Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 291 (2006) 278–290

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Atrophin contributes to the negative regulation of epidermal growth factor receptor signaling in *Drosophila*

Bernard Charroux [a], Matthew Freeman [b,*], Stephen Kerridge [a,*], Antonio Baonza [b,1]

[a] *Laboratoire de Génétique et Physiologie du Développement, UMR 6545 CNRS-Université, IBDM-CNRS-Université de la Méditerranée, Marseille Cedex 09 13288, France*

[b] *MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK*

Received for publication 8 September 2005; revised 21 November 2005; accepted 6 December 2005
Available online 27 January 2006

Abstract

Dentato-rubral and pallido-luysian atrophy (DRPLA) is a dominant, progressive neurodegenerative disease caused by the expansion of polyglutamine repeats within the human Atrophin-1 protein. *Drosophila* Atrophin and its human orthologue are thought to function as transcriptional co-repressors. Here, we report that *Drosophila* Atrophin participates in the negative regulation of Epidermal Growth Factor Receptor (EGFR) signaling both in the wing and the eye imaginal discs. In the wing pouch, Atrophin loss of function clones induces cell autonomous expression of the EGFR target gene *Delta*, and the formation of extra vein tissue, while overexpression of Atrophin inhibits EGFR-dependent vein formation. In the eye, Atrophin cooperates with other negative regulators of the EGFR signaling to prevent the differentiation of surplus photoreceptor cells and to repress *Delta* expression. Overexpression of Atrophin in the eye reduces the EGFR-dependent recruitment of cone cells. In both the eye and wing, epistasis tests show that Atrophin acts downstream or in parallel to the MAP kinase *rolled* to modulate EGFR signaling outputs. We show that *Atrophin* genetically cooperates with the nuclear repressor Yan to inhibit the EGFR signaling activity. Finally, we have found that expression of pathogenic or normal forms of human Atrophin-1 in the wing promotes wing vein differentiation and acts as dominant negative proteins inhibiting endogenous fly Atrophin activity.

Keywords: *Drosophila*; Atrophin; DRPLA; EGFR signaling; Cell signaling; Yan

Introduction

Dentato-rubral and pallido-luysian atrophy (DRPLA) is an autosomal dominant, progressive neurodegenerative disease with symptoms similar to those of Huntington's disease (HD) such as chorea, ataxia, incoordination and dementia (Naito and Oyanagi, 1982; Ross et al., 1997a,b; Smith et al., 1958). DRPLA is caused by expansion of a CAG repeat in the coding region of the *atrophin-1* gene. The Atrophin-1 protein is detected in both the cytoplasm and the nucleus, where it acts as a transcriptional co-repressor (Wood et al., 2000). Truncated fragments of Atrophin-1, containing the expanded polyglutamine tract, have been shown to accumulate in populations of neuronal nuclei, both in a mouse model of DRPLA and in human DRPLA brain tissue (Schilling et al., 1999). Several observations indicate that these fragments may underlie the pathogenesis of the disease (Nucifora et al., 2001, 2003). Little is known, however, about the physiological role of Atrophin-1.

The *Drosophila Atrophin* (*Atro*) homologue acts as a transcriptional co-repressor in multiple developmental processes. Segmentation is abnormal in $Atro^{-}$ mutant embryos, resulting from the lack of repression of the segmentation genes (Erkner et al., 2002; Zhang et al., 2002). In addition, $Atro^{-}$ embryos display a ventralized phenotype and neurogenic defects, suggesting a requirement for *Atro* in both dorso-ventral patterning and neurogenesis (Zhang et al., 2002). In the adult, $Atro^{-}$ clones

* Corresponding authors. M. Freeman is to be contacted at fax: +44 1223 412142. S. Kerridge, Laboratoire de Genetique, Centre Universitaire Marseille, Case 907, Marseille Cedex 9 F-13288, France. Fax: +33 91 82 06 82.

E-mail addresses: mfl@mrc-lmb.cam.ac.uk (M. Freeman), kerridge@lgpd.univ-mrs.fr (S. Kerridge).

[1] Present address: Universidad Autónoma de Madrid, Centro de Biologia Molecular-Severo Ochoa C-X Laboratorio 504, Cantoblanco, 28049 Madrid, Spain.

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.12.012

promote ectopic vein differentiation and notching of the wings (Zhang et al., 2002). In the eye, Atro activity is required to ensure proper planar polarity, ommatidial rotation and photoreceptor (PR) cell recruitment (Fanto et al., 2003; Zhang et al., 2002). *Atro*$^{-}$ clones located in the proximal region of the legs develop distal structures, cause defects in leg segmentation and patterning especially in ventral parts (Erkner et al., 2002). In all these tissues, Atro is also required for proper planar cell polarity (Fanto et al., 2003; Zhang et al., 2002). Remarkably, many of these adult phenotypes resemble those caused by upregulation of Epidermal Growth Factor Receptor (EGFR) signaling (Brown and Freeman, 2003; Campbell, 2002; Diaz-Benjumea and Hafen, 1994; Dominguez et al., 1998; Freeman, 1996; Galindo et al., 2002; Strutt and Strutt, 2003), raising the possibility that Atro plays a role in the EGFR pathway for proper patterning of the imaginal discs. In support of this idea, we have isolated *Atro* mutations as genetic modifiers of *Drosophila* EGFR signaling (this work).

The *Drosophila* EGFR signaling pathway controls multiple processes during development, such as cell fate, proliferation, survival, migration, adhesion and ommatidial rotation (Freeman, 1996; Shilo, 2003; Wolff, 2003). Despite its widespread function, EGFR signaling usually involves a common cytoplasmic cascade including Ras, and the serine–threonine kinases Raf, MAPKK/MEK and the MAPK *rolled* (*rl*) (reviewed by (Perrimon and Perkins, 1997). Activated MAPK transmits the RAS cascade signal into the nucleus by phosphorylating two members of the ETS family of transcription factors, Yan (also called Aop-Flybase, Rebay and Rubin, 1995) and Pointed (Pnt) (Brunner et al., 1994a; O'Neill et al., 1994). Yan is a transcriptional repressor that moves to the cytoplasm for degradation upon phosphorylation induced by EGFR signaling (Rebay and Rubin, 1995). The *pnt* gene encodes for two related proteins, PntP1 and PntP2, with PntP1 acting as a constitutive activator of transcription, while the activity of PntP2 is stimulated by the Ras/MAPK pathway (O'Neill et al., 1994). The role of EGFR signaling in cell differentiation has been extensively studied during development of both the eye and wing of *Drosophila*, where EGFR controls cell recruitment and vein differentiation, respectively. In both tissues, EGFR promotes the expression of several target genes including *Delta* (*Dl*) and *argos* (Golembo et al., 1996b; Martin-Blanco et al., 1999; Sawamoto et al., 1994; Tsuda et al., 2002).

We investigated the possibility that Atro plays a major role in the EGFR pathway during imaginal disc development of *Drosophila*. We found that Atro inhibits EGFR signaling both in the wing and eye imaginal discs from the third instar onwards (i.e. not for the earlier proximal distal wing patterning; Baonza et al., 2000; Wang et al., 2000; Zecca and Struhl, 2002a,b). In the wing pouch, *Atro* loss of function clones induce cell autonomous expression of the EGFR target gene *Dl*, and the formation of extra vein tissue, while overexpression of Atro inhibits EGFR-dependent vein formation. Atro also acts as a negative regulator of EGFR in the eye. Overexpression of Atro in the eye reduces the EGFR-dependent recruitment of cone cells. In both the eye and wing, epistasis tests show that Atro acts downstream or in parallel to the MAP kinase *rolled* to modulate EGFR signaling output. Interestingly, expression of a truncated form of human Atrophin-1 in the wing causes similar phenotypes to those produced by the ectopic activation of EGFR signaling.

Materials and methods

Genetic strains

Atro alleles are described in Erkner et al. (2002), except for *Atro*^{14967}. The P-element *P(w*$^{+}$*)14967* is located in the 5′ UTR of the *Atro* gene, at position 8428981. The P element came from the lethal insertions on the 3 chromosome of Szeged collection. The exact position of the insertion was analyzed following the standard protocols for inverse PCR for sequencing of P element insertions available in http://www.fruitfly.org/about/methods/. The 3′ flanking sequence of the Pelement is: TCATCATGGTACTAAGGGACTAATGGTAACGGACACA-CACACATACACACACACACCCAGAGAGAGAGAGAGAGAGA-GAAAAAGCCGCAGACTACAGAATTGGCTGGATACTTATGGTG-CAAAAAGGGGGGGATGGAGGTTGTGAACAAGAGGATGTGGATGTG-GATGAGGAAAAGGCGGACGGGGGGACGGCGTGCAAAATGAT-GAAGGTATCTACACCGACAATCTGTTTAACAACTTCATGAGCAAATC-CAACAAATCGTATGAAACGAAGCCGACAGCAGGCCAACAGCACA-GACACGAGCGGACGTCGTTGTATTACGATTTGGACTCGGATTCG-GATTCAGATTCAGATTCGGATTCGGATTCAGTTTCGGATGCAGAC-GAG. The following are the sources of the stocks used in this study: *rl*^{iSem}, *sty*^{A5}, *UAS-Yan*^{WT}, *pnt*$^{\Delta 88}$, *pnt*^{1277}, *pnt*^{07825} (Bloomington stock center), *cic*^{2}, *cic*^{bwkA11} (Roch et al., 2002), *UAS-EGFR*^{ACT} (Queenan et al., 1997), *UAS-EGFR*^{DN} (Freeman, 1996), *dpp-Gal4* (Staehling-Hampton et al., 1994), *UAS-Atro* (this work), *GMR-argos*, *sev-rho*, *rho*$^{P\Delta 5}$, *vn*^{10567}, *ru*^{1}, *rho*^{7M43} and *argos*$^{1\Delta 7}$. All these alleles are described in Flybase (http://flybase.bio.indiana.edu/).

Clone induction

Mitotic clones were generated by FLP-mediated mitotic recombination (Xu and Rubin, 1993). In all cases, recombination was induced in second instar larvae (60h AEL) by a 90 min heat shock at 37°C. In the eye, mutant clones for *Atro*^{14967}, *argos*$^{1\Delta 7}$, *argos*$^{1\Delta 7}$ *Atro*^{14967}, *ru rho*^{7M43} *argos*$^{1\Delta 7}$ *Atro*^{14967} and *sprouty Atro*^{14967} were marked by the absence of GFP, using *y w hsp-FLP122; ubi-GFP M(3)i*^{55} *FRT80B / TM6B* stock. These flies were crossed to: *Atro*^{14967} *FRT80B/TM6B*, or *w hsp-FLP122; argos*$^{1\Delta 7}$*FRT80B/TM6B*, or *argos*$^{1\Delta 7}$ *Atro*^{14967} *FRT80B/TM6B*, or *ru rho*^{7M43} *argos*$^{1\Delta 7}$ *Atro*^{14967}*FRT80B/TM6B*. Clones in adult wings were obtained by crossing *y w hsp-FLP122; mwh Atro*^{X} *FRT2A/TM6B* females (where X = 1207D6, 35, PZ3928, 14967) to *y w; Dp(1;3)sc*^{J4}, *y*$^{+}$ *M(3)i*^{55} *FRT2A/TM3* males. For induction of *Atro*$^{-}$ clones in wing discs: female *y w hsp-FLP122; ubi-GFP FRT2A/ubi-GFP FRT2A* were crossed to male *y w hsp-FLP122; vgBE-lacZ/CyO wg-LacZ; Atro*^{35} *FRT2A/TM6B* or male *y w hsp-FLP122; vgQ-lacZ/CyO wg-LacZ; Atro*^{35} *FRT2A/TM6B*. Mutant clones for *rho*$^{P\Delta 5}$*vn Atro*^{14967} were obtained by crossing *y w hsp-FLP122; ubi-GFP M(3)i*^{55} *FRT80B /TM6B* flies to *mwh rho*$^{P\Delta 5}$*vn Atro*^{14967}*FRT80B/TM6B*. In all cases, mutant clones were identified by absence of GFP or the *multiple wing hair* (*mwh*).

Induction of clones lacking Atro and expressing Nintra was obtained by crossing *y w hsp-FLP122; arm-GAL4/arm-GAL4; mwh Atro*^{35}*FRT2A/TM6B* flies to *y w hsp-FLP122; UAS-Nintra/UAS-Nintra; tubP-GAL80 FRT2A/TM6B*. Progeny were heat shocked at 36°C for 2 h between 48 and 72 or 72 and 96 h after egg laying. Clones lacking Atro and expressing Nintra were identified by the *mwh* marker. *Su(H)*$^{-}$ mutant clones were obtained by crossing females *y w hsp-FLP122; ubi-GFP FRT40A/ubi-GFP FRT40A* to male *Su(H)*^{del47} *FRT40A/CyO wg-LacZ*.

Histology

Scanning electron micrographs were prepared as described by Kimmel et al. (1990). Whole adult eyes were examined using a Leica MZFLIII stereomicroscope and processed with ACT-1.LNK image analysis software from Nikon. Adult wings and legs were dissected in 100% ethanol, rinsed twice in xylene, mounted in DPX and observed under a Zeiss AxioPhot microscope using Normarski optics. Immunohistochemical staining of imaginal discs and pupal discs (45 h after puparium formation) was performed as follows. Imaginal discs were dissected in PBS, 0.1% Triton X100 (PBT), and fixed for 15 min in 4% paraformaldehyde in PBT, washed (3 × 15 min each), blocked in PBT, 0.1%BSA for 1 h and incubated in the

same buffer for 4 h at room-temperature or overnight at +4°C with one of the following antibodies: mouse anti-Arm (1/100) (hybridoma—from DSHB), rat anti-Elav (1/10) (hybridoma-supernatant 7E8A10 from DSHB), rabbit anti-Atonal (1/500) (Jarman et al., 1993), mouse anti-Cut (1/10) (hybridoma-supernatant ZB-10 from DSHB), mouse anti-Delta (1/10) (hybridoma-supernatant C594-9B from DSHB), rabbit anti-β-galactosidase (1/1000) (Cappel ICN-Pharmaceuticals Inc.), mouse anti-dpERK (1/100) (SIGMA), rabbit anti-human Atrophin-1 APG840 (1/500) (Wood et al., 2000), rabbit anti-Atro (1/500) (Erkner et al., 2002), rabbit anti-Distal-less (1/200) (a gift from S. Carroll), rabbit anti-Vestigial (1/200) (a gift from S. Carroll) or mouse anti-Wingless (1/10) (hybridoma-supernatant 4D4 from DSHB). Secondary antibodies were TRITC-conjugated AffiniPure Donkey anti-IgG from Jackson ImmunoResearch used at a 1/400 dilution. A Zeiss confocal microscope was employed for capture of images.

Plasmid construction

A 6 kb cDNA containing the entire Atro open reading frame was cloned in the *pUAST* transformation vector (Brand and Perrimon, 1993) to generate *UAS-Atro*. Ubiquitous expression of *UAS-Atro* (with *arm-GAL4*) rescues the embryonic lethality of $Atro^{35}/Atro^{PZ3928}$ mutants. To create *ubi-GFP-Atro*, a *GFP-Atro* fusion cDNA was cloned into the transformation vector *pCaSpeR-UP* (Davis et al., 1995). The embryonic lethality of homozygous $Atro^{35}$ or $Atro^{PZ3928}$ is rescued in presence of the *ubi-GFP-Atro* transgene. Truncated *Atrophin-1* cDNAs encoding the first 917 amino acids (At-N917-26Q or At-N917-65Q) were generated by PCR using full-length human *Atrophin-1* cDNAs (At-FL-26Q or At-FL-65Q, a gift from David R. Borchelt; Schilling et al., 1999) as templates, and cloned into *pUAST* to generate *UAS-At-N917-26Q* or *UAS-At-N917-65Q*. For each construct, several transgenic lines were generated by P-element-mediated transformation (Spradling and Rubin, 1982). UAS lines expressing similar amounts of human Atrophin 1 proteins under *GMR-Gal4* control were identified and used here.

Detailed construct information is available upon request.

Results

Atrophin inhibits EGFR signaling

We used a genetic modifier screen to identify new components of EGFR signaling. A P-element induced mutation, $P(w^+)14967$, was found to enhance the rough eye caused by the misexpression of *rho* (Figs. 1B–C), a specific activator of EGFR signaling (Bier et al., 1990; Golembo et al., 1996a; Lee et al., 2001; Urban et al., 2001). $P(w^+)14967$ is located in the 5′ UTR of the *Atro* gene and fails to complement other *Atro* alleles, $Atro^{35}$, $Atro^{PZ3928}$ and $Atro^{12O7D6}$ (Erkner et al., 2002). All these *Atro* loss of function alleles also behave as enhancers of *rho* misexpression in the eye (see Table 1). This enhancement suggests that *Atro* is an inhibitor of EGFR signaling. Further genetic tests provide evidence that *Atro* interacts with the EGFR pathway. First, halving the dose of *Atro* suppresses the rough eye caused by overexpressing Argos (Figs. 1D–E), a negative regulator of the EGFR signaling (Freeman et al., 1992; Schweitzer et al., 1995; Vinos and Freeman, 2000). Second, halving *Atro* enhances the extra wing-vein phenotype caused by the constitutively active MAPK mutation $rolled^{Sevenmaker}$ (rl^{Sem}) (Figs. 1G–H and Brunner et al., 1994b), and ubiquitous overexpression of *Atro* weakly suppresses vein differentiation (Fig. 1I). Third, ectopic wing-veins differentiate in wings transheterozygous for *Atro* and mutations in genes acting as negative regulators of EGFR signaling (*sprouty* (*sty*) and *capicua* (*cic*), data not shown). Fourth, simultaneous expression of Atro and Yan, a nuclear repressor of EGFR signaling, cooperates genetically to counteract EGFR signaling in both the wing and the eye (see below). These results indicate that Atro inhibits EGFR signaling.

Atrophin regulates photoreceptor cell recruitment in the eye

The *Drosophila* compound eye is composed of approximately 800 ommatidia, each of which contains eight photoreceptor (PR) cells (R1–R8), four non-neuronal cone cells and eight accessory cells arranged in a highly ordered pattern. The EGFR signaling pathway is required for the recruitment and differentiation of cone cells and all PR cell types in the eye, with the exception of R8 (Dominguez et al., 1998; Freeman, 1996). Loss of function alleles of negative

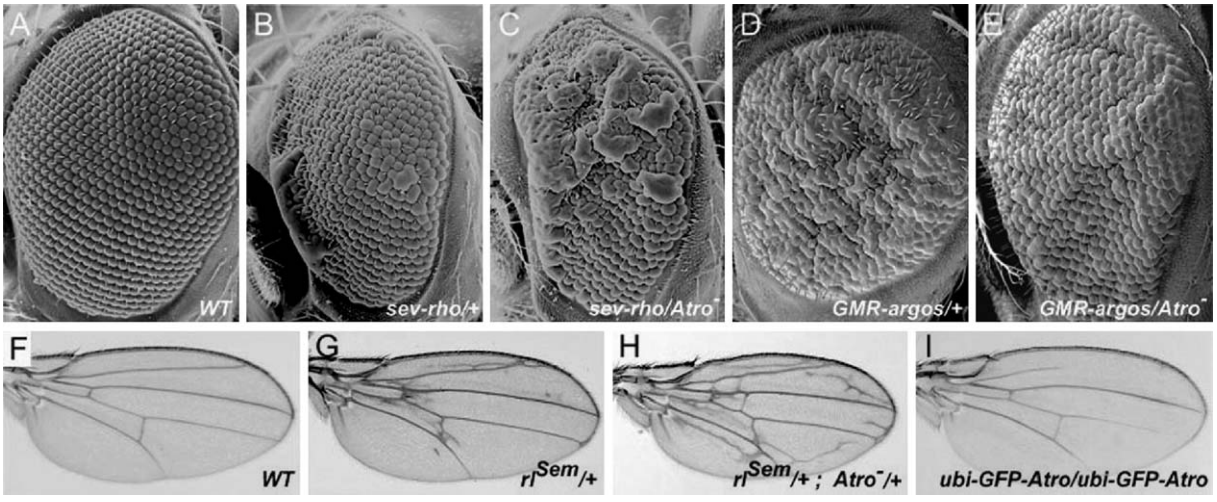


Fig. 1. *Atro* interacts with mutations of the EGFR pathway. (A) Wild-type eye. (B) Overexpression of *rhomboid* under the control of the *sevenless* promoter causes a rough eye phenotype (*sev-rho*/+). (C) $Atro^{35}$ heterozygotes enhance the *sev-rho*, excess EGFR signaling, phenotype (*sev-rho*/$Atro^{35}$). (D) Overexpression of *argos* under the control of the GMR enhancer causes a rough eye phenotype (*GMR-argos*/+). (E) $Atro^{35}$ heterozygotes suppress the *GMR-argos*, loss of EGFR signaling, phenotype (*GMR-argos*/$Atro^{35}$). (F) Wild-type wing. (G) The gain-of-function $rolled^{Sevenmaker}$ (rl^{Sem}) mutation induces the formation of ectopic vein material. (H) $Atro^{PZ03928}$ heterozygotes enhance the ectopic vein phenotype of rl^{Sem}/+(rl^{Sem}/+; $Atro^{PZ03928}$/+). (I) Partial vein suppression is observed following expression of GFP-Atro under the control of the ubiquitin promoter (*ubi-GFP-Atro*/*ubi-GFP-Atro*).

Table 1
Genetic interactions between *atrophin* and members of the *Egfr* pathway

Genotype	*sev-rho*/+	*GMR-argos*/+	rl^{sem}/+
	Eye	Eye	Wing
$Atro^{PZ3928}$/+	++	nd	++
$Atro^{1207D6}$/+	++	nd	++
$Atro^{35}$/+	++	− −	+
$Atro^{14967}$/+	+	−	+
UAS-Atro/GMR-Gal4	− −	++	nd
UAS-Atro/dpp-Gal4	nd	nd	− −
Ubi-GFP-Atro/Y	−	nd	− −

Enhancement (+). Supression (−). Not determined (nd).

regulators of the EGFR pathway promote the formation of extra cone cells and PRs (Casci et al., 1999; Freeman et al., 1992). For instance, clones mutant for *argos* develop extra PRs: in $argos^-$ clones, we observed 46.2% ommatidia with 8 PRs, 40.7% with 9 PRs, 11.1% with 10 PRs and 1.8% with 11PRs (Figs. 2A and D and Freeman et al., 1992). This phenotype is strongly enhanced in clones mutant for both *Atro* and *argos*, where numerous additional PRs differentiate: in $argos^-$ $Atro^-$ clones, there are 11.1% ommatidia with less than 6 PRs, 7.4% with 7 PRs, 14% with 8 PRs, 19.2% with 9 PRs, 14% with 10 PRs, 11.8% with 11 PRs, 8.1% with 12 PRs and 14% with more than 12 PRs (Figs. 2C and E). This phenotype is similar to the phenotype observed in double mutant clones for *argos* and *sty*, two negative regulators of the EGFR (Casci et al., 1999). This result indicates that *Atro* and *argos* genetically cooperate to antagonize EGFR activity. A similar result is obtained in mutant clones for both *Atro* and *sty* (data not shown). Note that no additional PR cells are formed in $Atro^-$ clones (see Fig. 2B). To exclude that the extra PRs observed in the $argos^-$ $Atro^-$ clones are R8 cells, which are independent of EGFR signaling (Dominguez et al., 1998; Freeman, 1996), third instar eye discs with these clones were stained with anti-Atonal, an R8-specific antibody. Single R8 cells are seen in each mature ommatidium in $argos^-$ $Atro^-$ clones (Fig. 2F). In addition, we found that mutant clones for *Atro* can occasionally induce ectopic cone cells, which are also recruited by EGFR signaling (Freeman, 1996): 65.2% of

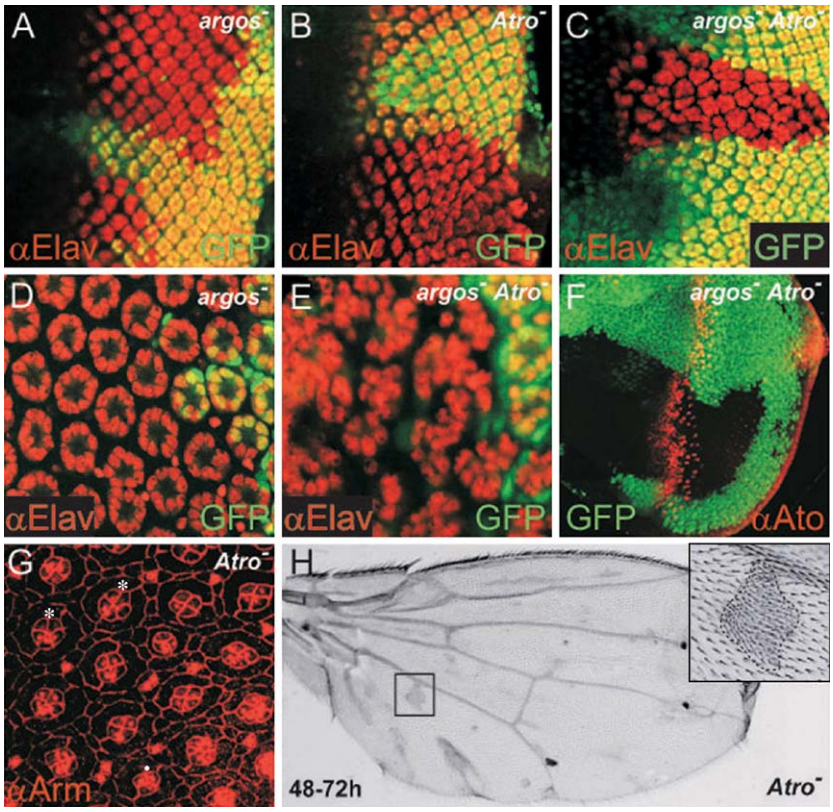


Fig. 2. *Atro* loss of function phenotypes. (A–E) *Atro* cooperates with *argos* to repress recruitment of PR cells. (A–C) Clones homozygous for $argos^-$ (A), $Atro^-$ (B) or $argos^-Atro^-$ (C) in eye imaginal discs, detected by the absence of GFP (green), and stained with anti-Elav (red) to mark all PR cells. (C) Note the great excess of cell autonomous recruitment of PR cells in $argos^-Atro^-$ cells when compared to $argos^-$ (A) or $Atro^-$ (B) clones. (D) A pupal disc showing several ommatidia with excess PR cells (marked with Anti-Elav (red)) in an $argos^-$ clone (detected by the absence of GFP). (E) A pupal disc showing several ommatidia with a massive excess of PR cells (marked with Anti-Elav (red)) in an $argos^-Atro^-$ clone (detected by the absence of GFP). (F) Recruitment of the PR R8 is not affected in $argos^-Atro^-$ cells. Eye imaginal disc with an $argos^-Atro^-$ clone (marked by absence of GFP, green) stained with anti-Atonal (red) to mark R8 PRs. Single R8 type PRs (red in F) are detected in $argos^-Atro^-$ mutant cells in each ommatidium. (G) Ectopic cone cells are formed in absence of *Atro* activity. Pupal eye disc with an $Atro^-$ clone stained with anti-Arm to outline the cone cells. The whole panel shows mutant cells for *Atro*. Ommatidia with 5 cone cells are labeled with an asterisk. The dot indicates an ommatidium with 3 cone cells. (H) Loss of *Atro* activity in the wing promotes ectopic vein formation. An adult wing with $Atro^-$ clones induced at 48–72 h after egg deposition show extra wing-vein material (H). Inset: magnification of the area showed in panel H showing the autonomous effect of $Atro^-$ clones on vein differentiation. $Atro^-$ clones are marked with *multiple wing hairs* (*mwh*).

ommatidia have 4 cone cells, as in wild-type eyes, but 20.1% have 5 cone cells, 14.7% only have 3 (Fig. 2G). Consistent with these data in the eye, Atro also acts as a suppressor of EGFR signaling in the wing. *Atro*^{35} clones marked with *multiple wing hairs* (*mwh*) develop extra vein tissue autonomously in the adult wing (Fig. 2H). We obtained similar results with other alleles: *Atro*^{PZ3928}, *Atro*^{12O7D6} and *Atro*^{14967} (data not shown). Autonomous differentiation of vein tissue is also observed in clones induced later during development (48 to 72h, Fig. 2H, and 72 to 96 h AED, data not shown). Thus, both in the eye and in the wing, *Atro*$^{-}$ clones show phenotypes diagnostic of excessive EGFR activity, implying a normal function for Atro in restricting EGFR signaling.

Overexpression of Atrophin mimics loss of EGFR signaling

Our results imply that *Atro* negatively regulates EGFR signaling. In accordance with this hypothesis, overexpression of *UAS-Atro* in the eye, using the *GMR-Gal4* driver, suppresses the Rhomboid misexpression phenotype (Figs. 3A–C) and enhances the rough eye phenotype caused by Argos overexpression (Figs. 3D–F). In addition, overexpression of Atro in the eye reduces the number of cone cells that are recruited to each ommatidium, while PR recruitment is normal: 76% of the ommatidia contain 3 cone cells, 8% have 2 cone cells and 15% are normal (Figs. 3G–I and data not shown). This is consistent with previous work showing that recruitment of cone cells is more sensitive than recruitment of PRs to deregulation of EGFR signaling (Freeman, 1996). In the wing, the overexpression of Atro in prospective veins driven by the line *580-Gal4* causes a substantial loss of wing vein tissue and a reduction in wing size (Fig. 3K). In legs, overexpression of Atro using the *dpp-Gal4* driver promotes fusion of the tarsus whereas the rest of the leg is normal (Fig. 3M). Reduction of EGFR activity in legs displays a similar phenotype (Campbell, 2002; Galindo et al., 2002). Atrophin therefore acts as a negative regulator of EGFR signaling in a variety of processes during imaginal disc development. Note that ubiquitous expression of *UAS-Atro* with *arm-Gal4* rescues the embryonic lethality of *Atro*^{PZ3928}/*Atro*^{35} transheterozygous (data not shown), showing that *UAS-Atro* produces a functional Atro protein.

Atrophin is a repressor of the EGFR target gene Delta

In third instar imaginal discs, the expression of *Dl* is positively regulated by the EGFR signaling pathway, both in the wing pouch and in PR cells of the eye (Tsuda et al., 2002). We investigated whether *Atro* represses the expression of *Dl*. In the eye disc, whereas the expression of *Dl* is unchanged (or slightly increased) in *Atro*$^{-}$ clones (Fig. 4A), *Atro*$^{-}$ *argos*$^{-}$ clones display high levels of *Dl* expression (Fig. 4C) when compared to wild-type cells or *argos*$^{-}$ clones (Fig. 4B). Atro activity therefore contributes to the repression of *Dl* in the eye in a cell autonomous manner. This result is consistent with the effect of *Atro*$^{-}$ clones on PR recruitment (see above) and indicates that Atro has a partially redundant role in the eye. In the wing, reduction of *Atro* activity in mutant clones causes cell autonomous high level expression of *Dl* (and *Serrate*, data not shown) in clones localized in any part of the wing pouch (Fig. 4D). Hence, both in the eye and in the wing imaginal disc, Atro is acting as a repressor of the EGFR target gene *Dl*. Note that wing notches caused by *Atro*$^{-}$ clones (Zhang et al., 2002) can be explained by the high levels of *Dl* expression in these clones (de Celis and Bray, 1997; Micchelli et al., 1997).

Atrophin acts downstream or in parallel to the MAPK Rolled

To further investigate the role of *Atro* in EGFR signaling, we analyzed the distribution of the activated form of MAPK (dpERK) in *Atro*$^{-}$ clones (Figs. 5A–B). Activated MAPK shows a prominent localization along the veins including those each side of the wing margin, in the wing pouch (Gabay et al., 1997). In the eye, high levels of expression are observed in clusters of cells within the morphogenetic furrow (Gabay et al., 1997). This activated MAPK distribution reflects the activity of the EGFR via the RAS signaling cascade. No obvious changes in dpERK staining are observed in *Atro*$^{-}$ clones located in the eye imaginal disc (Fig. 5A). Similarly, in *Atro*$^{-}$ clones located in the wing pouch, the distribution of dpERK is unaffected (Fig. 5B). These results suggest that Atro acts downstream or in parallel to MAPK in the EGFR pathway. Moreover, we found that overexpression of Atro suppresses the formation of extra wing-vein tissue caused by the constitutive activated MAPK mutation *rl*^{Sem} (Figs. 5C–D).

Next, we investigated whether signaling from the EGFR influences the *Atro*$^{-}$ phenotype. To do so, we analyzed the phenotype of *Atro*$^{-}$ clones in which EGFR signaling was blocked. We eliminated EGFR signaling in the wing by removing factors required for EGFR activation. In clones mutant for both *vein* (*vn*) (Schnepp et al., 1996) and *rho* (Sturtevant et al., 1993; Urban et al., 2001), veins fail to differentiate. In triple mutant clones for *vn*, *rho* and *Atro*, vein differentiation is also blocked, in contrast to *Atro*$^{-}$ clones, in which ectopic veins are induced (compare Figs. 5G and 2H). Consistent with this observation, no ectopic expression of the EGFR target *Dl* is seen in *rho*$^{-}$*vn*$^{-}$*Atro*$^{-}$ mutant clones when located in the wing pouch (Fig. 5F) contrary to *Atro*$^{-}$ mutant clones (Fig. 4D). Thus, a positive input by EGFR signaling is necessary for the phenotypes seen in *Atro* loss of function clones. This requirement is also observed in the eye. When the EGFR signaling is abolished in double mutant cells for *rho* and *roughoid* (*ru*) (Urban et al., 2001; Wasserman et al., 2000), PRs do not develop (data not shown and Wasserman et al., 2000). We found that *ru*$^{-}$*rho*$^{-}$*Atro*$^{-}$*argos*$^{-}$ mutant clones lack PR cells, in contrast to *Atro*$^{-}$ *argos*$^{-}$ clones where numerous PR are recruited (compare Figs. 2C and 5E). Hence, in both the eye and wing pouch, loss of Atro function is not sufficient to induce surplus EGFR signaling; EGFR signaling is required to induce target gene output in *Atro*$^{-}$ clones.

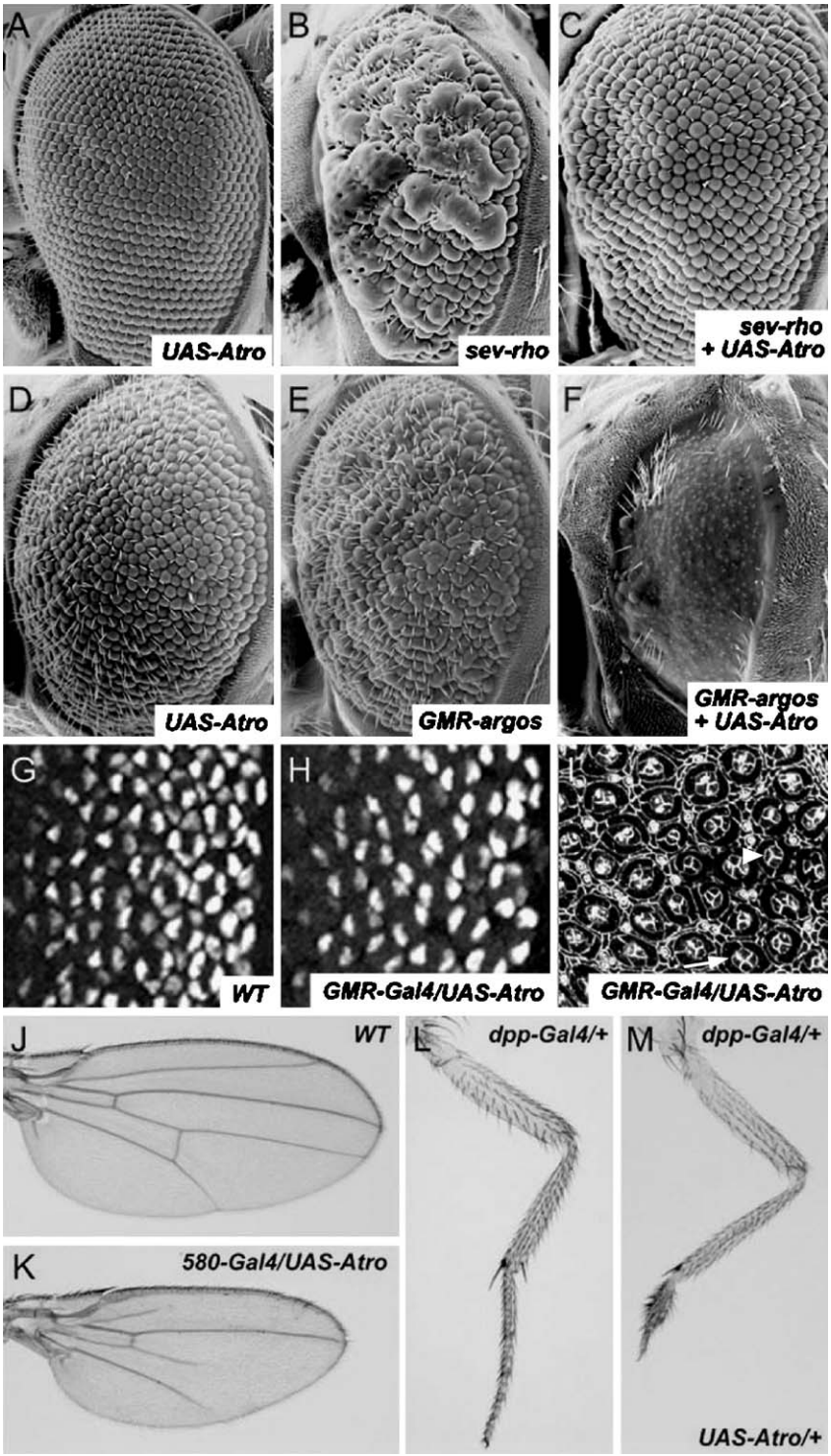


Fig. 3. Phenotypes of Atro overexpression. Overexpression of Atrophin antagonizes the EGFR signaling pathway. Flies were raised at either 18°C (A–C) or 25°C (D–J). *GMR-Gal4/UAS-Atro* eyes (A and D) have weak (A) or medium (D) rough eye phenotypes. (B) Overactivation of EGFR signaling causes a strong rough eye phenotype (*sev-rho/sev-rho*). (C) This phenotype is suppressed by overexpressing Atro (*GMR-Gal4/UAS-Atro; sev-rho/sev-rho*). (E) Overexpression of the EGFR antagonist Argos under the control of the GMR enhancer (*GMR-argos/+*) gives a rough eye phenotype. (F) This phenotype is enhanced when Atro is co-expressed with Argos (*GMR-Gal4/UAS-Atro; GMR-argos/+*). (G–I) Overexpression of Atro in the eye reduces the number of recruited cone cells. Third instar eye imaginal discs of wild-type (G) or *GMR-Gal4/UAS-Atro* (H) larvae stained with anti-Cut to mark the cone cells. *GMR-Gal4/UAS-Atro* eyes have a reduced number of cone cells (H), when compared to a wild-type eyes (G). Eye discs of *GMR-Gal4/UAS-Atro* (I) pupae stained with anti-Arm to outline the cone cells. (I) *GMR-Gal4/UAS-Atro* eye discs have a reduced number of cone cells. The arrowhead indicates an ommatidia with 3 cone cells; an ommatidia with 4 cone cells, as in wild-type eyes, is indicated by the arrow. (J–K) Overexpression of Atro in the wing causes wing-vein suppression and reduction of the wing size. (J) Wild-type wing. Wing from *580-Gal4/UAS-Atro* flies raised 29°C (K) show loss of veins and reduced wing size. (L–M) Overexpression of Atro in the leg causes fusion of the tarsus. (M) A leg from *dpp-Gal4/UAS-Atro* flies showing fusion of the tarsus. A *dpp-Gal4/+* leg is shown as a control (L).

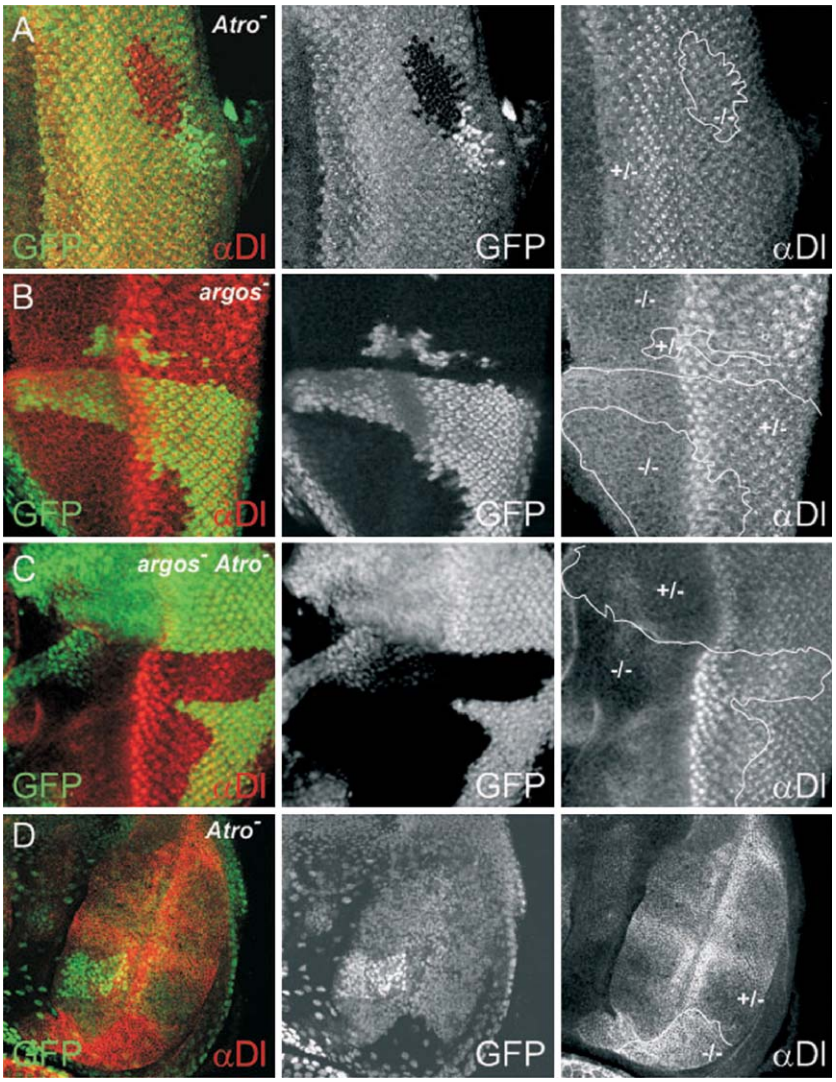


Fig. 4. Atro negatively regulates *Dl* expression. (A–C) *Atro* cooperates with *argos* to repress expression of *Dl* in the eye. Clones of $Atro^{-}$ (A), $argos^{-}$ (B) and $argos^{-}Atro^{-}$ cells (C) in eye imaginal discs, are detected by the absence of GFP (green) and stained with anti-Dl (red). (A) The expression of *Dl* is weakly increased in $Atro^{-}$ (A) and unaffected in $argos^{-}$ (B) cells, when compared to wild-type neighboring cells. (C) Ectopic expression of *Dl* is detected at high levels in $argos^{-}Atro^{-}$ cells. (D) *Atro* represses *Dl* expression in the wing pouch. Clones of $Atro^{-}$ mutant cells (D) in a wing imaginal disc, are detected by the absence of GFP (green) and stained with anti-Dl (red). The Dl protein is ectopically produced in $Atro^{-}$ clones, when located in the wing pouch (D). (A–D) Each disc is visualized in different channels and merged (left panel).

As a whole, our data suggest that *Atro* acts downstream and/or in parallel to MAPK to modulate the target genes that are activated by EGFR signaling.

Atrophin interacts genetically with Yan and Pointed to repress EGFR signaling

A variety of studies support a model where the balance between the activities of Yan and Pnt mediates the induction of cell differentiation via direct transcriptional control of specific target genes (Brunner et al., 1994a; O'Neill et al., 1994; Rebay and Rubin, 1995; Tootle et al., 2003; Xu et al., 2000).

Atro is thought to act as a transcriptional co-repressor (Zhang et al., 2002), hence, it could cooperate with Yan to counteract Pnt activity in both the eye and wing pouch. We analyzed possible genetic interactions between *yan*, *pnt* and *Atro* in overexpression experiments. Overexpression of *UAS-Atro* with *GMR-Gal4* enhances the phenotype caused by the ectopic expression of Yan^{WT} in the eye (Figs. 6B–D). Note that third instar eye discs overexpressing Yan^{WT} plus Atro are normal (i.e. these eyes display a normal number of PRs, data not shown), hence, the effects observed in adult eyes are likely due to an interaction occurring during the pupal stage. Next, we asked whether Atro cooperates with Yan to counteract EGFR signaling in the wing. To do so, we expressed an activated form of the EGF receptor ($EGFR^{ACT}$) in the wing, using the leaky activity of *GMR-Gal4* in this tissue, and analyzed the consequence of co-expressing Atro and Yan^{WT}. As expected, expression of *UAS-*$EGFR^{ACT}$ in the wing causes extra-wing vein differentiation (Fig. 6I). Overexpression of *UAS-Atro* or *UAS-*Yan^{WT} alone has no effect on this phenotype (Figs. 6J, K).

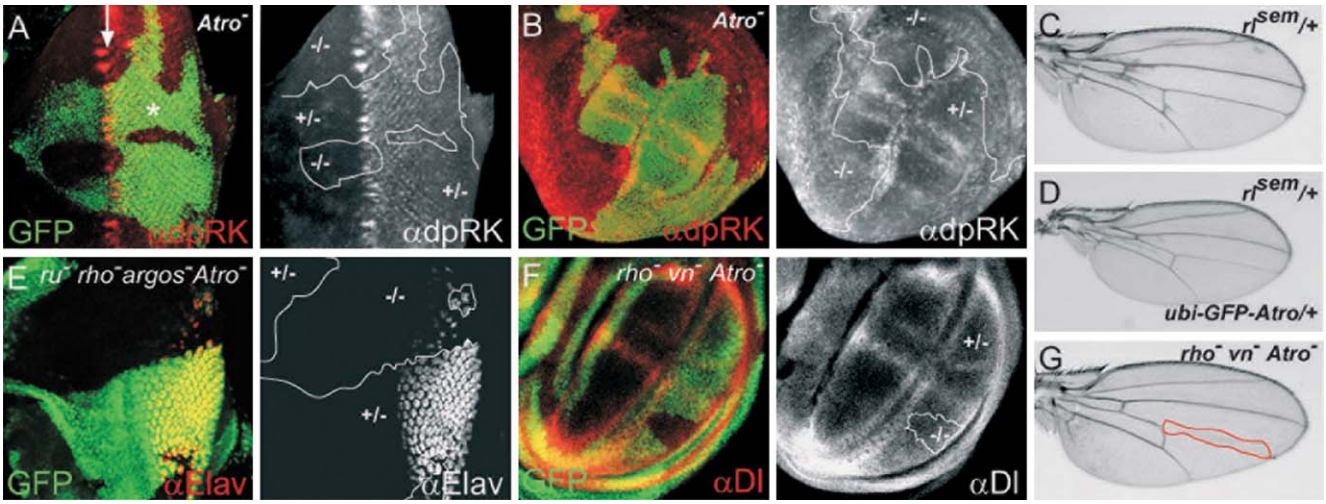


Fig. 5. Atro is acting downstream of the MAPK rolled. (A–B) The pattern and level of MAPK activation is not perturbed in the absence of *Atro* activity. Clones of *Atro*⁻ cells (A–B) in a third larval instar eye (A) or wing (B) imaginal disc, detected by the absence of GFP (green) and stained with anti-dpERK (red). (A) In the eye, neither the intense dpERK staining seen in the morphogenetic furrow (arrow in A), nor the weak staining in differentiating cells (asterisk in A), is affected in *Atro*⁻ cells. (B) In a wing pouch containing *Atro*⁻ cells, dpERK is detected along the veins including those each side of the wing margin as in wild type (data not shown). (C–D) Overexpression of Atro suppresses the phenotype caused by constitutive activation of MAPK. (C) The gain-of-function rl^{Sem} mutation induces the formation of ectopic veins. (D) Ubiquitous overexpression of Atro under the control of the ubiquitin promoter (*ubi-GFP-Atro*/+) suppresses the ectopic vein phenotype caused by rl^{Sem}/+. (E–G) In both the eye (E) and the wing pouch (F and G), loss of Atro function is not sufficient to induce surplus EGFR signaling. (E) Clones of *ru*⁻*rho*⁻*Atro*⁻*argos*⁻ cells (marked by absence of GFP, green) in the eye imaginal disc, and stained with anti-Elav (red). *ru*⁻*rho*⁻*Atro*⁻*argos*⁻ mutant clones lack PR cells, in contrast to *Atro*⁻*argos*⁻ clones where extra PRs are recruited (compare with Fig. 2C). (F) *Dl* is not ectopically expressed in clones of *rho*⁻*vn*⁻*Atro*⁻ mutant cells. Mutant clones, in the wing imaginal disc, are detected by the absence of GFP (green) and stained with anti-Dl (red). (F) In a *rho*⁻*vn*⁻*Atro*⁻, mutant clone located in the wing pouch, Dl is not detected. (G) Clones mutant for *vn*, *rho* and *Atro* do not differentiate veins in contrast to *Atro*⁻ clones where they do (compare with Fig. 2H).

However, when *UAS-Atro* and $UAS\text{-}Yan^{WT}$ are co-expressed, we observed a suppression of the extra-vein phenotype caused by $UAS\text{-}EGFR^{ACT}$ (Fig. 6L). Note that two copies of either $UAS\text{-}Yan^{WT}$ or *UAS-Atro* do not suppress the $UAS\text{-}EGFR^{ACT}$ phenotype (data not shown). This indicates that Atro and Yan cooperate genetically to inhibit signaling by the EGFR in the wing. Furthermore, we found that reducing *pnt* activity clearly enhances the rough eye phenotype caused by Atro overexpression (Figs. 6E–H), showing that Atro and Pnt have antagonistic activities during eye development. However, we have not been able to co-immunoprecipitate Yan and Atro, or Pnt and Atro, from *Drosophila* S2 cells (data not shown), indicating that these proteins may not directly bind to each other, at least in cultured S2 cells.

Expression of mutant human Atrophin-1 mimics loss of Atro activity in the fly

To get more insight into the function of human Atrophin-1 in vivo, we expressed in *Drosophila* an NH2-terminal truncation of expanded human Atrophin-1 (At-N917-65Q), a pathogenic form similar to a fragment seen in a transgenic mouse model (Nucifora et al., 2001; Schilling et al., 1999). The over-expression of *UAS-At-N917-65Q* under the regulation of *GMR-Gal4* driver induced progressive eye pigment loss (Figs. 7A, B), a phenotype often associated with expression of vertebrate polyQ containing proteins in the *Drosophila* eye (Takeyama et al., 2002; Warrick et al., 1998). We found that human At-N917-65Q is exclusively detected in nuclei, as shown in third instar larvae eye imaginal discs (Fig. 7C). No nuclear inclusions were observed at this developmental stage. This is in agreement with previous work showing that overexpression of human Atrophin-1 following transient transfection do not form aggregates (Nucifora et al., 2003).

When expressed in the wing using the *MS1096* driver or by the leaky activity of *GMR Gal4* in this tissue, *UAS-At-N917-65Q* promotes ectopic vein differentiation (Figs. 7D, E). This phenotype resembles the *Atro* loss of function phenotype and is caused by overactive EGFR signaling since halving the dose of negative regulators of EGFR pathway (such as *sty*, Fig. 7G, and *cic*, data not shown), or heterozygotes for rl^{Sem} (Fig. 7I), enhances this phenotype and co-expression of $UAS\text{-}EGFR^{DN}$ (a dominant negative form of the EGFR) suppresses it (Fig. 7K). We controlled the specificity of this phenotype by overexpressing a polyQ peptide (containing 48Q) or the exon 1 of Huntingtin with expanded polyQ (93Q). In both cases, wing vein differentiation was not affected (data not shown). Note, however, that overexpression of *UAS-At-N917-26Q* in the wing results in a similar phenotype to *UAS-At-N917-65Q* (data not shown), showing that polyQ expansion is not essential for the phenotype caused by overexpression of human Atrophin-1. The fact that overexpression of human At-N917-26Q/65Q leads to a similar phenotype as loss of *Drosophila Atro*, coupled with the characteristic interactions with the EGFR signaling, raises the possibility that human At-N917-26Q/65Q is acting as a dominant negative protein in the fly. In support of this hypothesis, we found that halving

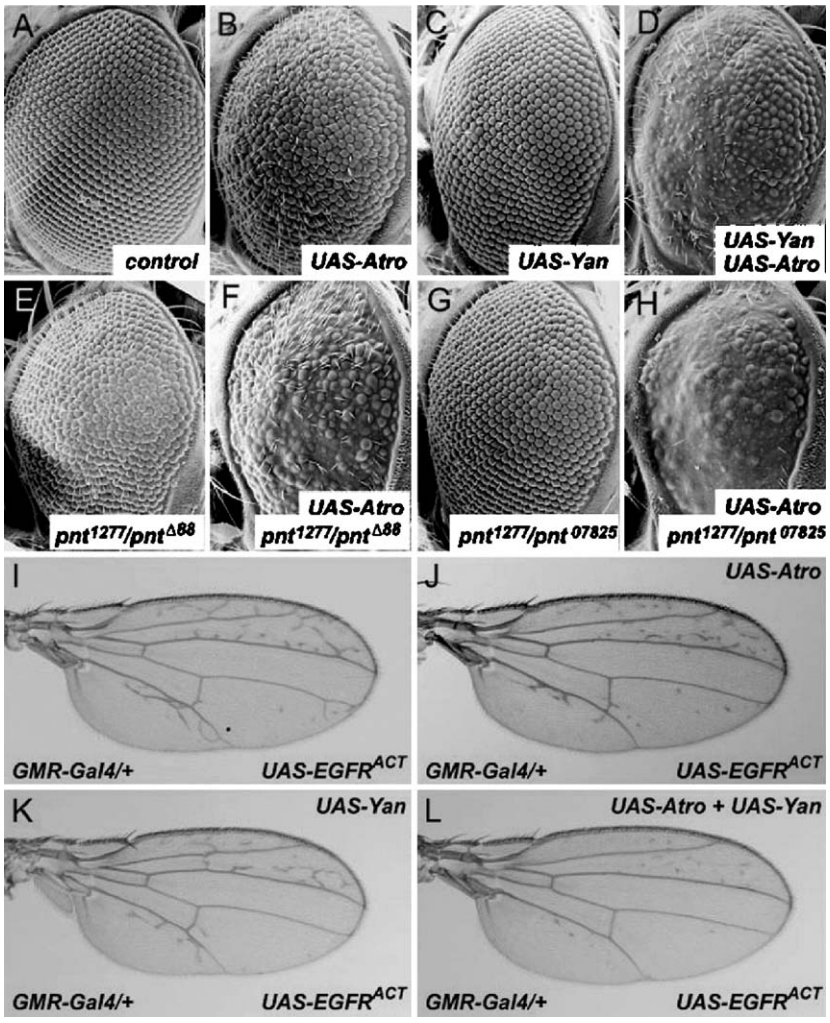


Fig. 6. *Atro* genetically interacts with *yan* and *pointed*. Flies were raised at 25°C for all panels. (A) Adult eye from *GMR-Gal4/+* flies. (B-D) Atro cooperates with Yan to inhibit eye development. (B) The eyes of *GMR-Gal4/UAS-Atro* flies are rough. Eyes overexpressing Yan^{WT} (C: *GMR-Gal4 UAS-*Yan^{WT}*/+*) display a weak rough eye phenotype. These phenotypes are enhanced when Atro is overexpressed with Yan^{WT} (D: *GMR-Gal4 UAS-*Yan^{WT}*/UAS-Atro*). (E–H) Atro and Pointed show antagonistic activities during eye development. Reduction of Pointed activity enhanced the rough eye phenotype of *GMR-Gal4/UAS-Atro* flies. *GMR-Gal4/+;* pnt^{1277}*/*$pnt^{\Delta 88}$ (E) and *GMR-Gal4/+;* pnt^{1277}*/*pnt^{07825} (G) flies display weak rough and normal eyes, respectively. These phenotypes are enhanced in flies overexpressing Atro (F: *GMR-Gal4/UAS-Atro;* pnt^{1277}*/*$pnt^{\Delta 88}$ and H: *GMR-Gal4/UAS-Atro;* pnt^{1277}*/*pnt^{07825}). (I–L) Atro and Yan cooperate to block EGFR signaling in the wing pouch. (I) Wings from *GMR-Gal4/+UAS-*$EGFR^{ACT}$*/+* flies have extra-wing vein material. Overexpression of *UAS-Atro* (J) or *UAS-*Yan^{WT} (K) have little or no effect on this phenotype (J: *GMR-Gal4/UAS-Atro; UAS-*$EGFR^{ACT}$*/+* and K: *GMR-Gal4 UAS-*Yan^{WT}*/+; UAS-*$EGFR^{ACT}$*/+*). However, the extra-vein phenotype caused by *UAS-*$EGFR^{ACT}$ is suppressed when *UAS-Atro* and *UAS-*Yan^{WT} are co-expressed (L; *GMR-Gal4 UAS-*Yan^{WT}*/UAS-Atro; UAS-*$EGFR^{ACT}$*/+*).

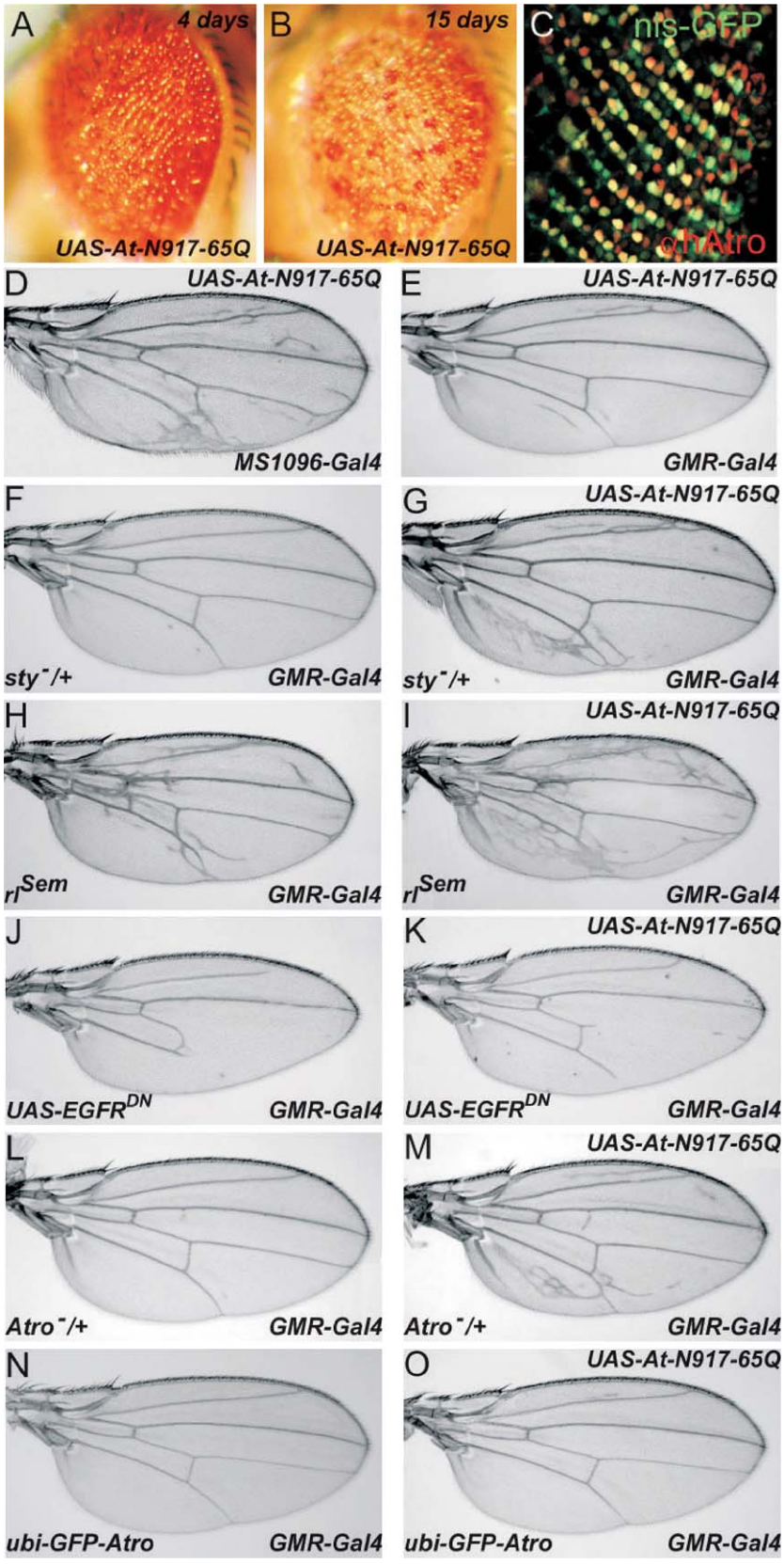
the dose of *Atro* enhances the ectopic vein phenotype caused by overexpression of *UAS-At-N917-26Q* in the wing (Fig. 7M), while ubiquitous expression of Atro suppresses it (Fig. 7O).

Discussion

Evidence that Atro contributes to the negative regulation of EGFR signaling is as follows. First, clones mutant for *Atro*

Fig. 7. Phenotypes of human Atrophin overexpression. Flies were raised at 29°C for all panels. (A–B) Eyes expressing At-N917-65Q (*GMR-Gal4/UAS-At-N917-65Q*), at 4 days (A) or 15 days (B). The eyes of 4-day-old flies expressing At-N917-65Q appeared relatively normal (A), with only slight disruption of the lattice. However, over time (B), the eye showed progressive pigment loss. Note that *GMR-Gal4* directs expression to pigment cells as well as PR neurons. (C) Human Atrophin-1 At-N917-65Q accumulates in nuclei of *Drosophila* cells. Eye imaginal disc from *GMR-Gal4/UAS-At-N917-65Q; UAS-nlsGFP/+* flies is stained with anti-human Atrophin-1 antibody APG840 (red in panel C). The At-N917-65Q protein (red) co-localizes with the nls-GFP fusion (green) in nuclei. (D–E) Overexpression of human Atrophin-1 At-N917-65Q promotes wing-vein differentiation. Wings of *MS1096-Gal4/+*; *UAS-At-N917-65Q/+* (D) or *GMR-Gal4 UAS-At-N917-65Q/+* (E) have extra wing-vein material when compared to Wild-type (see Fig. 1F). This phenotype is enhanced by sty^{-} heterozygotes (G: *GMRGal4 UAS-At-N917-65Q/+;* $sty^{\Delta 5}$*/+*), by rl^{Sem} heterozygotes (I: *GMRGal4 UAS-At-N917-65Q/*rl^{Sem}) and by $Atro^{-}$ heterozygotes (M: *GMRGal4 UAS-At-N917-65Q/+;* $Atro^{1207D6}$*/+*). *GMR-Gal4/+;* sty^{-}*/+* (F), *GMR-Gal4/*rl^{Sem} (H) and *GMR-Gal4/+;* $Atro^{-}$*/+* (L) wings are shown as controls. Overexpression of $EGFR^{DN}$ suppresses the ectopic vein phenotype caused by At-N917-65Q (*GMR-Gal4 UAS-At-N917-65Q/UAS-*$EGFR^{DN}$) (K). A *GMR-Gal4/UAS-*$EGFR^{DN}$ wing is shown as control (J). Ubiquitous expression of GFP-Atro suppresses the ectopic vein phenotype caused by At-N917-65Q (*GMR-Gal4 UAS-At-N917-65Q/+*; ubi-GFP-Atro/+) (O). A *GMR-Gal4/+*; ubi-GFP-Atro/+ wing is shown as control (N).

display phenotypes characteristic of overactive EGFR signaling and express high levels of the known EGFR target gene *Dl*. These effects are enhanced when negative regulators of EGFR signaling, such as Argos, are simultaneously removed in *Atro*$^{-}$ clones. Second, increased amounts of Atro reduce the activity of EGFR signaling. Third ectopic expression of



Atro enhances the effects of decreased EGFR signaling, whereas reduced Atro enhances the effects of ectopic signaling. Finally, Atro genetically interacts with *yan* suggesting that both repressors may cooperate to block EGFR signaling output.

The likely *C. elegans* orthologue of Atrophin, Egl27, has been shown to inhibit vulval development induced by the Ras signal transduction pathway (Solari and Ahringer, 2000; Solari et al., 1999). Thus, the role of Atro as a negative regulator of the RTK/EGFR pathway may have been conserved during evolution. Egl27 is a component of a repressor complex, the nucleosome remodeling and histone deacetylase (NURD) complex, which is composed of HDAC-1, HDAC-2, two proteins of the Mi-2/CHD family, and MTA1 or MTA2 (reviewed in Ahringer, 2000). During vulval induction, the NURD complex is proposed to interact with the sequence-specific transcription factors LIN-31, an Ets-related transcription factor and LIN-1, a winged-helix molecule. LIN-1 and LIN-31 are repressors of vulval development that are negatively regulated upon phosphorylation by the MAPK *mpk1/sur-1* (Tan et al., 1998).

MAPK-dependent phosphorylation of the ETS transcription factor Pnt is necessary for the activation of the EGFR target genes in third instar eye imaginal discs and in embryos (Brunner et al., 1994a; Gabay et al., 1996; O'Neill et al., 1994). We found that Yan and Atro show synergistic genetic interaction, suggesting that both are required for the repression of EGFR signaling function. Thus, by analogy with EGL-27 and LIN-31 from *C. elegans*, we propose a model where Yan cooperates with Atro in order to achieve tight repression. How does EGFR signaling counteract Atro-mediated repression? Localized downregulation (such as nuclear export and/or protein degradation) of specific repressors is a common mechanism for the activation of target genes by the EGFR pathway (Li et al., 1997; Mantrova and Hsu, 1998; Rebay and Rubin, 1995; Roch et al., 2002; Tsuda et al., 2002). Two observations argue against this mechanism for the co-repressor Atro. First, in cells with high levels of EGFR activity, such as either side of the dorso-ventral boundary in the wing pouch, or later in prospective veins of pupal wings, Atro protein is detected ubiquitously and at invariant levels in all nuclei (data not shown). Second, when EGFR signaling is overactivated in clones (by expressing the constitutive form of *EGFR, EGFR$^{\lambda CT}$*), the amount and/or subcellular localization of the co-expressed Atro protein is unchanged (data not shown).

Several lines of evidence show that, in the late phases of imaginal disc patterning, Atro plays a specific role for EGFR repression. We found that Atro does not contribute to other signaling pathways during imaginal disc development. For instance, expression of both *Distal-less* and the *vestigial* quadrant enhancer (*vgQE*), two known *wingless* (*wg*) target genes (Neumann and Cohen, 1996), is not affected in *Atro*$^{-}$ clones located in the wing pouch (Supplementary Fig. S1). Plus, we found that signaling from the Notch (N) receptor does not require Atro activity since *Atro*$^{-}$ clones expressing the constitutively active, intra-cellular fragment of the N receptor (*Nintra*) display identical phenotypes to *Nintra* control clones, when located in the wing pouch (see Supplementary Fig. S1).

Other signaling pathways are known to affect vein differentiation such as Decapentaplegic (DPP), which promotes vein differentiation in late pupae (Martin-Blanco et al., 1999), and N whose activity is necessary to restrict vein territories (de Celis and Bray, 1997). However, we favor the idea that Atro contributes mainly to EGFR signaling since Atro acts in third instar larvae and is dispensable for N activity in the wing (see Supplementary Fig. S1).

Differing modes of repression of EGFR targets in the nucleus

Despite the strong correlation of Atro repression of EGFR target genes in the imaginal discs, Atro is required for patterning where EGFR has not been implicated. For example, Atro is required for normal segmentation of the *Drosophila* embryo. However, we note that both EGFR signaling and Atro are required for cell survival during embryogenesis (Erkner et al., 2002; Urban et al., 2004). Additionally, Atro is not required for all EGFR-dependent events. For example, Atro is not involved in the function of the EGFR defining the identity of the proximal wing disc (Zecca and Struhl, 2002a,b) (data not shown). These observations indicate that variable mechanisms of control are implicated in the negative regulation of EGFR signaling in the nucleus.

This notion is supported even in different imaginal tissues. Tsuda et al. (2002) reported that EGFR signals via *Strawberry notch* (*Sno*) and *Ebi*, to inhibit the repressor activity of a Su(H)/SMRTER complex, leading to activation of *Dl* expression. Clones of cells mutant for the *Su(H)^{SF8}*, hypomorphic allele cause high level expression of Dl in PR cells, but not in the wing pouch (Tsuda, personal communication). We found that clones of cells mutant for the *Su(H)^{del47}*, null allele (Morel and Schweisguth, 2000) similarly do not show ectopic expression of Dl in the wing pouch (Supplementary Fig. S2). As expected, *Su(H)^{del47}* cells located at the D/V border abolish the expression of Cut (Supplementary Fig. S2). Thus, *Su(H)*, unlike *Atro*, is dispensable for *Dl* repression in the wing pouch. The reverse is true in the eye, where Su(H) activity is absolutely required to repress *Dl* expression (Tsuda et al., 2002) whereas Atro is less important (Fig. 4A). This is in agreement with the weak phenotype caused by the *Atro*$^{-}$ clones in the eye (i.e. no ectopic PRs, few extra cone cells), and indicates a redundancy with other negative regulators of EGFR signaling. This distinction between the relative requirements in different tissues for different regulators of EGFR signaling provides an interesting insight into tissue-specific control of ubiquitous signaling pathways. Regulators such as Atro, with functions restricted to some tissues, may contribute to the diverse outcomes of signaling through these common pathways.

Dentatorubral-pallidoluysian atrophy (DRPLA) is a dominant, hereditary malady typified by the degeneration of specific neurons in the brain. Although DRPLA has been mimicked in a mouse model, the molecular and cellular mechanisms leading to the disease remain obscure. Our data point to the role of Atro in the repression of EGFR signaling. We found that expression of human N917Atrophin-1 in the wing mimics the loss of Atro activity which raises the possibility that N917Atrophin-1 is acting as a dominant negative. Additionally, this phenotype is

independent of polyQ expansion and is sensitive to the dose of EGFR signaling components. Such effects are not seen following expression of polyQ repeats alone or the exon 1 of Huntingtin with expanded polyQ (93Q) in the wing, indicating that human N917Atrophin-1 has specific effects on this pathway. This mechanistic insight into the role of the fly gene may have broader implications concerning Atrophin function in other organisms.

Acknowledgments

Thanks are due to H. Bellen, S. Blair, D. Borchelt, S. Carroll, S. Cohen, K. Irvine, T. Klein, Mattias Mannervik, A. Martinez Arias, M. Milan, Randy Mottus, I. Rebay, F. Schweisguth and J. Wood who provided many of the materials used in this study as well as answering our queries over the e-mail. Special thanks to Michèle Delaage for technical assistance. A.B. was supported by a Marie Curie fellowship from the EU and a grant from Association for International Cancer Research. This work was possible with funds from the Centre National de la Recherche Scientifique, the Association de la Recherche contre le Cancer, the Fédération pour la Recherche sur le Cerveau and from a European Community grant to SK (HRPT2000).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.012.

References

Ahringer, J., 2000. NuRD and SIN3 histone deacetylase complexes in development. Trends Genet. 16, 351–356.

Baonza, A., Roch, F., Martin-Blanco, E., 2000. DER signaling restricts the boundaries of the wing field during *Drosophila* development. Proc. Natl. Acad. Sci. U. S. A. 97, 7331–7335.

Bier, E., Jan, L.Y., Jan, Y.N., 1990. rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. Genes Dev. 4, 190–203.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brown, K.E., Freeman, M., 2003. Egfr signalling defines a protective function for ommatidial orientation in the *Drosophila* eye. Development 130, 5401–5412.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H., Klambt, C., 1994a. The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway. Nature 370, 386–389.

Brunner, D., Oellers, N., Szabad, J., Biggs III, W.H., Zipursky, S.L., Hafen, E., 1994b. A gain-of-function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 76, 875–888.

Campbell, G., 2002. Distalization of the *Drosophila* leg by graded EGF-receptor activity. Nature 418, 781–785.

Casci, T., Vinos, J., Freeman, M., 1999. Sprouty, an intracellular inhibitor of Ras signaling. Cell 96, 655–665.

Davis, I., Girdham, C.H., O'Farrell, P.H., 1995. A nuclear GFP that marks nuclei in living *Drosophila* embryos; maternal supply overcomes a delay in the appearance of zygotic fluorescence. Dev. Biol. 170, 726–729.

de Celis, J.F., Bray, S., 1997. Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the *Drosophila* wing. Development 124, 3241–3251.

Diaz-Benjumea, F.J., Hafen, E., 1994. The sevenless signalling cassette mediates *Drosophila* EGF receptor function during epidermal development. Development 120, 569–578.

Dominguez, M., Wasserman, J.D., Freeman, M., 1998. Multiple functions of the EGF receptor in *Drosophila* eye development. Curr. Biol. 8, 1039–1048.

Erkner, A., Roure, A., Charroux, B., Delaage, M., Holway, N., Core, N., Vola, C., Angelats, C., Pages, F., Fasano, L., et al., 2002. Grunge, related to human Atrophin-like proteins, has multiple functions in *Drosophila* development. Development 129, 1119–1129.

Fanto, M., Clayton, L., Meredith, J., Hardiman, K., Charroux, B., Kerridge, S., McNeill, H., 2003. The tumor-suppressor and cell adhesion molecule Fat controls planar polarity via physical interactions with Atrophin, a transcriptional co-repressor. Development 130, 763–774.

Freeman, M., 1996. Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell 87, 651–660.

Freeman, M., Klambt, C., Goodman, C.S., Rubin, G.M., 1992. The argos gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. Cell 69, 963–975.

Gabay, L., Scholz, H., Golembo, M., Klaes, A., Shilo, B.Z., Klambt, C., 1996. EGF receptor signaling induces pointed P1 transcription and inactivates Yan protein in the *Drosophila* embryonic ventral ectoderm. Development 122, 3355–3362.

Gabay, L., Seger, R., Shilo, B.Z., 1997. In situ activation pattern of *Drosophila* EGF receptor pathway during development. Science 277, 1103–1106.

Galindo, M.I., Bishop, S.A., Greig, S., Couso, J.P., 2002. Leg patterning driven by proximal–distal interactions and EGFR signaling. Science 297, 256–259.

Golembo, M., Raz, E., Shilo, B.Z., 1996a. The *Drosophila* embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. Development 122, 3363–3370.

Golembo, M., Schweitzer, R., Freeman, M., Shilo, B.Z., 1996b. Argos transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. Development 122, 223–230.

Jarman, A.P., Grau, Y., Jan, L.Y., Jan, Y.N., 1993. atonal is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. Cell 73, 1307–1321.

Kimmel, B.E., Herbelein, U., Rubin, G.M., 1990. The homeo domain protein rough is expressed in a subset of cells in the developing *Drosophila* eye where it can specify photoreceptor cell subtype. Genes Dev. 4, 712–727.

Lee, J.R., Urban, S., Garvey, C.F., Freeman, M., 2001. Regulated intracellular ligand transport and proteolysis control EGF signal activation in *Drosophila*. Cell 107, 161–171.

Li, S., Li, Y., Carthew, R.W., Lai, Z.C., 1997. Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack. Cell 90, 469–478.

Mantrova, E.Y., Hsu, T., 1998. Down-regulation of transcription factor CF2 by *Drosophila* Ras/MAP kinase signaling in oogenesis: cytoplasmic retention and degradation. Genes Dev. 12, 1166–1175.

Martin-Blanco, E., Roch, F., Noll, E., Baonza, A., Duffy, J.B., Perrimon, N., 1999. A temporal switch in DER signaling controls the specification and differentiation of veins and interveins in the *Drosophila* wing. Development 126, 5739–5747.

Micchelli, C.A., Rulifson, E.J., Blair, S.S., 1997. The function and regulation of cut expression on the wing margin of *Drosophila*: Notch, Wingless and a dominant negative role for Delta and Serrate. Development 124, 1485–1495.

Morel, V., Schweisguth, F., 2000. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the *Drosophila* embryo. Genes Dev. 14, 377–388.

Naito, H., Oyanagi, S., 1982. Familial myoclonus epilepsy and choreoathetosis: hereditary dentatorubral-pallidoluysian atrophy. Neurology 32, 798–807.

Neumann, C.J., Cohen, S.M., 1996. A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the *Drosophila* wing. Development 122, 3477–3485.

Nucifora Jr., F.C., Sasaki, M., Peters, M.F., Huang, H., Cooper, J.K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V.L., et al., 2001. Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. Science 291, 2423–2428.

Nucifora Jr., F.C., Ellerby, L.M., Wellington, C.L., Wood, J.D., Herring, W.J.,

Sawa, A., Hayden, M.R., Dawson, V.L., Dawson, T.M., Ross, C.A., 2003. Nuclear localization of a non-caspase truncation product of atrophin-1, with an expanded polyglutamine repeat, increases cellular toxicity. J. Biol. Chem. 278, 13047–13055.

O'Neill, E.M., Rebay, I., Tjian, R., Rubin, G.M., 1994. The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. Cell 78, 137–147.

Perrimon, N., Perkins, L.A., 1997. There must be 50 ways to rule the signal: the case of the *Drosophila* EGF receptor. Cell 89, 13–16.

Queenan, A.M., Ghabrial, A., Schupbach, T., 1997. Ectopic activation of torpedo/Egfr, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development 124, 3871–3880.

Rebay, I., Rubin, G.M., 1995. Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell 81, 857–866.

Roch, F., Jimenez, G., Casanova, J., 2002. EGFR signalling inhibits Capicua-dependent repression during specification of *Drosophila* wing veins. Development 129, 993–1002.

Ross, C.A., Becher, M.W., Colomer, V., Engelender, S., Wood, J.D., Sharp, A. H., 1997a. Huntington's disease and dentatorubral-pallidoluysian atrophy: proteins, pathogenesis and pathology. Brain Pathol. 7, 1003–1016.

Ross, C.A., Margolis, R.L., Rosenblatt, A., Ranen, N.G., Becher, M.W., Aylward, E., 1997b. Huntington disease and the related disorder, dentatorubral-pallidoluysian atrophy (DRPLA). Medicine (Baltimore) 76, 305–338.

Sawamoto, K., Okano, H., Kobayakawa, Y., Hayashi, S., Mikoshiba, K., Tanimura, T., 1994. The function of argos in regulating cell fate decisions during *Drosophila* eye and wing vein development. Dev. Biol. 164, 267–276.

Schilling, G., Wood, J.D., Duan, K., Slunt, H.H., Gonzales, V., Yamada, M., Cooper, J.K., Margolis, R.L., Jenkins, N.A., Copeland, N.G., et al., 1999. Nuclear accumulation of truncated atrophin-1 fragments in a transgenic mouse model of DRPLA. Neuron 24, 275–286.

Schnepp, B., Grumbling, G., Donaldson, T., Simcox, A., 1996. Vein is a novel component in the *Drosophila* epidermal growth factor receptor pathway with similarity to the neuregulins. Genes Dev. 10, 2302–2313.

Schweitzer, R., Howes, R., Smith, R., Shilo, B.Z., Freeman, M., 1995. Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. Nature 376, 699–702.

Shilo, B.Z., 2003. Signaling by the *Drosophila* epidermal growth factor receptor pathway during development. Exp. Cell Res. 284, 140–149.

Smith, J.K., Gonda, V.E., Malamud, N., 1958. Unusual form of cerebellar ataxia; combined dentato-rubral and pallido-Luysian degeneration. Neurology 8, 205–209.

Solari, F., Ahringer, J., 2000. NURD-complex genes antagonise Ras-induced vulval development in *Caenorhabditis elegans*. Curr. Biol. 10, 223–226.

Solari, F., Bateman, A., Ahringer, J., 1999. The *Caenorhabditis elegans* genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning. Development 126, 2483–2494.

Spradling, A.C., Rubin, G.M., 1982. Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science 218, 341–347.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, A.H., Hoffmann, F.M., 1994. Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by decapentaplegic but not 60A. Cell Growth Differ. 5, 585–593.

Strutt, H., Strutt, D., 2003. EGF signaling and ommatidial rotation in the *Drosophila* eye. Curr. Biol. 13, 1451–1457.

Sturtevant, M.A., Roark, M., Bier, E., 1993. The *Drosophila* rhomboid gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. Genes Dev. 7, 961–973.

Takeyama, K., Ito, S., Yamamoto, A., Tanimoto, H., Furutani, T., Kanuka, H., Miura, M., Tabata, T., Kato, S., 2002. Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in *Drosophila*. Neuron 35, 855–864.

Tan, P.B., Lackner, M.R., Kim, S.K., 1998. MAP kinase signaling specificity mediated by the LIN-1 Ets/LIN-31 WH transcription factor complex during *C. elegans* vulval induction. Cell 93, 569–580.

Tootle, T.L., Lee, P.S., Rebay, I., 2003. CRM1-mediated nuclear export and regulated activity of the Receptor Tyrosine Kinase antagonist YAN require specific interactions with MAE. Development 130, 845–857.

Tsuda, L., Nagaraj, R., Zipursky, S.L., Banerjee, U., 2002. An EGFR/Ebi/Sno pathway promotes delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling. Cell 110, 625–637.

Urban, S., Lee, J.R., Freeman, M., 2001. *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107, 173–182.

Urban, S., Brown, G., Freeman, M., 2004. EGF receptor signalling protects smooth-cuticle cells from apoptosis during *Drosophila* ventral epidermis development. Development 131, 1835–1845.

Vinos, J., Freeman, M., 2000. Evidence that Argos is an antagonistic ligand of the EGF receptor. Oncogene 19, 3560–3562.

Wang, S.H., Simcox, A., Campbell, G., 2000. Dual role for *Drosophila* epidermal growth factor receptor signaling in early wing disc development. Genes Dev. 14, 2271–2276.

Warrick, J.M., Paulson, H.L., Gray-Board, G.L., Bui, Q.T., Fischbeck, K.H., Pittman, R.N., Bonini, N.M., 1998. Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. Cell 93, 939–949.

Wasserman, J.D., Urban, S., Freeman, M., 2000. A family of rhomboid-like genes: /r *Drosophila* rhomboid-1 and roughoid homboid-3 cooperate to activate EGF receptor signaling. Genes Dev. 14, 1651–1663.

Wolff, T., 2003. EGF receptor signaling: putting a new spin on eye development. Curr. Biol. 13, R813–R814.

Wood, J.D., Nucifora Jr., F.C., Duan, K., Zhang, C., Wang, J., Kim, Y., Schilling, G., Sacchi, N., Liu, J.M., Ross, C.A., 2000. Atrophin-1, the dentato-rubral and pallido-luysian atrophy gene product, interacts with ETO/MTG8 in the nuclear matrix and represses transcription. J. Cell Biol. 150, 939–948.

Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223–1237.

Xu, C., Kauffmann, R.C., Zhang, J., Kladny, S., Carthew, R.W., 2000. Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the *Drosophila* eye. Cell 103, 87–97.

Zecca, M., Struhl, G., 2002a. Control of growth and patterning of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. Development 129, 1369–1376.

Zecca, M., Struhl, G., 2002b. Subdivision of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. Development 129, 1357–1368.

Zhang, S., Xu, L., Lee, J., Xu, T., 2002. *Drosophila* atrophin homolog functions as a transcriptional corepressor in multiple developmental processes. Cell 108, 45–56.