Interaction between Drosophila bZIP proteins Atf3 and Jun prevents replacement of epithelial cells during metamorphosis

Petra Sekyrova1, Dirk Bohmann2, Marek Jindra1,* and Mirka Uhlirova3,*

SUMMARY
Epithelial sheet spreading and fusion underlie important developmental processes. Well-characterized examples of such epithelial morphogenetic events have been provided by studies in Drosophila, and include embryonic dorsal closure, formation of the adult thorax and wound healing. All of these processes require the basic region-leucine zipper (bZIP) transcription factors Jun and Fos. Much less is known about morphogenesis of the fly abdomen, which involves replacement of larval epidermal cells (LECs) with adult histoblasts that divide, migrate and finally fuse to form the adult epidermis during metamorphosis. Here, we implicate Drosophila Activating transcription factor 3 (Atf3), the single ortholog of human ATF3 and JDP2 bZIP proteins, in abdominal morphogenesis. During the process of the epithelial cell replacement, transcription of the atf3 gene declines. When this downregulation is experimentally prevented, the affected LECs accumulate cell-adhesion proteins and their extrusion and replacement with histoblasts are blocked. The abnormally adhering LECs consequently obstruct the closure of the adult abdominal epithelium. This closure defect can be either mimicked and further enhanced by knockdown of the small GTPase Rho1 or, conversely, alleviated by stimulating ecdysone steroid hormone signaling. Both Rho and ecdysone pathways have been previously identified as effectors of the LEC replacement. To elicit the gain-of-function effect, Atf3 specifically requires its binding partner Jun. Our data thus identify Atf3 as a new functional partner of Drosophila Jun during development.

KEY WORDS: Epithelial cell replacement, Cell adhesion, Epidermis, Metamorphosis, Abdomen, bZIP

INTRODUCTION
Metamorphosis of Drosophila larvae into pupae and adult flies provides remarkable examples of morphogenetic changes that involve replacement of entire cell populations. Epithelia that had served larval function undergo programmed cell death while imaginal cells proliferate and differentiate to take their position. The Drosophila abdomen is an attractive system for studying the developmental replacement of one epithelial cell population with another. Unlike the adult head and thorax with appendages, all forming from pre-patterned imaginal discs, the adult abdomen derives from histoblasts that reside in each abdominal segment (Madhavan and Madhavan, 1980; Madhavan and Schneiderman, 1977). Soon after the onset of metamorphosis, the diploid histoblasts undergo an initial phase of synchronized cell divisions; later the histoblasts expand while proliferating and replace the old polyplid larval epidermal cells (LECs) that cover the surface of the abdomen (Ninov et al., 2007; Bischoff and Cseresnyes, 2009). To free space for the histoblasts, LECs are extruded from the epithelial monolayer. In order to maintain integrity of the epithelia, changes in cell adhesion and cell migration must be precisely orchestrated during this tissue remodeling.

Ninov and colleagues have demonstrated that Rho kinase signaling, which stimulates constriction of the apical actomyosin cytoskeleton through myosin phosphorylation, is necessary for the extrusion and the ensuing apoptosis of LECs (Ninov et al., 2007). Perturbed myosin phosphorylation leaves the process of the epithelial exchange incomplete, with residual LECs obstructing closure of the adult abdominal epidermis at the dorsal midline. A similar defect results from compromised function of the ecdysone receptor (EcR), which is required for both the initial phase of histoblast proliferation (Ninov et al., 2009) and for the removal of LECs (Ninov et al., 2007). Other factors besides Rho signaling and EcR that regulate the epithelial cell replacement are unknown.

We have implicated Atf3 (A3-3 – FlyBase), the single Drosophila ortholog of the vertebrate Activating transcription factor 3 (ATF3) and Jun dimerization protein 2 (JDP2) in abdominal development. ATF3 and JDP2 belong among basic region-leucine zipper (bZIP) proteins, some of which play important roles in epithelial morphogenesis (Kockel et al., 2001; Xia and Karin, 2004). Particularly the functions of Jun (Jra – FlyBase) and Fos (Kayak – FlyBase) bZIP proteins in epithelial closure events during development are well understood owing to genetic studies in Drosophila (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar et al., 2003; Kawauchi et al., 2002; Lu et al., 2006; Mashima et al., 2001; Nakagomi et al., 2003; Nobori et al., 2002; Tamura et al., 2005; Yin et al., 2008) and JDP2 (Aronheim et al., 1997; Heinrich et al., 2004;
Jin et al., 2002; Ostrovsky et al., 2002; Piu et al., 2001). Atf3-/– mice are viable (Hartman et al., 2004) but suffer from altered glucose and immune homeostasis (Gilchrist et al., 2006; Qi et al., 2009; Rosenberger et al., 2008). Also Jdp2-/- mice survive but produce extra fat in their brown adipose tissue (Nakade et al., 2007). In vivo significance of the interaction between the ATF3 or JDP2 proteins and Jun remains unclear.

Here we show that Atf3 interacts biochemically and genetically with Jun in Drosophila. Temporal downregulation of af3 transcription during metamorphosis is crucial, as sustained atf3 expression alters adhesive properties of LECs, thus preventing their exposure and replacement by the adult epithmis. This effect of Atf3 expression alters adhesive properties of LECs, thus preventing their

MATERIALS AND METHODS

Fly strains and preparation of atf3 mutants

Fly strains obtained from the Bloomington Drosophila Stock Center included w1118, arm-Gal4, Esp1CD-Gal4, Cg-Gal4, Eip2-Gal4, T155-Gal4, P{EPgy}2.edu[37]-FRT-YAC103562, (UAS-Repo)1114, UAS-p35, UAS-diap1, hsFlp, act5c-FRT-2FRT-Gal4, UAS-EGFP and UAS-myr mRFp. Additional lines were esp-Gal4 (National Institute of Genetics, Mishima, Japan), ppl-Gal4 (Colombani et al., 2003), sGMCA (Kiehart et al., 2000), C7-Gal4 (from B. Edgar, Seattle, USA) and GMR-Gal4 (from M. Mlodzik, New York, USA). UAS-α-Cat.TG- and ubi-p65E-shgc; GFP (Oda and Tsukita, 2001) were obtained from the Drosophila Genetic Resource Center (Kyoto, Japan), and UAS-Rho1RNAi (stock v12734) was from the Vienna Drosophila RNAi Center. The UAS-JunRNAm and UAS-fosRNAm (Hyun et al., 2006) lines were a gift from C. Yanicostas (Paris, France). UAS-Jun, UAS-fos, UAS-fosZ2P and UAS-fosZ4RNAm lines were described previously (Ciapponi et al., 2001; Eresh et al., 1997; Zeitlinger et al., 1997).

Atf3 mutants were generated by imprecise excision of a P-element P{EPgy}2.edu[37]-FRT-YAC103562 from the second intron of atf3, and deletions were detected by nested PCR with primers flanking the original insertion site of the P-element in crude genomic DNA extracts. Sequencing of genomic DNA amplified from atf3 mutants with primers 5’-GTGCTGTAACCGTCAACGTCGACC-3’ and 5’-ATGCTGCTGTGCAATCAGTCGTTG3’ within the first and the third atf3 exons, respectively, determined the exact limits of the deletions.

atf3 misexpression

Transgenic flies were obtained by P-element mediated germline transformation (Genetic Services, Sudbury, USA). To express the entire Atf3 protein, the atf3 open reading frame (613 codons) was amplified with primers 5’-GGTGGTACCGTCAACCGTCAACGTCGACC-3’ and 5’-GGTCTAGATCCCCGCTCCTGCTG3’, and inserted into the pUAST vector. To express the Atf3 bZIP domain (amino acids 207-261), a cDNA fragment encoding residues 199-269 of Atf3 was subcloned into plasmids carrying PCR amplified genomic DNA from atf3 exons, respectively, determined the exact limits of the deletions.

atf3 expression analysis

N-terminal blots with 5 μg of total RNA per lane were hybridized with an atf3 antisense cRNA digoxigenin-labeled probe. For RT-PCR, cDNA was prepared with the Superscript II system (Invitrogen, Carlsbad, CA) from 2 μg of total DNA-treated RNA. cDNA samples were subjected to standard PCR reactions. Quantitative RT-PCR was performed with the iQ SYBR Green kit (BioRad, Hercules, CA) using the RotorGene RG 3000 cycler (Corbett Research, Sydney, Australia). In situ hybridization of atf3 mRNA was performed using a digoxigenin-labeled atf3 antisense probe as described (Tautz and Pfeifle, 1989).

Immunostaining and confocal microscopy

Dorsal pupal epidermis attached to the cuticle was fixed for 30 minutes with 4% formaldehyde in PBS, blocked in 0.1% BSA, 0.3% Triton X-100 in PBS for 1 hour, and incubated overnight at 4°C with primary antibodies against Drosophila Dlg (4F3; 1:1000), Armadillo (N27A1; 1:100) or RhoI (1D9; 1:100) from the Developmental Studies Hybridoma Bank (Iowa, USA). The tissue was washed with PBS, 0.3% Triton X-100, incubated for 2 hours with Cy3- or Alexa flour 488-conjugated antibodies, counterstained with 4 μg/ml DAPI, and analyzed under the Olympus FV1000 confocal microscope. For imaging of live pupae, animals were removed from the puparia, placed into a drop of halocarbon oil and analyzed using a Leica SP2 confocal microscope. The images were processed with IMARIS (Bitplane) and ImageJ (NIH) software.

Electrophoresis mobility shift assay

The full-length open reading frame was cloned into the pET-28a vector (Novagen) using Ndel and BamHI restriction sites. Using an N-terminal histidine tag, the bZIP domains of Atf3 and of the Jun and Fos proteins were purified from Escherichia coli and used in DNA-binding assays as described (Jindra et al., 2004). Double-stranded oligonucleotides comprising the ATF/CRE element (underlined) (Fawcett et al., 1999; Montminy et al., 1986), TGAGCCCG-AATGGACGTCAACCGGGGCGTGTGCAGG, or the AP-1 consensus site (Eresh et al., 1997; Perkins et al., 1990) were used as probes.

Immunoprecipitation

The full-length open reading frame was cloned into the pMT-V5-His B vector (Invitrogen) using Xhol and ApaI restriction sites. pMT-ATF3-His/V5 (5 μg) or the empty plasmid was co-transfected with a vector expressing a C-terminally histidine-tagged Fos protein into Drosophila S2 cells. Cells were harvested 30 hours after induction with 0.5 mM CuSO4, and processed as described (Jindra et al., 2004). The lysates (100 μl per assay) were incubated for 2 hours at 4°C with 20 μl of Protein G-conjugated Dynabeads (Invitrogen), combined with 1 μg of the mouse anti-V5 antibody (Invitrogen). Bound proteins were analyzed on western blots with anti-Jun (1:1500) (Bohmann et al., 1994) and anti-Fos (1:1000) (Zeitlinger et al., 1997) rabbit antibodies.

RESULTS

The Drosophila ortholog of ATF3/JDP2 interacts with Jun

Among Drosophila bZIP proteins, the predicted product of the CG11405 gene (also referred to as a3-3), located on the X chromosome, shows the closest similarity to the mammalian ATF3 and JDP2 proteins. The DNA-binding/dimerization bZIP domains of the human ATF3 and Drosophila Atf3 proteins are identical in 60% of their amino acids (Fig. 1A); there is 58% identity between Atf3 and JDP2 in this region.

Dimerization between Atf3 and Jun in Drosophila has been theoretically predicted (Fassler et al., 2002) and confirmed by results of a yeast two-hybrid screen (Giot et al., 2003). To demonstrate direct binding, we conducted co-immunoprecipitation experiments. The endogenous Jun protein from Drosophila S2 cells co-precipitated with a transiently expressed Atf3 whereas Fos did not (Fig. 1B). Next, we performed a DNA mobility-shift assay with recombinant bZIP domains of Atf3, Jun and Fos to test for their DNA-binding properties. We found that Atf3 specifically bound an ATF/CRE consensus element but not the AP-1 site, which was recognized by the Jun-Fos (AP-1) complex (Fig. 1C). Although Atf3 bound DNA by itself, presumably as a homodimer, the binding was enhanced in the presence of Jun (compare lanes 7 and 8 in Fig. 1C).
Fos did not synergize with Atf3 in DNA binding (lane 9). Excess unlabeled DNA bearing the ATF/CRE binding site competed for the Atf3 bandshift activity whereas the AP-1 binding element did not (data not shown). These results have shown that, like ATF3 or JDP2 in mammals, Atf3 in Drosophila selectively dimerizes with Jun, with which it cooperatively and specifically binds the ATF/CRE DNA element.

**Atf3 cooperates with Jun in vivo**

To test whether Atf3 and Jun interact in vivo, we conducted experiments in the *Drosophila* compound eye, the precise structure of which sensitively reflects genetic interactions. Overexpression of *atf3* under the GMR-Gal4 driver disrupted the ommatidial arrangement, resulting in smaller eyes with a glossy appearance (Fig. 2). This *atf3* misexpression phenotype could be completely suppressed by simultaneous RNAi-mediated knockdown of *jun* (Fig. 2) but not of *fos* (data not shown). Conversely, the phenotype was exacerbated when *jun* was overexpressed in the eye together with *atf3* (Fig. 2), suggesting that it is Atf3 in a complex with Jun that derails the normal eye development. Neither RNAi nor overexpression of *jun* alone had any effect on eye morphology. Interestingly, like depletion of Jun, co-expression of *fos* under the GMR-Gal4 driver completely averted the *atf3* misexpression phenotype, restoring the normal appearance of the eye (Fig. 2). Expression of *fos* (data not shown) or its mutant versions (see Fig. S1 in the supplementary material) alone had no effect. These data can be explained by the ability of the surplus Fos to bind Jun and thus reduce its availability for interaction with the Atf3 protein. This interpretation is further supported by experiments showing that expression of the truncated bZIP domain of Fos was sufficient to suppress the Atf3 gain-of-function phenotype, whereas its transcription activation domain or phosphorylation sites were dispensable (see Fig. S1 in the supplementary material).

Taken together, our results show that Atf3 cooperates with Jun, as Jun is specifically required for an effect caused by overexpression of Atf3 in the developing eye. Given the capacity of both Atf3 and Fos to bind Jun, and based on the ability of Jun to enhance and of Fos to suppress the Atf3 gain-of-function phenotype, we suggest that Atf3 and Fos compete for their common partner Jun in vivo.

**atf3 is an essential gene with transcription dynamically regulated during metamorphosis**

To find out whether Atf3 is required for Drosophila development and whether its absence might resemble a phenotype caused by loss of its partner Jun, we generated *atf3* mutant flies. The longest deletion (line *atf3*76) obtained by imprecise excision of a P element (see Fig. S2A in the supplementary material), removed the entire bZIP domain of Atf3, and *atf3*76 hemizygous (male) larvae lacked detectable *atf3* mRNA (see Fig. S2B in the supplementary material). Thus, *atf3*76 probably represents a null allele. Most *atf3*76 larvae died soon after hatching and during all three larval stages. Only a few (approximately 2%) reached the third instar but died before metamorphosis as defective pseudopuparia (see Fig. S2C in the supplementary material). Expression of *atf3* cDNA under the ubiquitous armadillo (*arm-Gal4*) driver rescued some *atf3*76 hemizygotes to adults, confirming that loss of *atf3* was the cause of the lethal phenotype. Interestingly, the moribund *atf3*76 larvae...
abnormally enlarged lipid droplets in their fat body (see Fig. S2D in the supplementary material), thus displaying a phenotype reminiscent of that in mice lacking one of the Atf3 orthologs, JDP2 (Nakade et al., 2007). However, in contrast to viable Jdp2 or Atf3 (Hartman et al., 2004) knockout mice, atf3 is an essential gene in Drosophila.

Fly embryos lacking the function of Jun or Fos die because of the failed dorsal closure (Hou et al., 1997; Kockel et al., 1997; Riesgo-Escovar and Hafen, 1997). However, atf3 embryos develop normally, without the dorsal open defect, even when derived from atf3-deficient germline clones induced in atf376/ovoD1 mothers (data not shown). Thus, unlike its partner Jun, Atf3 is not required for dorsal closure, suggesting that dorsal closure is regulated by Jun-Fos dimers and that the Atf3-Jun complex has another function later in development.

Consistent with the vital requirement for Atf3 during larval stages, atf3 mRNA was expressed in embryos and larvae (Fig. 3A). Expression then sharply declined by the late-third larval instar, and no atf3 mRNA was detected by northern blot hybridization in wandering larvae and during metamorphosis from the time of puparium formation until the second day of pupal development. Detailed RT-PCR analysis showed that atf3 downregulation coincided with the cessation of feeding and the onset of metamorphosis (0 hours after puparium formation (APF)) (Fig. 3B). A pulse of expression occurred at 6 hours APF. RT-PCR from isolated fat body and abdominal integuments, together with in situ hybridization performed on puparia at this stage, showed that atf3 mRNA was primarily present in the larval epidermis (LECs) during the expression peak at 6 hours APF (Fig. 3C, D). At the peak of expression at 6 hours APF (see panel B), atf3 mRNA occurs in the larval epidermis as shown by RT-PCR (C) performed on isolated integuments (Epi), fat bodies (FB) and whole prepupae (PP). In situ hybridization (D) shows atf3 mRNA in LECs. Scale bar: 100 μm.

![Fig. 3. Expression of atf3 mRNA is developmentally regulated.](image1)

---

**Fig. 4. Misexpression of atf3 causes incomplete fusion of abdominal epidermis.** (A) ppl>atf3 pharate adults usually die inside puparia with a dorsally open abdominal cleft in the adult cuticle. The scar is outlined with a brown line of necrotic tissue, and it is filled with the old pupal cuticle. Other body parts metamorphose normally and the unaffected regions of the abdomen produce adult cuticle with sensory bristles. (B) Few ppl>atf3 flies eclose, invariably bearing the scar, often with signs of bleeding. (C) Effect of atf3 expressed under the ubiquitous (arm) driver. (D) Expression of UAS-α-Catenin::GFP in posterior abdominal segments of a normally developing pupa at 27 hours APF shows that the ppl-Gal4 driver is active in LECs but not in the surrounding histoblasts that occupy the areas around and between LECs at this time. The punctate GFP signals probably come from LECs that had already been extruded and phagocytosed. (E) The Eip71CD driver is also expressed in LECs (large GFP-positive cells) but not in histoblasts. DAPI staining (magenta) shows cell nuclei. (F) Abdominal lesion in a rarely eclosing Eip71CD>atf3 adult. (G) Flip-out induction of atf3 in the polyploid larval cells reproduces the abdominal closure phenotype (for more severe defects see Fig. S4 in the supplementary material). The LECs that express atf3 and GFP persist in the adult abdomen and mainly localize to the dorsal cleft. Scale bars: 100 μm in D; 50 μm in E.

![Fig. 4](image2)
Sustained expression of atf3 in LECs disrupts abdominal development

The precise temporal control of atf3 expression (Fig. 3) suggested that the rise and subsequent fall of Atf3 during metamorphosis might be critical for the complex morphogenesis occurring in fly pupae. We tested this possibility by means of sustained expression of the full-length Atf3 protein using the UAS-Gal4 system with various drivers. A striking, fully penetrant metamorphic defect was observed with the pumpless (ppl) Gal4 driver. Although ppl>atf3 animals developed normally until the pupal stage, they failed to complete fusion of the adult abdominal epidermis (Fig. 4A). A dorsal cleft in the abdomen remained that could not be covered with the adult cuticle, and consequently 86% of the flies (n=913) died inside the puparium. All of the ppl>atf3 adults that did eclose showed abdominal lesions filled with the old pupal cuticle lacking adult pigmentation and bristles, often with a clot covering a bleeding wound (Fig. 4B). Adults with the same abdominal cleft (but otherwise normal) also emerged when atf3 was moderately and ubiquitously misexpressed under the arm-Gal4 driver (Fig. 4C), suggesting that abdominal morphogenesis was the process most sensitive to ectopic Atf3.

The adult fly abdomen derives from histoblasts that proliferate, replace LECs and finally differentiate, giving rise to the adult cuticle. Therefore, the observed abdominal defect suggested a compromised function of the epidermis, either LECs, histoblasts or both cell types. To distinguish between these possibilities, we first examined expression of the ppl-Gal4 driver in the epidermis. We found that ppl-Gal4 was active in LECs but not in histoblasts (Fig. 4D). Second, we used another driver, Eip71CD-Gal4, which was inactive in histoblasts but strongly expressed in LECs (Fig. 4E). Eip71CD-Gal4-driven misexpression of atf3 mostly produced lethal pupae lacking adult cuticle (data not shown), but it occasionally yielded adults with a dorsal abdominal cleft (Fig. 4F). In addition to being active in LECs, both ppl-Gal4 (Colombani et al., 2003) (see Fig. S3B in the supplementary material) and Eip71CD-Gal4 (data not shown) were also expressed in the fat body. However, no abdominal defects occurred when atf3 was misexpressed under either of three fat-body-specific Gal4 drivers, Lsp2, Cg or C7 (data not shown). Third, to rule out the possibility that ectopic Atf3 affected the imaginal epidermis, we directed its expression to histoblasts by using the flp-out technique. Owing to the timing of heat-shock induction to the mid-third instar, this method triggers expression in the polyploid larval cells but not in the diploid histoblasts (Ninov et al., 2007) (see Fig. S6 in the supplementary material). Misexpression of atf3 under the actin promoter following the flp-out event invariably led to an abdominal cleft (Fig. 4G). The lesions were often more severe than those observed in ppl>atf3 animals, affecting also lateral and ventral parts of the abdomen (see Fig. S4 in the supplementary material). Together, the above data demonstrate that the sustained expression of atf3 prevents fusion of the adult abdominal epidermis by acting upon LECs, suggesting that the replacement of these obsolete larval cells by adult histoblasts requires the developmental downregulation of atf3 expression.
Deregulated Atf3 prevents extrusion of LECs

To understand the cellular events underlying the incomplete epithelial closure in pupae, we visualized cell membranes by antibody staining of the septate junction component, Discs large 1 (Dlg1), or used a transgenic DE-cadherin::GFP fusion protein (shg::gfp). In wild-type animals 24 hours APF, LECs covering the surface of the abdomen gave way to the rapidly expanding nests of histoblasts (Fig. 5A) that began to fuse laterally and ventrally (Bischoff and Cseresnyes, 2009; Ninov et al., 2007). In pupae, the histoblast nests also spread (Fig. 5D), and at least at 16 hours APF, before their fusion, they comprised normal numbers of histoblasts (see Fig. S5 in the supplementary material). By 48 hours APF a control abdomen was fully covered with adult epidermis consisting exclusively of histoblasts, now forming sensory bristles (Fig. 5B). Histoblasts in pupae also differentiated the adult cuticle with sensory bristles, although polarity of the bristles near the dorsal cleft was altered (Fig. 4A and Fig. 5H; see Fig. S4C in the supplementary material). However, in contrast to the control, a large population of LECs remained in the dorsal abdomen of animals at 48 hours APF (Fig. 5E,G). The membranes of the persisting LECs accumulated the Dlg protein (Fig. 5F), and although these cells became severely deformed they survived throughout metamorphosis (Fig. 5E-I) to the adult stage (Fig. 4G). When visualized in live pupae, apical junctions of the remaining LECs displayed interdigitation and accumulation of DE-cadherin::GFP (Fig. 5G,H). Another adherens junction component, the Drosophila β-catenin Armadillo, was also enriched in LECs expressing Atf3 (see Fig. S6 in the supplementary material).

Cooperation between adherens junctions and the apical ring of actomyosin cytoskeleton is required for basal extrusion of LECs (Ninov et al., 2007). The altered pattern of DE-cadherin and β-catenin therefore suggests that excessive Atf3 might prevent LEC extrusion through stabilization of the cell-cell adhesion complex. To examine the effect of Atf3 on LECs in further detail, we employed the flip-out technique, which allows comparisons of misexpressing and control LECs within one tissue. Fig. 6 shows that membrane interdigitation occurred between LECs of control animals already at 18 and 24 hours APF, even in areas where the LECs had no contact with histoblasts. At 48 hours APF only LECs expressing Atf3 persisted, apparently being squeezed by the expanding histoblasts. The membrane-associated DE-cadherin::GFP signal was stronger in adjacent LECs compared with non-induced LECs (Fig. 6), and quantitative analysis of confocal images acquired at 18 hours APF (see Fig. S7 in the supplementary material) and at 24 hours APF (data not shown) both revealed a statistically significant 1.4-fold increase of the DE-cadherin::GFP signal intensity upon Atf3 induction. Enrichment of DE-cadherin on apical membranes of LECs was further confirmed on confocal cross sections (Fig. 7).

Although some LECs began the extrusion process, they could not detach from the apical surface even when entirely surrounded by histoblasts, possibly being tethered to it by the excessive adhesion protein (Fig. 7). By contrast, control LECs did completely separate from the epithelium (Fig. 7). In addition, LECs overexpressing Atf3 displayed apical enrichment of moesin, an actin-binding protein of the ERM (ezrin, radixin, moesin) family, which links transmembrane proteins to cortical actin filaments (see Fig. S8 in the supplementary material). Interestingly, prominent accumulation of DE-cadherin was also observed in LECs expressing
clones of epithelial cells within the hinge region of wing discs that form the adult thorax (see Fig. S9 in the supplementary material), indicating that the effect of Atf3 on cell adhesion components may not be limited to larval epithelia.

In summary, our results show that deregulation of atf3 expression causes marked changes of cell membranes, including interdigitation and accumulation of cell adhesion molecules, suggesting that LEC adhesiveness might be increased. Although some of the affected LECs initiate extrusion, this process stays incomplete. Consequently, the adhering LECs present a physical barrier for the migrating histoblasts.

**Effects of Rho1 signaling and 20-hydroxyecdysone**

Rho kinase (Rok)-dependent phosphorylation of myosin regulatory light chain was shown to be required for LEC extrusion (Ninov et al., 2007). To examine a possible relationship between the Rok-dependent cytoskeletal regulation and Atf3, we interfered with the function of the GTPase Rhô1 (also called RhoA), which acts immediately upstream of Rok. RNAi silencing of Rhô1 using the ppl-Gal4 driver produced a phenocopy of atf3 misexpression, causing a dorsal abdominal cleft in 100% of ppl>Rhô1(RNAi) adults (n=172), of which most died in the puparium and about 12% eclosed (Fig. 8A,B), similar to ppl>atf3 animals (Fig. 4A,B). However, when Rhô1 RNAi and misexpression of atf3 in LECs were combined, the abdominal defect became more severe (Fig. 8C), not allowing any pharate adults to eclose (n=77). Conversely, co-expression of a dominantly active Rhô1V14 protein suppressed the otherwise fully penetrant abdominal defect in some ppl-atf3 flies (Fig. 8D). Surprisingly, we found that the endogenous Rhô1 protein was mislocalized in atf3-misexpressing LECs, showing a diffuse cytoplasmic signal, compared with membrane localization in control LECs (Fig. 8E). These results suggest a genetic interaction between Rho signaling and atf3, and support the idea that excess Atf3 prevents extrusion of LECs by altering their cell adhesion properties.

**Jun is required for Atf3 effect on abdominal morphogenesis**

Atf3 interacts with Jun to form a DNA-binding complex (Fig. 1B,C) and genetically when overexpressed in the developing compound eye (Fig. 2). To see if this interaction is biologically relevant during abdominal morphogenesis, we tested whether Atf3 relies on the presence of Jun to cause the dorsal cleft phenotype. First, we confirmed that Jun is indeed expressed in LECs during metamorphosis (see Fig. S10 in the supplementary material). RNAi-mediated depletion of Jun in animals that misexpressed atf3 under the ppl-Gal4 driver restored viability of adults from 14% (atf3 alone) to 100% (n=565). Strikingly, 87% of the ppl>atf3, jun(RNAi) adults eclosed with a completely normal abdomen (Fig. 10A,B). By contrast, RNAi knockdown of Fos in ppl>atf3 background did not improve the abdominal defect (Fig. 10C). RNAi silencing of either jun or fos alone under the ppl-Gal4 driver had no effect on the abdomen (data not shown). These results demonstrate that Atf3 requires its partner Jun but not Fos to disrupt abdominal morphogenesis. Similar to the situation in the compound eye (Fig. 2 and Fig S1 in the supplementary material), the effect of misexpressed atf3 can be neutralized by simultaneously expressing Fos or its truncated bZIP domain under the ppl-Gal4 driver (data not shown). Therefore, the model in which Atf3 and Fos compete for their common partner Jun may be extended to the developing abdomen.

**DISCUSSION**

**Functional interaction between Atf3 and the AP-1 proteins**

We identified Atf3 as a new partner of Jun in *Drosophila*. Previously, Jun has only been known to dimerize with itself and with the *Drosophila* homolog of Fos (Perkins et al., 1988; Perkins et al.,...
Functional analysis of Atf3 has not yet been reported. Our biochemical data show that, similar to mammalian ATF3 and JDP2 (Aronheim et al., 1997; Chu et al., 1994; Hai and Curran, 1991; Hsu et al., 1991), the Atf3 protein selectively binds Jun but not Fos. Also consistent with the properties of ATF3 and JDP2 is the ability of Atf3 to bind the ATF/CRE response element alone or synergistically with Jun (Aronheim et al., 1997; Hsu et al., 1991). In contrast to its mammalian counterparts, however, neither Atf3 alone nor in complex with Jun bound to the AP-1 element under the same conditions. The selective interactions of Atf3 point to distinct biological roles for the Atf3-Jun and the Fos-Jun dimers, respectively.

Using the Drosophila model, we show a genetic interaction between Atf3 and Jun. The evidence is based on the ability of ectopic Atf3 to disturb morphogenesis of the adult abdomen and the compound eye, which strictly depends on the availability of Jun. Importantly, none of the Atf3 gain-of-function phenotypes could be induced by misexpression of the truncated bZIP domain of Atf3 (data not shown), suggesting that the functional Atf3 protein in complex with Jun is required. Based on the selectivity of Atf3 in our DNA-binding assay, we predict that the Atf3-Jun complex regulates specific target genes distinct from those targeted by Fos-Jun dimers.

Our data also reflect a relationship between the AP-1 and Atf3-Jun complexes. Although Fos does not dimerize or bind DNA with Atf3, its ability to suppress the Atf3 misexpression phenotype in the eye suggests that Fos and Atf3 compete in vivo for their common partner Jun. The fact that the same suppression can be achieved by overexpressing either the truncated Fos bZIP domain or Fos lacking phosphorylation sites indicates that the suppression does not rely on a transcriptional function of Fos but probably occurs through sequestering of Jun, even by a transcriptionally inactive Fos protein. Early in vitro studies have proposed a competition model for the AP-1 and Atf3 proteins to explain a temporal regulation of gene expression in the regenerating liver (Hsu et al., 1992). However, to date such a relationship among Fos, Jun and Atf3 has not been supported with direct genetic evidence.

Deregulated Atf3 increases adhesiveness of LECs

Removal of LECs is normally complete by 36 hours APF, at which time the sheets of histoblasts reach the dorsal midline (Bischoff and Cseresnyes, 2009; Ninov et al., 2007). Our data strongly support the argument that the temporal downregulation of atf3 expression during abdominal morphogenesis is necessary for LECs to be replaced by the adult epidermis. When experimentally sustained, atf3 activity in LECs interfered with this exchange by blocking extrusion and death of the LECs. This was evident as the atf3-expressing LECs survived within the epithelial layer for days after their scheduled destruction.

Intergeneration of cell membranes and accumulation of adherens junction proteins in LECs suggested that ectopic Atf3 caused adjacent LECs to reinforce their mutual contacts. This probably resulted from altered distribution of the proteins, as levels of the shg (DE-cadherin) mRNA remained unchanged in LECs of ppl>atf3 animals (data not shown). By contrast, junctions between atf3-expressing LECs and their normal neighbors or histoblasts were smooth and presumably less rigid. DE-cadherin was similarly enriched in clones of imaginal disc cells (see Fig. S9 in the supplementary material). These observations suggested that differential adhesion of atf3-expressing cells might have led to their sorting out from the surrounding epithelium. Even modest differences in cadherin levels have been shown to cause segregation of cells within a population by altering their adhesiveness (Duguay et al., 2003).

Recent live imaging data (Bischoff and Cseresnyes, 2009) have revealed that migrating histoblasts push the LECs ahead of themselves towards the dorsal midline, where histoblasts fuse last. The atf3-expressing LECs that adhered to each other were probably moved and pressed by the expanding histoblasts to the dorsal side, whereas non-induced LECs were eliminated. This explains why the abdominal lesions primarily occurred at the dorsal midline, although our flp-out experiments showed that atf3 misexpression could affect LECs in other areas as well (see Fig. S4 in the supplementary material). Strengthened contacts among persisting LECs probably blocked invasion of histoblasts in between them and inhibited LEC extrusion, eventually causing gaps in the adult epidermis.

In accord with the notion that extrusion from the epithelium is a prerequisite for LECs to undergo apoptosis (Ninov et al., 2007), we assume that sustained presence of Atf3 primarily enhanced adhesiveness of LECs, which only consequently prevented their death. This view is supported by the observation that membranes of atf3-expressing LECs interdigitated and accumulated DE-cadherin as early as 18-24 hours APF, even in areas of the larval epidermis that were far from histoblasts and where control LECs did not yet extrude (Fig. 6 and Fig. S7 in the supplementary material). In addition, the Atf3 gain-of-function phenotype was stronger than abdominal closure defects caused by caspase mutation or inhibition (Muro et al., 2006; Ninov et al., 2007). When we misexpressed the anti-apoptotic proteins p35 or DIAP1 (Thread – FlyBase) under the ppl-Gal4 driver, the resulting dorsal lesions were not lethal and were clearly milder than the broad, mostly fatal scars in ppl>atf3 animals (see Fig. S11A in the supplementary material). Compared with the
large contiguous populations of persisting LECs in ppla>atf3 pupae (Fig. 5), inhibiting apoptosis with p35 only allowed small islands of LECs to survive (see Fig. S1B in the supplementary material).

Ecdysone signaling promotes replacement of the abdominal epidermis by stimulating both the early histoblast proliferation and the extrusion of LECs (Ninov et al., 2007; Ninov et al., 2009). As atf3 misexpression affected LECs but did not impair early histoblast proliferation (see Fig. S5 in the supplementary material), we are left with the latter possibility that added 20E counteracted the effect of ectopic Atf3 by facilitating the extrusion process. As we detected normal 20E titers in ppla>atf3 larvae or prepupae (data not shown), the failure of the ecdysone extrusion was not a result of steroid deficiency. Also, 20E had no effect on atf3 mRNA levels, at least in Drosophila S2 cells or third-instar larvae (data not shown). Atf3 and ecdysone signaling therefore probably influence LEC extrusion by acting independently.

Although the mechanism through which ecdysone contributes to LEC removal is unknown, one attractive possibility is that it might cooperate with Rho signaling, which is required for LEC extrusion as well (Ninov et al., 2007). It has been demonstrated that genetic interaction between the 20E-response gene broad and components of the Rho pathway including RhoGEF2, Rho1 and myosin II is important for ecdysone-dependent epithelial cell elongation during Drosophila leg morphogenesis (Bayer et al., 2003; Ward et al., 2003). Our data show that Rho1 becomes mislocalized in LECs upon atf3 misexpression and that Rho1 silencing enhances the abdominal gain-of-function phenotype of atf3. The exact relationship between Atf3, Rho1 and ecdysone remains to be determined. However, Atf3 clearly represents a new intrinsic regulator of epithelial cell replacement during Drosophila metamorphosis.

Acknowledgements


