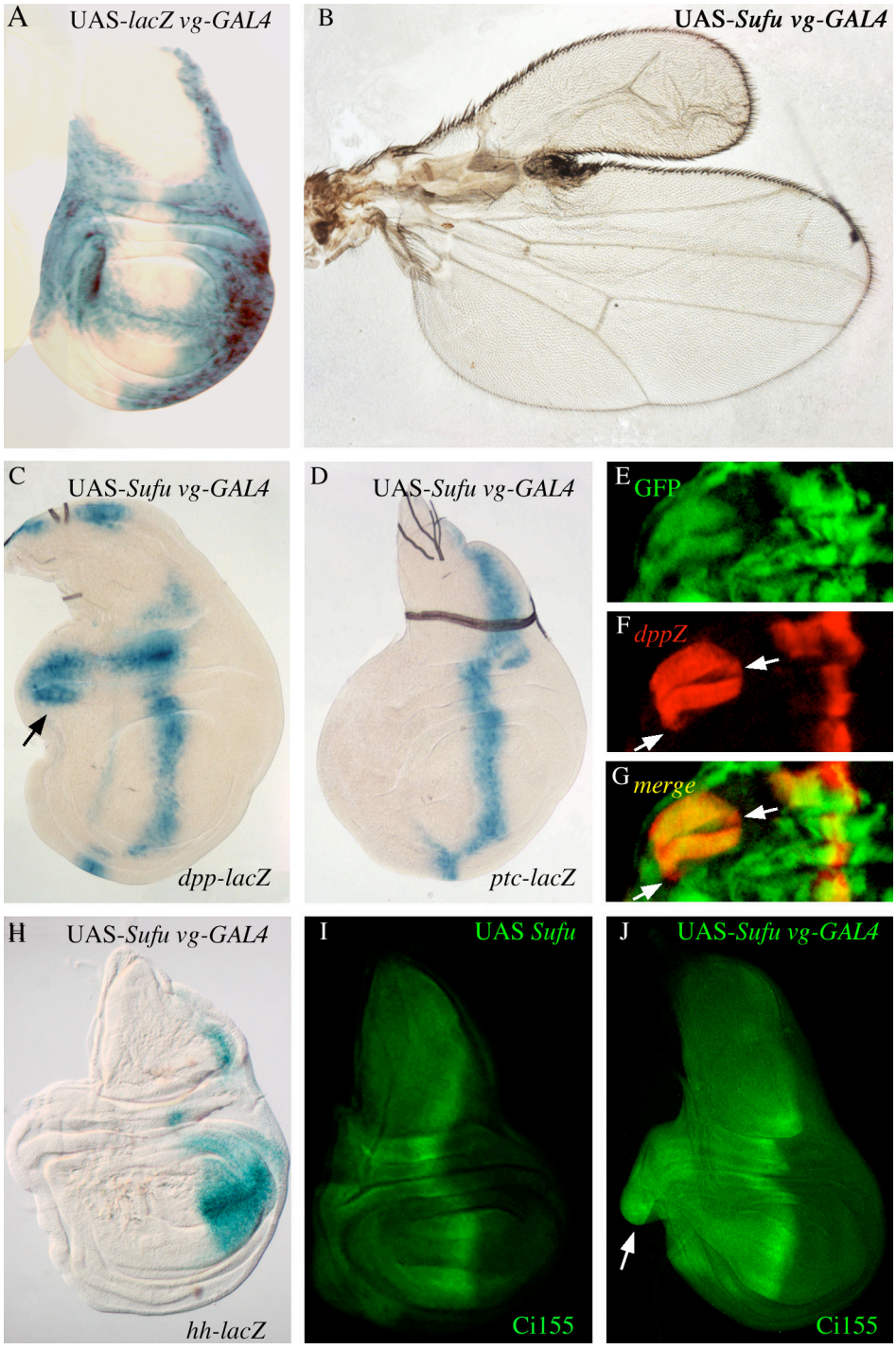


**Fig. 6:** Overexpression of *Su(fu)* outside the antero-posterior border in wing imaginal discs also drives ectopic activation of Hh target gene expression.
(A) Expression pattern of the *vg-GAL4* driver in *vg-GAL4 UAS-lacZ* imaginal wing disc. (B) wing of *UAS-Su(fu); vg-GAL4* fly raised at 25°C. (C, D) Expression of *dpp-lacZ* (C) and *ptc-lacZ* (D) in *UAS-Su(fu); vg-GAL4* discs raised at 25°C. *Su(fu)* overexpression in *vg-GAL4* domain leads to an anterior overgrowth and *dpp* anterior ectopic expression (arrow in C). (E-G) *Su(fu)* overexpression in clones generated in *UAS-Su(fu)/y w hs-flp; act5C>CD2>GAL4, UAS-GFP/ dpp-lacZ* flies. *GFP* (E) and *dpp-lacZ* (F) expressions are merged in G. *dpp-lacZ* expression is detected outside the *GFP*-expressing clones (arrows in F and G). (H) *hh-lacZ* expression in *UAS-Su(fu); vg-GAL4* discs. (I, J) $Ci^{155}$ localization in *UAS-Su(fu)* (I) and *UAS-Su(fu); vg-GAL4* (J) discs. In wild type discs, $Ci^{155}$ is detected in the anterior compartment along the antero-posterior border. When overexpressing *Su(fu)*, $Ci^{155}$ is also detected in the anterior outgrowth (arrow in J).

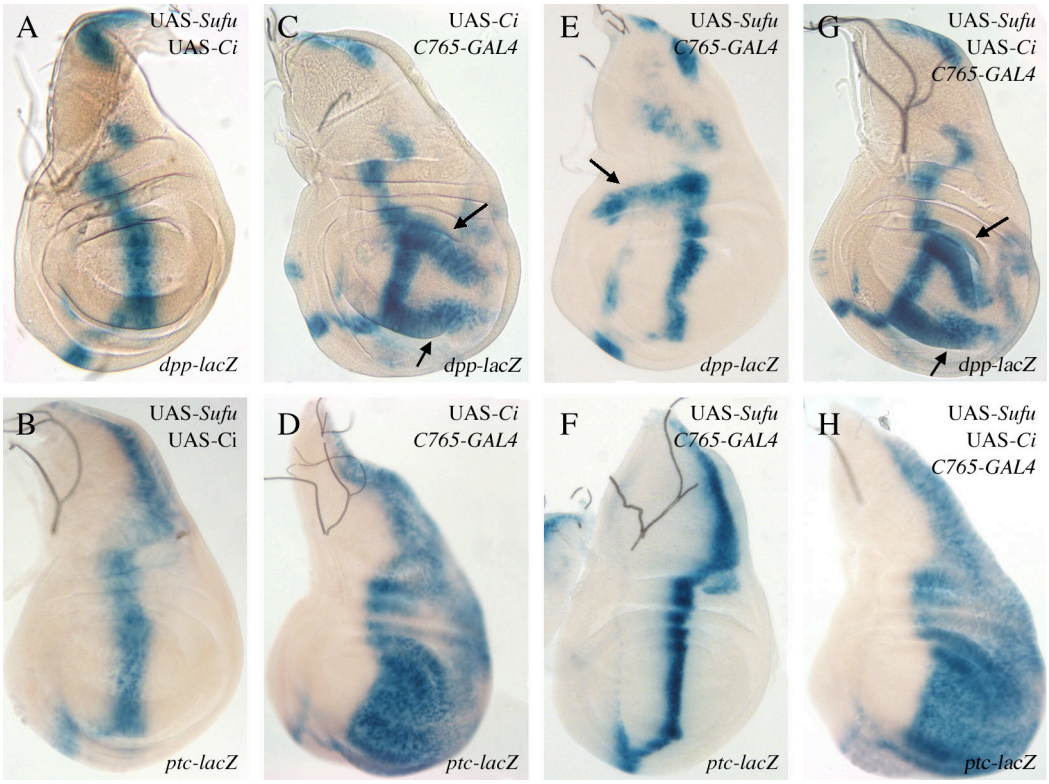


**Fig. 7:** Epistatic relationship between *ci* and *Su(fu)* overexpression effects.
Expression of *dpp-lacZ* (A, C, E, G) and *ptc-lacZ* (B, D, F, H), in *UAS-Su(fu); UAS-ci* control discs (A, B) and *UAS-ci; C765-GAL4* (C, D), *UAS-Su(fu); C765-GAL4* (E, F) and *UAS-Su(fu); UAS-ci; C765-GAL4* (G, H) wing discs, from flies raised at 25°C. Ubiquitous overexpression of *ci* under the *C765-GAL4* driver in the wing disc leads to two stripes of ectopic *dpp-lacZ* expression in the posterior compartment (arrows in C), and to ectopic *ptc-lacZ* expression in the whole posterior compartment (D). The overexpression of *Su(fu)*, with the same driver, leads to an anterior ectopic expression of *dpp-lacZ* (arrow in E), but not of *ptc-lacZ* (F). Co-over-expression of *ci* and *Su(fu)* gives patterns of *dpp-lacZ* and *ptc-lacZ* expression similar to those obtained overexpressing *ci* alone (compare G with C and H with D).

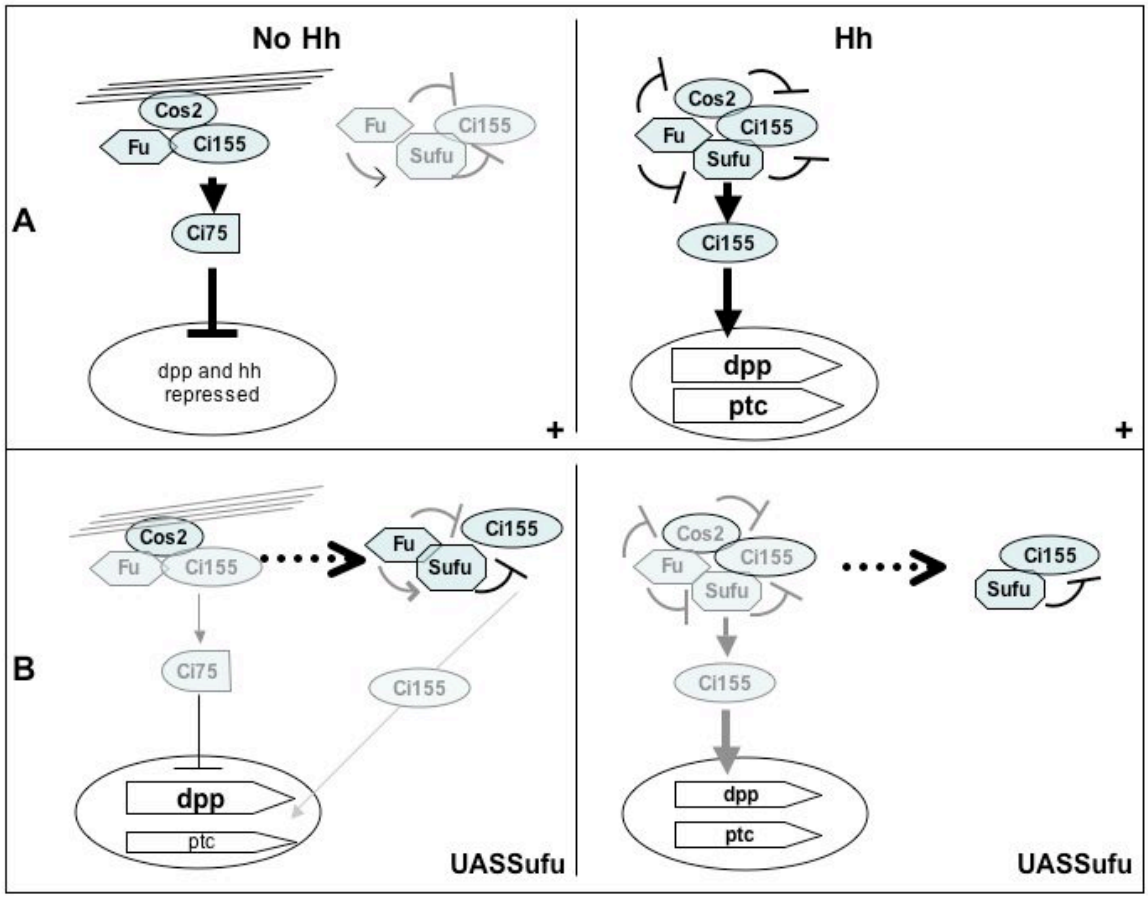


Fig. 8: Model for the action of Su(fu) in the wing imaginal disc. In this model, we propose that an equilibrium exists between different $Ci^{155}$-containing complexes and that Su(fu) is mainly involved in the cytoplasmic retention of $Ci^{155}$. In wild-type cells receiving the Hh signal (A-Hh), $Ci^{155}$ is mainly present in a cytoplasmic complex containing also Cos2, Su(fu) and Fu. In this complex, the Fu kinase activated in response to Hh exerts a negative effect upon Cos2 and Su(fu), thus allowing the release and activation of $Ci^{155}$. $Ci^{155}$ enters the nucleus where it activates the expression of *ptc* (high Hh level) and *dpp* (low Hh level). In *Su(fu)* overexpressing discs (B-Hh), the equilibrium is disrupted towards a $Ci^{155}$-Su(fu) complex in which Su(fu) exerts a major retention effect upon Ci, thus depleting the amount of $Ci^{155}$ available for activation. In discs co-overexpressing *Su(fu)* and *ci*, this effect is reversed as enough $Ci^{155}$ is available for it to enter the quadripartite complex and to be activated. In *fu* mutant discs overexpressing *Su(fu)*, Su(fu) and Cos2 negative effects on Ci in the quadripartite complex are no more antagonized by Fu, thus further reducing the activation of Ci. In wild-type anterior cells not receiving Hh (A-noHh), $Ci^{155}$ is mainly present in a microtubule bound complex which contains Cos2 and Fu but not Su(fu); $Ci^{155}$ retained in this complex is addressed to the proteasome and cleaved into $Ci^{75}$ which enters the nucleus where it represses the expression of *dpp* and *hh*. Su(fu) is present in a second cytoplasmic complex with Fu and a fraction of $Ci^{155}$. In Su(fu) over-expressing discs (B-noHh), the excess of Su(fu) disrupts the equilibrium towards the Su(fu)-$Ci^{155}$-Fu complex, thus depleting the quantity of $Ci^{155}$ available for cleavage. In this complex, we propose that Fu acts to reinforce the retention effect of Su(fu) upon $Ci^{155}$. This leads to a depletion in the amount of $Ci^{75}$ that accounts for the ectopic expression of *dpp*. In discs co-overexpressing *Su(fu)* and *ci*, this effect is reversed since enough $Ci^{155}$ is available for it to enter the microtubule bound complex necessary to address Ci for cleavage thus producing sufficient amount of $Ci^{75}$. In *fu* mutant discs over-expressing *Su(fu)*, the lack of Fu activity weakens the retention effect of Su(fu) upon $Ci^{155}$; free $Ci^{155}$ enters the nucleus where it activates strong ectopic *dpp* expression; the fact that strong ectopic *ptc* expression is also observed suggests that a high level of Ci activation can be reached.

Table 1: Effects of *Su(fu)* over-expression on the viability of wild type and *fu* mutant flies.

## Table 1: Effects of *Su(fu)* over-expression on the viability of wild type and *fu* mutant flies.

| | | 18°C | 21°C | 25°C | 29°C |
|---|---|---|---|---|---|
| (A) Females *UAS-Su(fu)* X Males *da-Gal4* | Adults | 277 | 140 | 0* | 0* |
| | Dead Pupae | 10 | 10 | 80 | 200 |
| (B) Females *FM3/w f fu$^{1}$, UAS-Su(fu)* X Males *w$^{1118}$* | Adults | 39 F*1/2B*<br>68 F<br>29 M *fu* | 41 F*1/2B*<br>38 F<br>17 M *fu* | 61 F*1/2B*<br>44 F<br>27 M *fu* | 51 F*1/2B*<br>48 F<br>3 M *fu* |
| | Dead Pupae | 0 | 0 | 0 | 41 |
| (C) Females *FM3/wf fu,$^{1}$ UAS-Su(fu)* X Males *da-Gal4* | Adults. | 48 F*1/2B*<br>51 F<br>4 M *fu* | 34 F*1/2B*<br>39 F<br>0 M *fu** | 60 F*1/2B*<br>4 F<br>0 M *fu* | 54 F*1/2B*<br>0 F<br>0 M *fu* |
| | Dead Pupae | 44 | 25 | 70 | 100 |
| (D) Females *FM3/w fu$^{A}$, UAS-Su(fu)* X Males *da-Gal4* | Adults. | 55 F*1/2B*<br>50 F<br>0 M *fu** | 51 F*1/2B*<br>61 F<br>0 M *fu** | 57 F*1/2B*<br>2 F<br>0 M *fu* | 58 F*1/2B*<br>0 F<br>0 M *fu* |
| | Dead Pupae | 23 | 50 | 80 | 100 |

The number of adults and dead pupae according to the temperature is given for each cross. *UAS-Su(fu); da-GAL4* flies did not hatch at 25°C nor 29°C dying as pharate adults (cross (A). The progeny of crosses (B), (C), (D), gives two kinds of fu$^{+}$ females: FM3/+ females of *1/2 B* phenotype (F*1/2B*) and *wf fu,$^{1}$ UAS-Su(fu)/+*, or *wfu$^{A}$, UAS-Su(fu/+* females (F) and one kind of *fu* mutant males: *w f fu$^{1}$, UAS-Su(fu)* or *wfu$^{A}$, UAS-Su(fu)* (M *fu*). Cross (B) is a control which shows the thermosensitivity of *fu$^{1}$* males which were almost completely lethal at 29°C, but consistently viable at 25°C, 21°C and 18°C; heterozygous *fu$^{+}$/fu$^{1}$* females were fully viable at all temperatures. Similar results were obtained with *fu$^{A}$* allele (data not shown). In crosses (C) and (D), *fu$^{1}$* or *fu$^{A}$* males overexpressing *Su(fu)* displayed a strongly reduced viability at 25°C, 21°C and 18°C, whereas their *fu$^{+}$/fu* heterozygous sisters overexpressing *Su(fu)* displayed a reduced viability at 25°C and 29°C but were fully viable at 21°C and 18°C.

*: rare escapers were obtained in similar crosses allowing wing observation at the temperatures indicated (see Figure 3B for cross (A) and Figure 3D for cross (D)). Legs could be observed at all temperatures by dissecting pharate adults.