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The tumor suppressor gene *fat* modulates the EGFR-mediated proliferation control in the imaginal tissues of *Drosophila melanogaster*

Flavio Garoia[a,*,1], Daniela Grifoni[b,1], Vincenzo Trotta[b], Daniela Guerra[b], Maria Cristina Pezzoli[b], Sandro Cavicchi[b]

[a]*Alma Mater Studiorum, Università di Bologna, CIRSA-GenMAP Lab, via S.Alberto 163, 48100 Ravenna, Italy*
[b]*Alma Mater Studiorum, Università di Bologna, Dipartimento di Biologia Evoluzionistica Sperimentale, via Selmi 3, 40126 Bologna, Italy*

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Abstract

Molecules involved in cell adhesion can regulate both early signal transduction events, triggered by soluble factors, and downstream events involved in cell cycle progression. Correct integration of these signals allows appropriate cellular growth, differentiation and ultimately tissue morphogenesis, but incorrect interpretation contributes to pathologies such as tumor growth. The Fat cadherin is a tumor suppressor protein required in *Drosophila* for epithelial morphogenesis, proliferation control and epithelial planar polarization, and its loss results in a hyperplastic growth of imaginal tissues. While several molecular events have been characterized through which *fat* participates in the establishment of the epithelial planar polarity, little is known about mechanisms underlying *fat*-mediated control of cell proliferation. Here we provide evidence that *fat* specifically cooperates with the epidermal growth factor receptor (EGFR) pathway in controlling cell proliferation in developing imaginal epithelia. Hyperplastic larval and adult *fat* structures indeed undergo an amazing, synergistic enlargement following to EGFR oversignalling. We further show that such a strong functional interaction occurs downstream of MAPK activation through the transcriptional regulation of genes involved in the EGFR nuclear signalling. Considering that *fat* mutation shows *di per se* a hyperplastic phenotype, we suggest a model in which *fat* acts in parallel to EGFR pathway in transducing different cell communication signals; furthermore its function is requested downstream of MAPK for a correct rendering of the growth signals converging to the epidermal growth factor receptor.

Keywords: *Drosophila*; *fat*; EGFR; Proliferation control; Transcription regulation; Tumor suppressor

1. Introduction

Cell–cell interaction involving adhesion molecules regulates many important developmental processes that lead to species-specific organ size and patterned cell proliferation. Many adhesion molecules have been found to be conserved between *Drosophila* and vertebrates, suggesting an early settlement long before the evolutionary divergence between arthropods and chordates (Hortsch and Goodman, 1991). Among these proteins, the cadherin family mediates calcium-dependent cell–cell adhesion regulating several morphogenetic processes (see Takeichi, 1995; Perez-Moreno et al., 2003 for review). Although classic cadherins are composed from 5 to 7 cadherin domains, some non-classic members of this family are huge proteins showing a large number of cadherin repeats. The *Drosophila fat* (*ft*) belongs to this latter category, indeed encodes for a transmembrane protein characterized by an extraordinarily long extracellular domain that shows a very strong homology with the human protocadherin hFAT (Dunne et al., 1995). Ft extracellular domain is composed of 34 cadherin domains, five EGF-like repeats and two laminin A motifs. It has a novel intracytoplasmic tail with a bipartite β-catenin binding domain, suggesting a possible role in the interaction with the cytoskeleton (Mahoney et al., 1991).

ft acts as a tumor suppressor gene, since its loss of function causes loss of proliferation control resulting in

* Corresponding author. Tel.: +39 0544 600390; fax: +39 0544 600411.
E-mail address: garoia@cib.unibo.it (F. Garoia).
[1] These authors contributed equally to this work.

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a hyperplastic growth of imaginal discs (Bryant et al., 1988; Mahoney et al., 1991). The study of cell behavior of lethal allelic conditions has been carried out in wing genetic mosaics. Mosaic patches showed autonomous overgrowth and enlarged territories, with more and smaller cells with respect to controls. Mosaic extra-growth was locally restricted to the clones that showed minor differentiation phenotypes, reflecting failure in cell adhesiveness and cell recognition (Garoia et al., 2000). Recently the *ft* gene has been extensively studied for its role in establishment of planar cell polarity (Yang et al., 2002; Rawls et al., 2002; Fanto et al., 2003). However, its role in the control of cell proliferation remains poorly understood.

Particularly interesting is the evidence that *ft* interacts with components of the EGFR pathway in *Drosophila* (Garoia et al., 2000), thus suggesting a possible role in growth factors-mediated cell cycle events. The EGFR signalling cascade plays a central role in different processes in *Drosophila*, such as differentiation, proliferation control and regulation of events that shape the adult structures (reviewed in Schweitzer and Shilo, 1997; Rebay, 2002; Shilo, 2003). The Drosophila EGFR pathway plays a pivotal role in the wing and eye imaginal discs growth (Diaz-Benjumea and Hafen, 1994; Simcox, 1997; Baker and Yu, 2001; Baonza et al., 2002). Its role in proliferation control has been verified by experiments in the imaginal discs, where the activation of Ras1 GTPase, a component of the signalling transduction pathway of EGFR, provokes a dramatic enhancement of the proliferation rate (Karim and Rubin, 1998).

The results presented in this paper demonstrate a strong functional interaction between *ft* and the EGFR pathway in the proliferation control. Since *ft* mutations show *di per se* a hyperplastic phenotype, we suggest a model in which Ft acts in parallel to EGFR pathway in transducing different cell communication signals and downstream of MAPK for the growth signals converging on the EGFR to be correctly interpreted. This function is exerted, at least in part, through the transcriptional regulation of genes involved in the EGFR nuclear signalling.

2. Results

2.1. EGFR signalling modulates ft mutant phenotypes in mosaics

We induced ft^{18} mitotic clones in a background in which EGFR signalling was enhanced using the MS1096-GAL4 driver (expressed in the wing pouch, Capdevila and Guerrero, 1994) in combination with UAS-*rho* and UAS-raf^{GOF}. The results showed that clones induced at 72 ± 2 h were significantly larger (two tailed student *t*-test $P < 0.01$ in both backgrounds) than ft^{18} clones in wild-type (Fig. 1A,G,H), otherwise clones induced in an EGFR reduced background (UAS-raf^{DN}) were significantly smaller ($P < 0.05$), showing a proliferation rate similar to wild type cells (Fig. 1A); interestingly the size of *ft* clones induced in a MS1096-GAL4/UAS raf^{GOF} background were three times larger than those induced in a wild type background even if the wing was severely reduced (Fig. 1D). The number of *ft* clones evaginating from the wing surface as a result of overproliferation was higher in clones induced in UAS-*rho* and UAS-raf^{GOF} backgrounds, where we found respectively the 77% ($n = 56$) and 65% ($n = 37$) of overgrown clones with respect to the 6% ($n = 50$) observed in wild type background. Conversely in a UAS-raf^{DN} background we found no overgrowth in the 40 analyzed clones. To verify if the increased proliferation advantage of *ft* cells in the presence of activated EGFR signal may result from the reduced competition of the background mutant cells, we induced wild type clones in the same backgrounds. The result showed that the proliferation rate of the wild type cells did not significantly differ from that observed in wild type background (Fig. 1A,I), confirming that the increased proliferation of *ft* cells was not the result of a simple additive effect.

With the aim to analyze the allocation of *ft* clones along the wing we induced earlier clones (60 ± 12 h) using the same GAL4 driver in combination with the above mentioned UAS lines and, in addition, in combination with a UAS line expressing a dominant negative form of the EGFR receptor (UAS-$EGFR^{DN}$). While *ft* clones in wild type background grow preferentially in the proximal regions of the wing (Fig. 1B), when we induced an EGFR oversignalling with UAS-raf^{GOF} and UAS-*rho* (Fig. 1D,F) the clones showed an almost homogeneous distribution along the wing blade. Interestingly the pattern of clone allocation is very similar to that observed in wing clones induced in viable loss of function double mutant *vein veinlet* (*vn, ve*) wings (Garoia et al., 2000), whereas in UAS-raf^{DN} and UAS-$EGFR^{DN}$ background the distalization of clones is less marked (Fig. 1C,E). This discrepancy in the clone distribution with respect to the *vn, ve* wings is probably a result of the weaker suppression of the EGFR activity by the transgenes utilized, also resulting in a very partial inhibition of vein differentiation observed in these wings.

In all the analyzed combinations we found that polarity defects previously observed in *ft* clones induced in a wild-type background (Garoia et al., 2000) were not modified (Fig. 1G,H).

2.2. ft interacts with EGFR in the imaginal cells proliferation control

Using the *dpp*-GAL4 driver we induced the expression of UAS-*rho*, UAS-raf^{GOF} and UAS-raf^{DN} in $ft^{G\text{-}rv}$ mutant eye and wing imaginal discs. The results showed that overexpression of UAS-*rho* induced a significant enhancement of overgrowth in the *dpp* expression territory (Fig. 2H,T), whereas overexpression of UAS-raf^{GOF} induced a strong

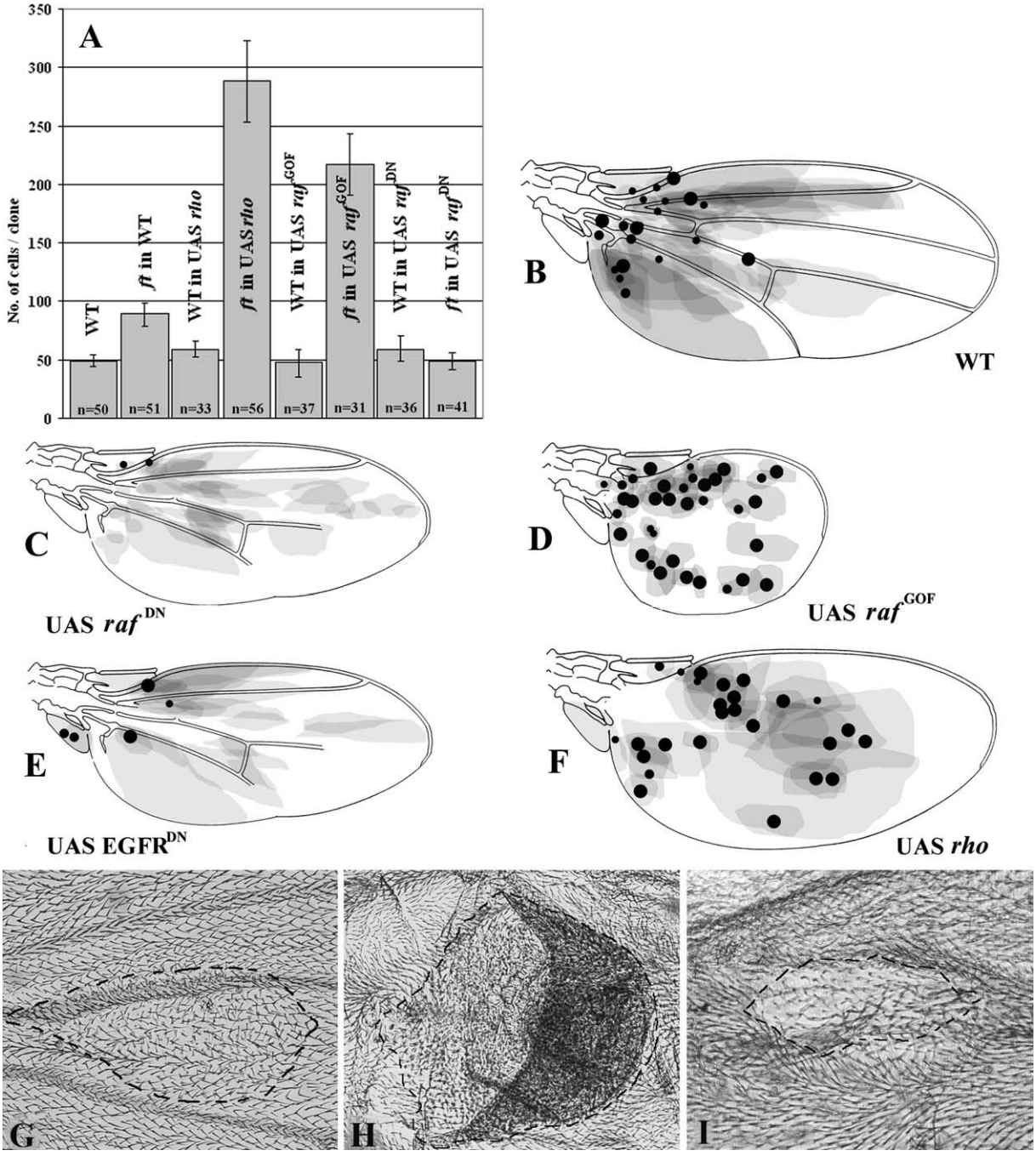
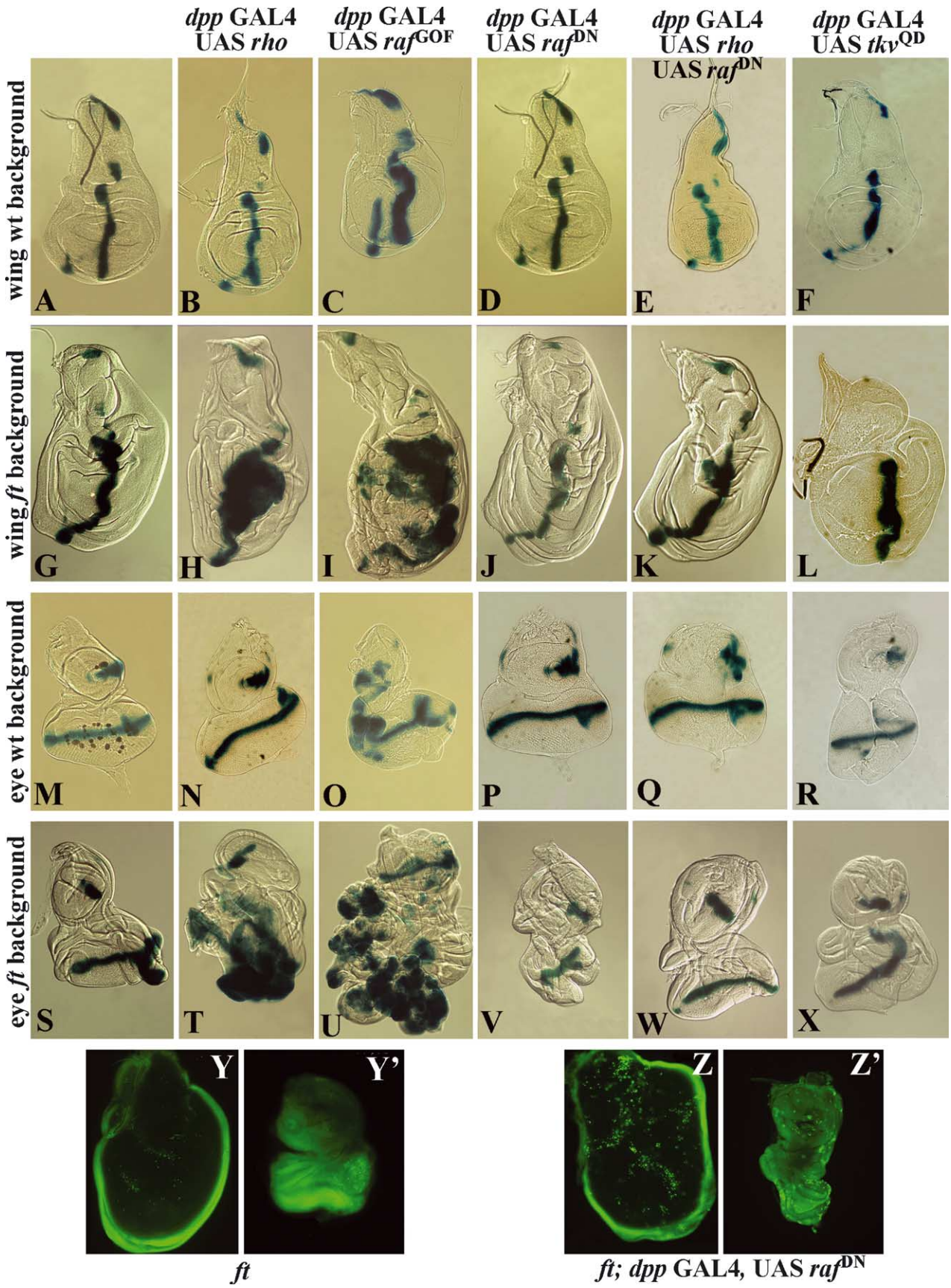


Fig. 1. Clonal analysis of *ft* clones in EGFR mutant backgrounds. (A) Comparison of the number of *ft* mutant cells in late induced clones (70–74 h AEL). (B–F) Plots of early induced ft^{18} clones (48–72 h AEL) in wild type background (B), and in EGFR mutant backgrounds, respectively: (C) MS1096-GAL4; UAS raf^{DN}; (D) MS1096-GAL4; UAS raf^{GOF}; (E) MS1096-GAL4; UAS $EGFR^{DN}$ and (F) MS1096-GAL4; UAS *rho*. The dots indicate the position of *ft* clones causing tissue outgrowth in the wing surface. (G,H) Phenotypes of late ft^{18} clones (outlined profiles) in wild type (G) and MS1096-GAL4; UAS *rho* background (H). Polarity defects and enlargement of vein are visible both in the small clone induced in a wild type background (G) and in UAS *rho* background (H), also if in UAS *rho* background the proliferation of *ft* mutant cells is dramatically increased and causes outgrowth from the wing surface (H). Wild type clones induced in the same background did not show proliferation advantage (I).

enhancement of growth of the whole disc with dramatic misexpression of the *dpp*-lacZ reporter (Fig. 2I,U, note the different magnification of the images). The overexpression of UAS-raf^{DN} led to a significant reduction of the whole eye disc whereas no effects were observed in the wing disc (Fig. 2J,V). To address whether this reduction could result from cell death, we stained $ft^{G\text{-}rv}$, UAS-raf^{DN} imaginal discs with acridine orange. We observed an increase of cell death in the whole eye and wing discs (Fig. 2Z,Z′) with respect to that observed in $ft^{G\text{-}rv}$ control tissues (Fig. 2Y,Y′). However, when the acridine staining was performed in the eye and wing discs of the other $ft^{G\text{-}rv}$/UAS combinations, the same


dpp GAL4 UAS rho
dpp GAL4 UAS rafGOF
dpp GAL4 UAS rafDN
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dpp GAL4 UAS tkvQD
wing wt background
wing ft background
eye wt background
eye ft background
A
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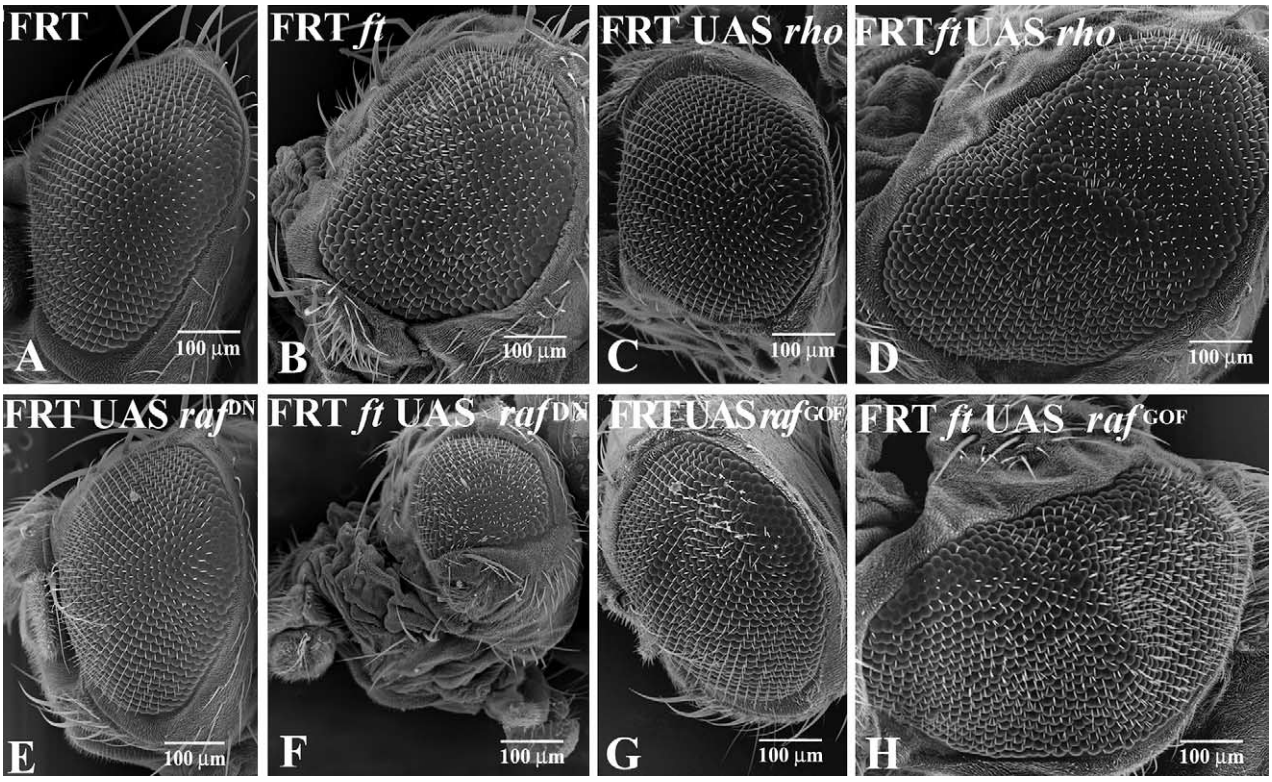

Fig. 3. Ectopic expression of EGFR pathway genes in *ft* mutant eyes obtained using the EGUF system. The genotypes are as follows: (A) FRT40A/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/+; (B) FRT40A, $ft^{G\text{-}rv}$/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/+; (C) FRT40A/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/UAS *rho*; (D) FRT40A, $ft^{G\text{-}rv}$/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/UAS *rho*; (E) FRT40A/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/UAS raf^{DN}; (F) FRT40A, $ft^{G\text{-}rv}$/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/UAS raf^{DN}; (G) FRT40A/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/UAS raf^{GOF}; (H) FRT40A, $ft^{G\text{-}rv}$/FRT 40A, GMR-*hid*, 2L CL2L; EGUF/UAS raf^{GOF}.

increase in non-autonomous cell death was observed (data not shown).

To understand if the different effects on proliferation enhancement were mediated from different effectors or if *rho*-induced overproliferation required the Ras/Raf signalling cascade, we co-expressed UAS-*rho* and UAS-raf^{DN} constructs. The *dpp* presumptive expression territory in both wing and eye discs appeared to be similar to *ft* control when UAS-*rho* and UAS-raf^{DN} were simultaneously expressed under the control of *dpp*-Gal4 driver (Fig. 2K,W), indicating that Raf activity is required even for *rho* induced proliferation enhancement.

When UAS-*rho*, UAS-raf^{GOF} and UAS-raf^{DN} were overexpressed in a wild-type background (Fig. 2A–E,M–Q) no effect on disc growth or misexpression of the *dpp* transgene was seen, even if the *dpp*-GAL4; UAS-raf^{GOF} occasionally showed a little increase of the *dpp* expression territory (Fig. 2O).

2.3. ft modulates the EGFR morphogenetic signals during the eye formation

To examine thoroughly the relationship between EGFR and *ft* in proliferation control we used the EGUF system for producing flies in which the eye is composed exclusively of $ft^{G\text{-}rv}$ homozygous cells (Stowers and Schwarz, 1999). This method uses the *ey*-GAL4 driver to induce UAS-FLP expression and FRT-mediated recombination in the presumptive eye territory. We took advantage of this system to induce simultaneously mitotic recombination and overexpression of UAS-*rho*, UAS-raf^{GOF} and UAS-raf^{DN} transgenes. The results showed that *ft* eye is slightly larger than wild-type eye and shows duplicated bristle cells and severe morphological defects (Fig. 3A,B). When we induced overexpression of UAS-*rho* or UAS-raf^{GOF} in a *ft* background the eye resulted significantly enlarged (Fig. 3D,H), whereas UAS-raf^{DN} transgene severely

Fig. 2. Ectopic expression of the EGFR pathway genes in *ft* imaginal discs. X-gal staining to detect *dpp* expression from the BS3.0 transgene in wing (A–L) and eye (M–X) discs. (A,M) wild type; (G,S) $ft^{G\text{-}rv}$; (B,N) *dpp*-GAL4/UAS *rho*; (H,T) $ft^{G\text{-}rv}$; *dpp*-GAL4/UAS *rho*; (C,O) *dpp*-GAL4/UAS raf^{GOF}; (I,U) $ft^{G\text{-}rv}$, *dpp*-GAL4/UAS raf^{GOF}; (D,P) *dpp*-GAL4/UAS raf^{DN} (J,V) $ft^{G\text{-}rv}$, *dpp*-GAL4/UAS raf^{DN}; (E,Q) *dpp*-GAL4/UAS raf^{DN}, UAS *rho*; (K,W) $ft^{G\text{-}rv}$, *dpp*-GAL4/UAS raf^{DN}, UAS *rho*; (F,R) *dpp*-GAL4/UAS tkv^{QD}; (L,X) $ft^{G\text{-}rv}$; *dpp*-GAL4/UAS tkv^{QD}. Acridine orange staining reveals apoptosis in $ft^{G\text{-}rv}$ wing and eye discs respectively (Y,Y′) that is increased in a non-autonomous manner in $ft^{G\text{-}rv}$, *dpp*-GAL4/UAS raf^{DN} background (Z,Z′). All the discs are displayed at 200× magnification except for I and U that are shown at 100×.

reduced the ommatidial number (Fig. 3F). Even in these strongly reduced eyes, however, we found bristle duplication, suggesting that, as previously observed in wing clone phenotypes, the defects observed in *ft* mutants are not dependent from the EGFR signalling. No significant morphogenetic defects were indeed found when UAS-*rho*, UAS-*raf*^{DN} and UAS-*raf*^{GOF} were overexpressed in the wild type eye using the same GAL4 line (Fig. 3C,E,G).

2.4. EGFR activity is unaffected in ft mutant cells

To investigate the effect of *ft* mutation in EGFR signalling we induced null *ft*$^{G\text{-}rv}$ mitotic clones in wing and eye discs. In the third instar wing and eye imaginal discs, the distribution of the activated form of MAPK detected with antibodies that recognize the phosphorylated Rolled protein (anti-dpERK), reflects the activity of EGFR via the Ras/Raf signalling cascade (Gabay et al., 1997). The results showed that there is no significant increase or misexpression of EGFR signalling in the clones, whereas morphological defects due to overproliferation were observed (Fig. 4A–F). This result is not amazing since *ft* clones did not show the differentiation defects associated with MAPK activation (Martin-Blanco et al., 1999; Prober and Edgar, 2000).

2.5. ft modulates the transcription of genes involved in EGFR signalling

The EGFR pathway controls cell proliferation and growth in different ways. In the wing disc EGFR increases Dmyc protein levels via the Ras/Raf signalling cascade, that promotes G1–S transition by increasing levels of CycE protein (Prober and Edgar, 2000, 2002). In the eye disc EGFR activity phosphorylates a splice variant of Pointed (Pnt) ETS domain transcriptional activator (PntP2), that triggers G2–M transition increasing *stg* transcription (Baonza et al., 2002). EGFR activity is also involved in the degradation of *yan* transcriptional repressor, which functions as a fairly general inhibitor of differentiation (Lai et al., 1997). To better understand the interaction between *ft* and EGFR in the control of cell proliferation we studied the transcriptional profile of the above mentioned genes in *ft*$^{G\text{-}rv}$ mutant discs using semi-quantitative RT-PCR (Fig. 5A). The results showed that in both wing and eye discs the failure of *ft* function enhanced the transcription of two genes

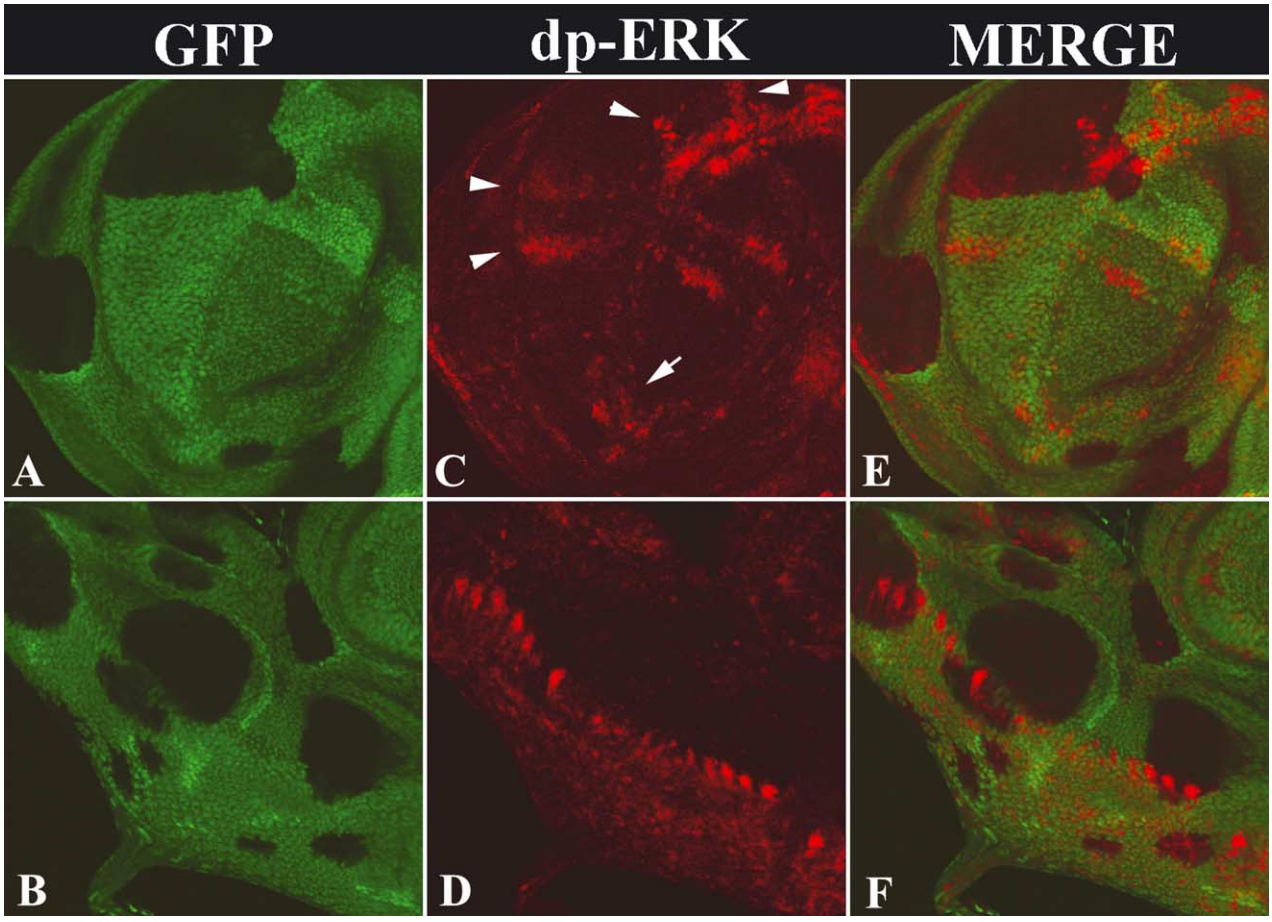

Fig. 4. The EGFR activity is not affected in *ft* clones. *ft* GFP^{-} clones in imaginal wing (A) and eye (B) discs. The EGFR activity visualized with the anti-dpERK antibody is normally displayed in the wing presumptive margin (C, arrow) and veins (C, arrowheads), as well in the eye disc (D). No alterations in the anti-dpERK staining are visible in *ft* mutant territories (E,F).

involved in EGFR signalling (*yan* and *dmyc*), whereas *pnt* transcription was unaffected.

The findings concerning *dmyc* are particularly interesting, as this gene acts as a major regulator of cell growth in the *Drosophila* imaginal tissues (Prober and Edgar, 2000, 2002). Since *dmyc* is involved in the G1–S cell cycle transition in the wing disc via the increase of CycE protein (Prober and Edgar, 2000), we used fluorescence-activated cell-sorting analysis (FACS) to study the cell cycle profile in *ft* wing mutant discs. The results showed that the distributions of cell cycle phases and cell size in *ft* mutant discs were undistinguishable from wild type (Fig. 5B,C). This result appears to be in contradiction with previous observations (Garoia et al., 2000) indicating that cell size in *ft* mutant clones was smaller than wild type, however, the measure of cell size in *ft* wing discs obtained from confocal sections resulted rather similar to wild type (Garoia et al., 2000), despite the strong reduction observed in adult wings.

To corroborate the possible role of *dmyc* activity in *ft* proliferation control we induced ft^{18} wing clones in *dmyc* hypomorphic background (using the dm^{P0} viable allele), in which the autonomous extra cell proliferation could be quantitatively ascertained using a twin test. In this test *ft* cells are labelled with *f* and the twin control (generated from the same recombination event) with *ck*. The result showed that *ft* clones induced at 72 ± 2 h in this background were severely reduced respect to those induced in wild type background (Fig. 6A), in a *f*/*ck* ratio of 1.5 ± 0.2, significantly lower than *f*/*ck* ratio induced in wild type wings (6.6 ± 0.9). Furthermore the allocation of *ft* clones in dm^{P0} background was almost uniform along the wing blade (Fig. 6E), similarly to *ck* control clones (Fig. 6B). These results confirm that *dmyc* activity is involved in the generation of extra-growth advantage of *ft* mutant cells.

The role of *ft* in the control of the imaginal disc growth is dependent on its interaction with the EGFR pathway, since no interaction was found with mutants involved in *Notch* and *wingless* signalling pathways (Garoia et al., 2000). However, there are observations that Dpp-signalling may affect cell proliferation by stabilizing Dmyc protein levels (see Prober and Edgar, 2002). To verify a possible synergistic effect we first induced the expression of a constitutively activated form of the *dpp*-receptor *thickveins* (UAS-tkv^{QD}), using the *dpp*-GAL4 driver in $ft^{G\text{-}rv}$ mutant eye and wing imaginal discs (Fig. 2L,X). The *dpp* presumptive expression territory in those discs did not result enlarged respect to the *ft* control discs (Fig. 2G,S). Furthermore, a twin test was carried out inducing ft^{18} wing clones in *tkv* hypomorphic background using the tkv^{1} viable allele. Even in this case the size (Fig. 6A) and the allocation of the clones (Fig. 6D) were not modified respect to those induced in wild type background (Fig. 6C), suggesting that *dpp*-signalling is not involved in *ft*-mediated control of proliferation.

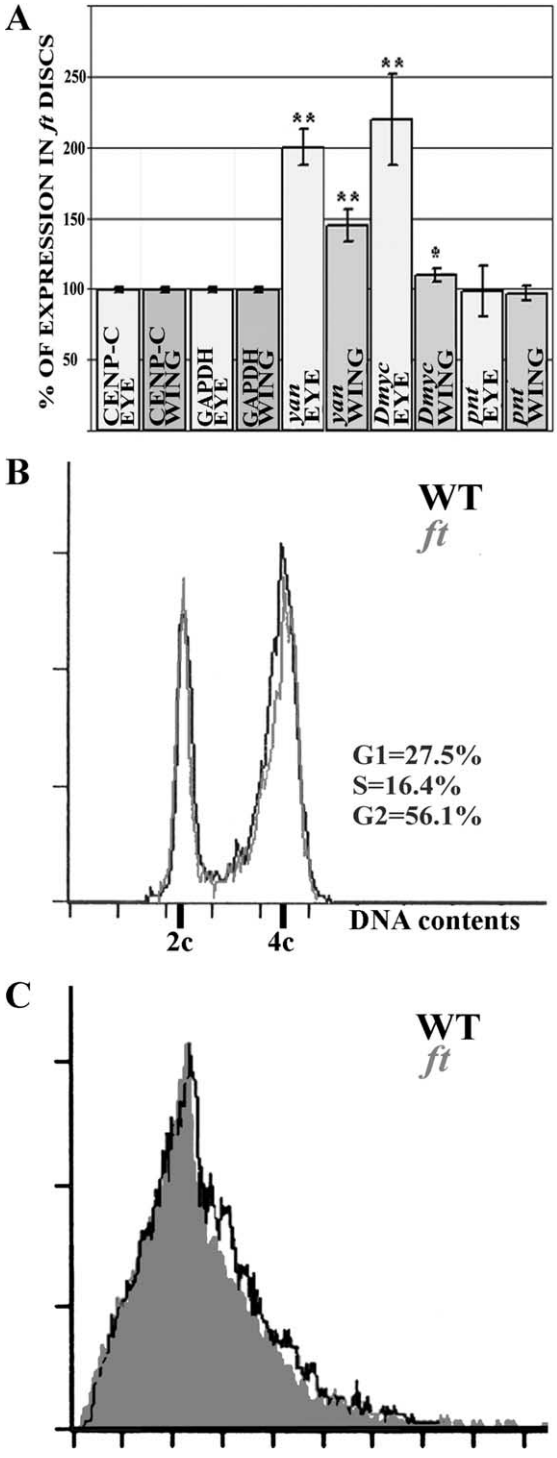

Fig. 5. Effect of the *ft* mutation on transcription and cell cycle. (A) Percentage of expression of *yan*, *Dmyc* and *pnt* genes in *ft* mutant eye and wing discs respect to wild type. To test the reliability of the RT-PCR we used two different controls: an internal control (GAPDH expression) that allows to assess the effect of metabolic status on transcription levels in discs of different sizes, and an external control (CENP-C, see experimental procedures) to assess the efficiency of the reverse transcriptase reaction. Significant differences respect to CENP-C expression are denoted as follows: $*P<0.05$, $**P<0.01$. FACS analysis shows that cell cycle phasing (B) and cell size (C) are unaffected in 120 h AEL *ft* wing discs (gray) respect to wild type (black).

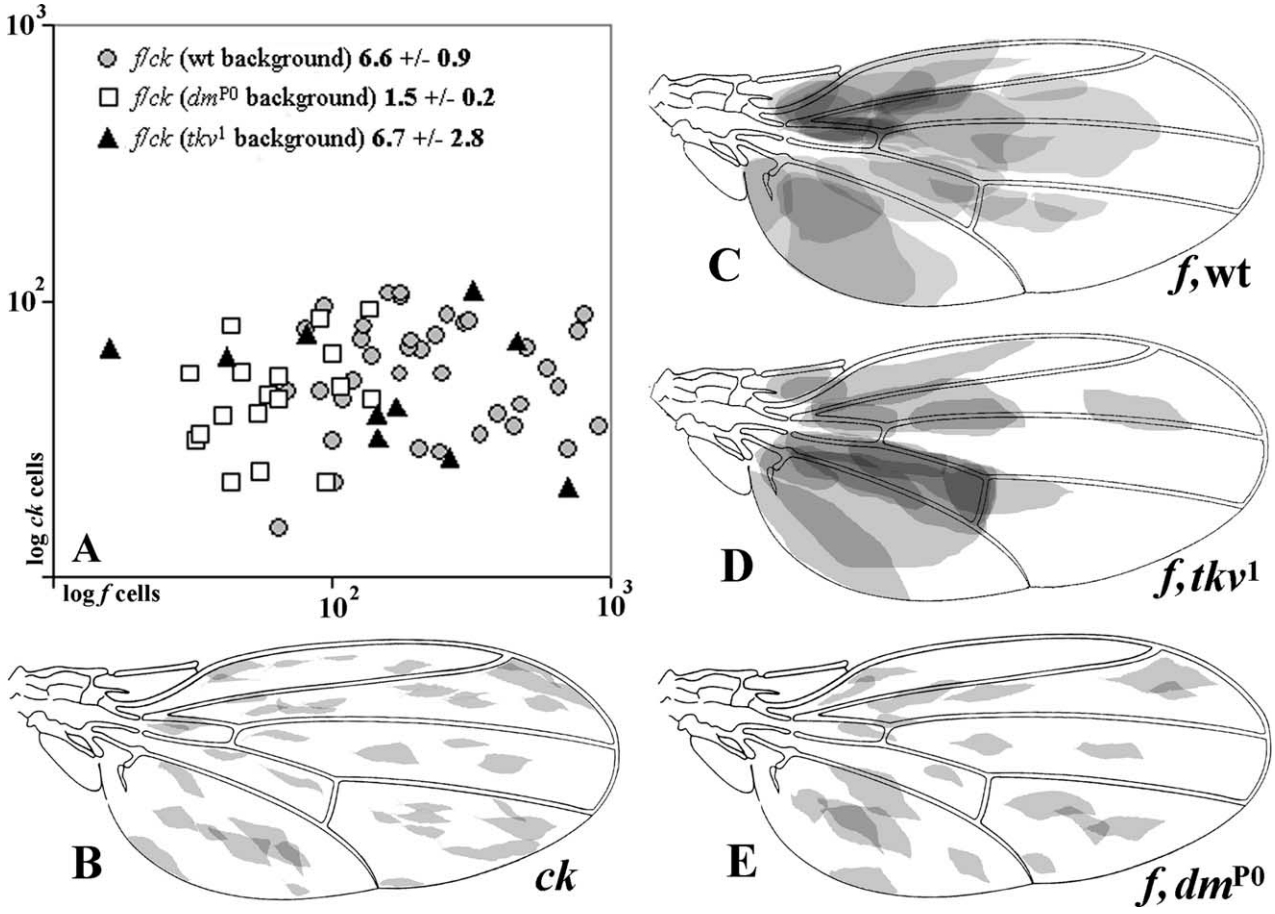

Fig. 6. Twin analysis of *ft* clones in hypomorphic backgrounds. (A) Comparison of the number of cells (in logarithmic scale) of twin clones and *f/ck* average ratio in wild type, dm^{P0} and tkv^{1} backgrounds. (B–E) Plots of *ft* clones in different mutant backgrounds. (B) *ck* twin control clones; forked *ft* in (C) wild type, (D) tkv^{1} and (E) dm^{P0} backgrounds. *ft* clones in wild type (C) and tkv^{1} (D) backgrounds occupy more frequently proximal positions in the wing blade, whereas in dm^{P0} background (E) the clones are smaller and distalize similarly to *ck* control clones.

3. Discussion

The tumor suppressor gene *fat (ft)* controls cell proliferation and polarity, according to the hyperplastic growth and loss of polarity of tissues lacking its function (Bryant et al., 1988; Mahoney et al., 1991; Garoia et al., 2000; Yang et al., 2002; Rawls et al., 2002; Fanto et al., 2003). The hyperplastic growth of *ft* imaginal tissues is allometric, resulting in regularly shaped organs that stop growing when a critical mass is reached. This allometric growth was confirmed by the evidence that the expression profile of genes with territorial specificity *(ap, en, dpp* and *wg)* does not show relevant variations in mutant imaginal discs (Garoia et al., 2000).

The experiments described in this paper verified and characterized the genetic interactions between *ft* and genes of the EGFR pathway. This signalling cascade plays a pivotal role in different processes in *Drosophila*, such as proliferation control and regulation of events that shape the adult structures (Diaz-Benjumea and Hafen, 1994). Its role in proliferation control results evident from experiments in the wing imaginal disc, where activation of *ras*1 provokes a dramatic enhancement of the proliferation rate (Karim and Rubin, 1998). The role of the EGFR signalling pathway, however, is not restricted to the control of local cell proliferation; its cascade is also involved in determining the positional values which in turn regulate the final size of the wing (Diaz-Benjumea and Garcia-Bellido, 1990).

3.1. ft interacts with the EGFR signalling pathway in the modulation of proliferation signals

Our results support the hypothesis of a relevant functional interaction between *ft* and genes of the EGFR pathway. When EGFR signalling is raised in fact, an amazing, non-additive increase in *ft*-induced proliferation is observed. *ft* clones in UAS-*rho* or UAS-raf^{GOF} wings where an EGFR oversignalling is induced show the same distinctive features of those induced in a wild-type background (tissue hyperplasia, reduced cell size, loss of planar polarity), with a phenotype much more severe concerning outgrowth number and dimensions. On the contrary, in experiments where the EGFR signalling was reduced, *ft*-induced hyperproliferation resulted partially suppressed.

The same trend was observed in the adult eye; the *ft* head-capsule where *rho* or raf^{GOF} were ectopically expressed was particularly enlarged, whereas resulted reduced with the ectopic expression of raf^{DN}.

The most dramatic effects were however observed in the eye and wing imaginal discs where the EGFR signalling was increased in the presumptive *dpp* expression domains. The controls did not show significant phenotypes; conversely, in *ft* discs we observed severe effects, including non-autonomous aberrations in the disc morphology. In the *ft* raf^{DN} eye disc we observed a strong non-autonomous reduction of the eye presumptive territory that may be caused by decrease of proliferation rate and/or by increase of cell death. Non-autonomous cell death may result from cell competition (Morata and Ripoll, 1975), ectopic cell proliferation or experimentally induced apoptosis (Milan et al., 1997). An increase in non-autonomous cell death was observed not only in *ft* raf^{DN} discs but in all the *ft*/UAS combinations analyzed. Thus, cell death does not seem to be the mechanism through which the growth deficit occurs in the *ft* raf^{DN} eye disc, suggesting a more complex interaction between *ft* and EGFR activity in the modulation of eye disc growth.

ft hence interacts with the EGFR pathway modulating its proliferative signal; *ft* function would so be involved in growth regulation, allowing the structures to correctly interpret signals incoming from EGFR that are essential for eye and wing to acquire the final shape. No interaction was found with mutants involved in pathways other than the EGFR cascade (*N, dpp* and *wg*; Garoia et al., 2000; present paper), making the role of *ft* in the control of the imaginal disc growth specifically dependent on its interaction with the EGFR pathway.

3.2. *ft* interacts with the EGFR pathway in the wing shape determination

The proximal *ft* clone allocation along the wing blade arises from an alteration in the growth direction and not from a different viability of the cells relative to their layout in the proximo-distal axis (Garoia et al., 2000); it is then obvious that *ft* cells show a greater "affinity" for the proximal region of the wing. The *ft* mutant phenotype seems anyway to be influenced from EGFR signalling also concerning the proximalization of the clones; their distribution results indeed more homogeneous along the wing blade if the EGFR signalling is altered. It is interesting to notice that an EGFR signalling reduction or increase produces, in this case, the same biological effect, while the proliferative phenotype is directly correlated to the activity of the EGFR effectors. The activity of the EGFR cascade is spatially and temporally modulated during development and in the wing disc it is gathered in the hinge and vein presumptive regions (Martin-Blanco et al., 1999; Zecca and Struhl, 2002). Even if there are no evidences that the EGFR signalling plays a role in the P/D patterning of the wing blade, recent studies showed that a gradient of EGFR activity is required for the correct P/D development of the leg (Galindo et al., 2002). In our experimental conditions the MS1096-GAL4 driver creates an almost homogeneous EGFR signal along the wing blade, so determining a *quasi* wild-type distribution of *ft* clones. This role in the modulation of the differential distribution of *ft* mutant cells along the P/D wing axis suggests for the EGFR pathway an involvement in the morphogenetic events that control the final shape of the wing.

3.3. *ft* regulates the transcription of genes involved in the EGFR signalling

The *ft*-induced hyperplasia is associated with an abnormal pattern of gene expression, as visualized in two-dimensional protein gels of *ft* mutant imaginal discs (Santarén et al., 1998). Although we had no data to hypothesize a cytoplasmic interaction between *ft* and the EGFR signalling, we could not exclude an alteration in MAPK expression. The *ft* mutant phenotypes, however, do not include the differentiation defects typical of the EGFR pathway genes, whose activity is indeed not altered since we did not detect significant modifications of the activated dpERK levels or patterns in the *ft* tissues with respect to the wild-type.

The results shown in this paper suggest that the interaction between *ft* and EGFR takes place at the proliferation level, while differentiation signals controlled by the EGFR pathway appear unaffected. With the aim to find some mechanisms that could explain the synergic phenotype of *ft* and EGFR mutations, we studied in *ft* and wild-type imaginal tissues the transcriptional levels of *yan*, *dmyc* and *pnt*, genes involved in proliferation control whose function is regulated by the EGFR cascade. The results of semi-quantitative RT-PCR trials showed in *ft* tissues an increase of the transcription levels of *yan* and *dmyc*, whereas *pnt* was unaffected. The Dmyc transcription factor, the unique *Drosophila* homologue of the Myc family of proto-oncogenes, plays a central role in the control of cell growth in *Drosophila* (Johnston et al., 1999). Overexpression of *ras* is capable to increase post-transcriptionally the Dmyc protein levels, promoting the G1–S transition via the increase of CycE translation (Prober and Edgar, 2000). The increase in the Dmyc levels, however, affects growth rate but not proliferation, since the shortening of the G1 phase is balanced by the compensatory lengthening of G2, resulting in an increase in cell size but not in cell number (Johnston et al., 1999). *ft* mutation otherwise induced an increase of cell proliferation without altering the cell size. Taken together, these results indicate that *ft* mutation affects not only the G1–S transition via *Dmyc* but also the G2–M transition, since the coordinated stimulation of the two cell-cycle checkpoints is necessary to increase the proliferation rate in *Drosophila* imaginal discs (Neufeld et al., 1998). Interestingly, the transcription level of *pnt* was unaffected in

ft mutant discs. *pnt* is an ETS transcriptional activator that plays a central role in the mitosis control mediated by the EGFR signalling cascade (Baonza et al., 2002); several data however suggest the presence of additional Pnt-independent effectors in the EGFR-mediated mitosis control (Yang and Baker, 2003). The *ft* control of the G2–M transition may involve EGFR effectors other than *pnt*, or molecules functioning through different signalling pathways. The *yan* gene is another component of the ETS transcriptional regulator family involved in the EGFR signalling. Phosphorylation by MAPK affects stability and subcellular localization of Yan, resulting in a rapid down-regulation of its activity (Rebay and Rubin, 1995). Yan functions as a fairly general inhibitor of differentiation, allowing both neuronal and non-neuronal cell types to choose between cell division and differentiation in multiple developmental contexts (Rogge et al., 1995) and recent studies indicate that the mammalian homologue of the *Drosophila yan*, TEL, is overexpressed in tumors (Galang et al., 2004). In the *Drosophila* developing eye *yan* is expressed in all undifferentiated cells and is down regulated as cells differentiate (Rebay and Rubin, 1995), so a high *yan* activity in *ft* mutant discs is correlatable with the observed proliferative advantage of *ft* cells.

There are several indications that EGFR signalling can trigger different responses by different activity levels: in the *Drosophila* eye disc, differentiation requires high signalling levels, whereas lesser EGFR activity promotes mitosis and protects against cell death (Baker and Yu, 2001; Halfar et al., 2001). These findings indicate that EGFR signalling may coordinate partially independent processes, transferring graded activity to the nucleus, rather than triggering 'all or none' responses (Yang and Baker, 2003). The simultaneous increase of activity in both growth promoters (*dmyc*) and differentiation repressors (*yan*) in *ft* mutant imaginal discs suggests the presence of a mechanism that shifts the EGFR nuclear equilibrium towards a level insufficient to induce differentiation but adequate for promoting cell growth and proliferation.

These results indicate that, in the *Drosophila* imaginal discs, *ft* function is necessary for the correct interpretation of the multiple EGFR signals that coordinate proliferation, and that its loss causes misinterpretation of proliferation stimuli leading to tissue overgrowth. This effect may be due, at least in part, to the transcriptional regulation of genes involved in EGFR signalling. Nevertheless, the hyperplastic phenotype of *ft* mutations cannot be completely ascribed to its role in modulating signals transduced by EGFR, according to the very partial rescue observed utilizing dominant-negative alleles of the pathway. These results suggest that the *ft* function is not restricted to the modulation of EGFR signals, but controls different developmental events involved in imaginal discs morphogenesis.

Several findings indicate that cadherin–catenin complexes may interact with growth factors receptors (see Vleminckx and Kemler, 1999; Comoglio et al., 2003 for reviews). The association of cadherins with growth factor receptors allows the assembly of a locally active apparatus that is essential for the generation of correct cell–cell signalling, as suggested by the downregulation of E-cadherins observed in mammalian tumors (Hirohashi, 1998; Perl et al., 1998). Furthermore, E-cadherins were found to be a direct biochemical target of the EGFR pathway, suggesting a close relation of these molecules with the modulation of cell–cell communication (Hoschuetzky et al., 1994; Fujita et al., 2002). The only partial homology between the Ft protocadherin and the classic E-cadherins, and the lack of data about interactors for the cytoplasmic domain of *ft* makes a direct comparison of their function very difficult. Taken together, our data suggest a novel mechanism through which *ft* tumor suppressor gene and EGFR pathway cooperate in the control of proliferation and morphogenesis in *Drosophila* imaginal tissues.

4. Experimental procedures

4.1. Fly strains

The flies were reared at 25 °C on a standard medium. The following mutations and transgenes were used: at the *ft locus*, the homozygous lethal alleles $ft^{G\text{-}rv}$ (Bryant et al., 1988) and ft^{18} (Garoia et al., 2000); at the *myc locus*, the hypomorphic dm^{PO} mutation (Bloomington stock 11298); at the *tkv locus*, the hypomorphic tkv^{1} mutation (Bloomington stock 427). The GAL4 lines used were MS1096-GAL4 (Capdevila and Guerrero, 1994), expressed in the wing pouch, and *dpp*-GAL4 (Bloomington stock 1553). To detect the *dpp* expression in the imaginal discs we used, in combination with the *ft* mutation, the $dpp^{BS3.0}$ transgene, a construct that contains the coding region for bacterial β-galactosidase under the transcriptional control of the 3′ *dpp* enhancer (Blackman et al., 1991).

The UAS lines used were UAS *rho* (de Celis et al., 1997), that expresses a wild type form of the *rho* gene; UAS raf^{DN} (Martin-Blanco et al., 1999) that acts as a dominant negative form of *raf*; UAS raf^{GOF} (Bloomington stock 2033), a constitutively activated form of the *Drosophila raf* gene; UAS-$EGFR^{DN}$ (Bloomington stock 5364), a dominant negative form of EGFR, with a stop codon introduced 20 amino acids C-terminal to the transmembrane domain; UAS-tkv^{QD} (Nellen et al., 1996) that expresses a constitutively activated form of the Dpp receptor *thickveins*.

4.2. Generation of mutant clones

Mitotic recombination in wings was induced by X-rays (1000 R; 300 R/min, 180 kV, 11 mA, 2 mm Al filter). Irradiated larvae were aged at 4 h and 24 h intervals after egg laying (AEL). We studied clones initiated at 70–74 h and 48–72 h AEL. As mutant cell markers for clonal analysis we used the ck^{1} allele of the *crinkled* gene, located in the same 2L chromosome arm as *ft*. Mutant clones were

induced in female flies of the following genotypes: (1) ft^{18}, *ck*/+; (2) MS1096-GAL4/+; ft^{18}, *ck*/+; UAS-raf^{DN}/+; (3) MS1096-GAL4/+; ft^{18}, *ck*/+; UAS-raf^{GOF}/+; (4) MS1096-GAL4/+; ft^{18}, *ck*/+; UAS-*rho*/+; (5) MS1096-GAL4/+; ft^{18}, *ck*/+; UAS-$EGFR^{DN}$/+.

Wild type clones in mutant background were induced at 70–74 h AEL in the following genotypes: (1) MS1096-GAL4/f^{36a}; UAS-raf^{DN}; (2) MS1096-GAL4/f^{36a}; UAS-raf^{GOF}; (3) MS1096-GAL4/f^{36a}; UAS-*rho*.

For twin analysis, mutant clones in the 2L arm were induced at 70–74 h AEL in female flies of the following genotypes: (1) f^{36a}; ft^{18}/f^{+30B}, *ck*, *pr*, *pwn*; (2) f^{36a}; ft^{18}, tkv^{1}/f^{+30B}, tkv^{1}, *ck*, *pr*, *pwn*; (3) dm^{P0}, f^{36a}; ft^{18}/f^{+30B}, *ck*, *pr*, *pwn*.

Mitotic recombination in imaginal discs was induced using the FLP–FRT technique (Golic, 1991), FRT40, $ft^{G\text{-}rv}$/FRT40, GFP; hs-FLP/+ larvae were heat-shocked for 1 h at 44–52 h AEL.

Whole mutant eyes were obtained using the EGUF system (Stowers and Schwarz, 1999). The *yw*; FRT 40 A, GMR *hid*, 2L CL2L/Cy0; EGUF/EGUF strain was crossed with the following strains: (1) FRT40, $ft^{G\text{-}rv}$/CyO; (2) FRT40, $ft^{G\text{-}rv}$/CyO; UAS-*rho*/TM6b; (3) FRT40, $ft^{G\text{-}rv}$/CyO; UAS-raf^{GOF}/TM6b; (4) FRT40, $ft^{G\text{-}rv}$/CyO; UAS-raf^{DN}/TM6b.

4.3. Immunohistochemistry and histology

Immunohistochemistry was performed as described in Cubas et al. (1991). The primary antibody utilized was mouse anti-dpERK (Sigma). The secondary Cy3 anti-mouse antibody (Jackson ImmunoResearch) was used at 1:200 dilution. Discs were mounted in Vectashield (Vector Laboratories) and imaged by a Leica TCS confocal microscope. Cell death was visualized with acridine orange as described in Neufeld et al. (1998). Scanning electron micrograph was performed as described in Lai et al. (1997).

4.4. Semi-quantitative RT-PCR

120 h AEL wild type and homozygous $ft^{G\text{-}rv}$ larvae were dissected in diethylpyrocarbonate-treated PBS in ice, wing and eye discs were isolated and immediately stored in RNAlater (Ambion). The RNAeasy kit (Qiagen) was used to isolate total RNA (approximately 200 discs for each experiment) following the manufacturer instructions.

Since the reliability of results of semi-quantitative RT-PCR is very sensitive to the efficiency of RT reaction, we added a standard RNA (human CENP-C RNA, obtained as described in Zhang et al., 1997) as internal control prior to perform the RT reaction. This technique provides greater accuracy in the following product quantification than techniques based upon amplification of housekeeping genes (Zhang et al., 1997). 12.5 fmol of standard RNA were added to five hundred ng of total RNA; the mixture was incubated at 70 °C for 5 min and then added to the cDNA master mixture (0.5 mM dNTPs, 5 μM random hexamers, 0.3 unit Reverse Transcriptase (Promega), 1 unit RNase inhibitor (Promega), 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 4 mM DTT and 10 mM $MgCl_2$) in a final volume of 10 μl. The cDNA synthesis reaction was performed at 37 °C for 60 min. The reaction was stopped by denaturing at 95 °C for 5 min and then 90 μl of distilled water were added.

The PCR reactions (50 μl total volume) included 5 μl of cDNA solution, 1.5 mm $MgCl_2$, 0.5 μM of each primer, 200 μM dNTPs, 10 mM Tris–HCl (pH 9), 50 mM KCl, 0.1% Triton X-100 and 1 unit of *Taq* Polymerase (Promega). Amplifications were performed on a GeneAMP PCR System 9700 (Applied Biosystems) as follows: initial denaturation at 94 °C for 3 min; cycling conditions: 94 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s.

The following primers were used: CENP-C, fwd 5′-ccagaaatcacatccaaagt-3′and rev 5′-ctctttttggcactagatgg-3′; *yan*, fwd 5′-catctgtccatgaattacga-3′ and rev 5′-ccgaaaggtagtggaagg-3′; *pnt*, fwd 5′-cttccaacatcacctcaaag-3′ and rev 5′-ggaaggattgagcatcatag-3′; *dmyc* fwd 5′-cgggagtcaataacaaagtg-3′ and rev 5′-gctgcatactaagctccttc-3′; GAPDH fwd 5′-gtcaacgatcccttcatc-3′ and rev 5′-tcgaccttagccttgattt-3′.

The linear range of amplification was determined increasing the PCR cycles from 20 to 30, then 5 μl of the reaction were loaded on a 2% agarose gel. For all genes 26 cycles of amplification were found to be within the linear range and used in all the amplification experiments, each reaction was run at least three times. The DNA bands were quantified using NIH Image software and band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be quantified and CENP-C was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation in all experiments performed were calculated after normalization to CENP-C; the statistical significance between mean intensity levels was calculated using a two tailed Student's *t*-test implemented in the Microsoft Excel software.

4.5. Flow cytometry

Wild type and homozygous $ft^{G\text{-}rv}$ 120 h AEL wing discs were dissociated for 2–4 h in Tripsin–EDTA (Sigma). FACS analysis was performed as described in Crissman et al. (1975), using a Coulter Elite Cytometer. Each experiment was performed at least three times.

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