

# Developmental role for ACF1-containing nucleosome remodellers in chromatin organisation

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## SUMMARY

The nucleosome remodelling complexes CHRAC and ACF of *Drosophila* are thought to play global roles in chromatin assembly and nucleosome dynamics. Disruption of the gene encoding the common ACF1 subunit compromises fly viability. Survivors show defects in chromatin assembly and chromatin-mediated gene repression at all developmental stages. We now show that ACF1 expression is under strict developmental control. The expression is strongly diminished during embryonic development and persists at high levels only in undifferentiated cells, including the germ cell precursors and larval neuroblasts. Constitutive expression of ACF1 is lethal. Cell-specific ectopic expression perturbs chromatin organisation and nuclear programmes. By monitoring heterochromatin formation during development, we have found that ACF1-containing factors are involved in the initial establishment of diversified chromatin structures, such as heterochromatin. Altering the levels of ACF1 leads to global and variegated deviations from normal chromatin organisation with pleiotropic defects.

**KEY WORDS:** CHRAC, Chromosome, Epigenome, Histone modification, Nucleosome remodelling, *Drosophila*

## INTRODUCTION

The differentiation of cell identities during embryogenesis involves a step-wise diversification of the epigenome, which most obviously manifests through histone modification and an accompanying maturation of higher order chromatin structure. Pluripotent cells differ from their differentiated offspring by many chromatin features, including histone and DNA modification, nucleosome density and dynamics, linker histone stoichiometry, heterochromatin distribution, and chromosome arrangements in interphase nuclei (de la Serna et al., 2006; Giadrossi et al., 2007; Lin and Dent, 2006; Probst and Almouzni, 2008). For example, the chromatin of embryonic stem cells appears to be in a hyperdynamic state, in which the basic chromatin constituents, the core and linker histones, as well as heterochromatin protein 1 (HP1), are relatively mobile. Chromatin in lineage-committed cells is more rigid than that of pluripotent cells. Hyperdynamic chromatin may contribute to the maintenance of pluripotency and to the timely formation of higher order chromatin structures during differentiation (Meshorer and Misteli, 2006), but the molecular basis for these processes is still unclear.

The peculiarities of *Drosophila* embryogenesis permit a detailed resolution of the gradual implementation of chromatin structures from a hyperdynamic to a fully structured state. Starting from the zygotic nucleus, the first 14 mitotic divisions occur in a syncytium. After eight divisions, the nuclei migrate to the periphery of the embryo where they continue to divide until cellularisation during division 14. This is a turning point for chromatin structure and

function in several respects. During cleavage, chromatin appears largely decondensed, unstructured and unpatterned by histone modifications (Rudolph et al., 2007). In preblastoderm embryos, the nuclear volumes are 20-fold larger than those in embryos at later stages, which is explained, at least in part, by the absence of histone H1. The linker histone is gradually assembled into chromatin from nuclear division ten onwards and reaches the appropriate stoichiometry around the cellular blastoderm stage (Ner and Travers, 1994). At this time, the first signs of diversification of the unstructured chromatin into domains with distinct functional organisation are observable: the first heterochromatin foci can be visualised (James et al., 1989; Vlassova et al., 1991). This structuring of the epigenome coincides with cell fate determination (Simcox and Sang, 1983).

Preblastoderm embryos are particularly rich in nucleosome remodelling factors, especially those of the ISWI type (Corona and Tamkun, 2004). In *Drosophila*, ISWI is known to be the ATPase subunit of the four nucleosome remodelling factors ACF, CHRAC, NURF and RSF (Hanai et al., 2008; Ito et al., 1999; Tsukiyama and Wu, 1995; Varga-Weisz et al., 1997). These evolutionary conserved regulators of dynamic chromatin transitions alter histone-DNA contacts in an ATP-dependent manner in order to incorporate core and linker histones into chromatin or to move nucleosomes on DNA. The nucleosome sliding factors CHRAC and ACF were purified from *Drosophila* embryos following assays for chromatin assembly and disruption, respectively (Ito et al., 1999; Varga-Weisz et al., 1997). These nucleosome remodellers are distinct from other ISWI-containing complexes because of their large ACF1 subunit. ACF consists only of ISWI and ACF1, whereas CHRAC additionally contains two small histone-fold subunits (Eberharter et al., 2001; Ito et al., 1997). In vitro, ACF can promote the assembly of regular arrays of nucleosomes or chromatosomes (Lusser et al., 2005), and can catalyse the movement of nucleosomes and chromatosomes in arrays (Eberharter et al., 2004; Maier et al., 2008). Biochemically, CHRAC and ACF have very similar properties (Hartlepp et al., 2005).

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It is unclear whether CHRAC and ACF are separate entities with different physiological functions *in vivo*. Absence of CHRAC/ACF due to depletion of the shared ACF1 subunit severely compromises fly viability. Flies surviving the ACF1 depletion, presumably due to compensatory mechanisms, show defects in chromatin assembly, alterations of cell cycle progression and developmental delay (Fyodorov et al., 2004). Because the phenotypes are observable through all stages of the life cycle of the fly, it was assumed that CHRAC/ACF were general chromatin remodelling factors. To our surprise, we have now found that the expression of CHRAC/ACF is tightly restricted during development. ACF1 is expressed early in embryogenesis where it plays an important role in the initial diversification of the epigenome. Later in development, ACF1 expression is retained in some undifferentiated precursor cells, including the germ line precursors. Ectopic expression of ACF1 perturbs chromatin organisation, which results in faulty proliferation and differentiation decisions. Remarkably, high levels of ACF1 are required only for the initial establishment of chromatin structure diversity during development, but appear to be dispensable for its subsequent propagation.

## MATERIALS AND METHODS

### Fly strains

*Acfl* mutant flies, *Acfl*[2]/*Acfl*[2] and *Acfl*[1]/*Acfl*[1], were provided by D. Fyodorov (Albert Einstein College of Medicine, Bronx, USA) and J. T. Kadonaga (University of California, San Diego, La Jolla, USA) (Fyodorov et al., 2004). *Acfl* alleles were kept balanced and the characterisation of the *Acfl* phenotype was performed with homozygote *Acfl* alleles of flies from generations III-V. Flies mutant for *Su(var)205* [(In(1)*w*<sup>4h</sup>; *Su(var)205*/In(2L)Cy, In(2R)Cy, Cy<sup>1</sup>) were obtained from the Bloomington Stock Centre (stock number 6234). For generating transgenic UAS-ACF1-flag fly lines, the coding sequence for *Drosophila* ACF1 was amplified by PCR from a cDNA clone using the primers 5'-GCTATGCTATGCGCCGCATGCC-ATTGCAAGCG-3' and 5'-CACTAGTACTTGTCTGTCGTCGTCCTTGTAGTCGAAGCTTTGACTTCCC-3' containing the flag epitope sequence. The PCR product was gel purified and cloned in the pCR2.1-TA Topo vector (Invitrogen). PHD1 or PHD2 domains were deleted from pCR2.1-ACF1-flag using the Site-directed Mutagenesis Kit (Stratagene). The following pairs of oligonucleotides were used to delete the PHD1 or PHD2, respectively: PHD1F, 5'-ACCAATAAGTCATTAGTCGACGTAAGAG-TCTGGGTCTCAGC-3'; PHD1R, 5'-ACCCAGACTCTTTACGTCGAC-TAATGACTTATTGGTGAACGC-3'; PHD2F, 5'-GATGAGGAAAAG-GTGCTCGAGAAGCCGATGCCACAACGAC-3'; and PHD2R, 5'-GTGGCATCGGCTTCTCGAGCACCTTTTCTCATCTGACTC-3'. The inserts were mobilised with *Spe*I and *Not*I, and subcloned into pUASp vector (Rorth, 1998). The correct sequence was verified and the constructs were used to generate a *y*[1]*w*[1118] strain bearing a homozygous viable insertion by P element-mediated transformation. To induce ACF1 expression in eye discs, these flies were crossed to homozygote flies with the following genotype [*yw*; eye-Gal4, GFP] on the third chromosome (Schmitt et al., 2005). For expression behind the morphogenetic furrow, flies carrying [*w*\*]; P(*w*+mC)=GAL4-ninaE.GMR)12] on the second chromosome were used (Bloomington stock number 1104). To induce ACF1 expression in the salivary glands, homozygote UAS-ACF1-flag lines were crossed to *yw*; [*w*+, Gal4<sup>2314</sup>] (*sgs3*-Gal4) (Isogai et al., 2007). As a control for ACF1 ectopic expression, UAS-*lacZ* [N1 line (Zink and Paro, 1995)] was crossed to the same driver lines. To drive the targeted depletion of ACF1 in eye discs and salivary glands, flies carrying inverted repeats of the *Acfl* gene under control of UAS sequences [Vienna *Drosophila* RNAi Center stock number 33447 (Dietzl et al., 2007)] were crossed to *ey*-Gal4 or *sgs3*-Gal4 driver lines. Unless otherwise specified, all fly crosses were carried out at 25°C.

### Embryo collection and staining

Mini collection chambers, made by filling 35 mm Petri dishes with 1.8% Bacto Agar, 2% sucrose and 0.1% acetic acid, were placed as lids on bottles containing three- to ten-day-old adult flies (*y*[1] *w*[1118]). Plates

were exchanged every 90 minutes and embryos were either immediately collected or allowed to develop further. Embryos were rinsed twice with PBS-T (1× PBS + 0.1% Tween 20), and once with milli-Q water, and dechlorinated by immersion in 25% bleach for 3 minutes. After extensive washes with PBS-T and milli-Q water, embryos were transferred into glass jars containing 1-2 ml heptane and an equal volume of freshly added 3.7% paraformaldehyde (PFA; BDH) and the two solutions were vigorously mixed for 30 seconds. Embryos were fixed at the interphase between heptane and PFA for 20 minutes at room temperature. The phase containing PFA was carefully removed and replaced by two volumes of methanol. After vortexing for 15 seconds, embryos were collected in methanol and incubated overnight at 4°C. Embryos were then re-hydrated by successive incubations in 80%, 50% and 20% methanol for 5 minutes each, followed by two 5 minute PBS-T washes.

Following a 30 minute incubation in blocking buffer (0.3% BSA, 1% NGS, 0.1% Triton X-100 in 1× PBS), embryos were incubated with primary antibodies (diluted in blocking buffer) for 16-72 hours at 4°C then with secondary antibodies (Jackson ImmunoResearch Laboratories) diluted in blocking buffer for 3 hours at 26°C. Following each antibody incubation, embryos were subjected to four 15-minute washes with PBS-T.

Anti-mouse antibodies conjugated with Alexa 488 were used for detection of the anti-HP1 (1:100; C1A9, a kind gift from S. Elgin, Washington University in St Louis, St Louis, USA), Alexa 488-conjugated anti-rabbit antibodies were used for detection of ISWI (1:100; provided by J. Tamkun, University of California, Santa Cruz, USA), H2Av [1:500; a gift from R. Glaser, State University of New York, Albany, USA (Leach et al., 2000)], and H3K9me2 (1:25; Upstate; catalogue number 07-212). Anti-rat antibodies labelled with Rhodamine Red-X were used to detect ACF1 antibodies. The ACF1 monoclonal antibodies were raised against full-length ACF1-flag expressed in Sf9 cells (Eberharter et al., 2001). Monoclonal 8E3 was used throughout the study.

Following incubations and extensive washes, samples were counterstained with 1 μM TO-PRO3 (Molecular Probes) in 1× PBS for 10 minutes at 26°C. After washing twice with PBS-T, samples were mounted onto slides using Vectashield mounting medium (Vector Laboratories). Images were acquired with a Zeiss LSM 510 META confocal microscope equipped with an argon and two helium ion lasers and images processed with Zeiss LSM 510 META Software.

### Polytene chromosome staining

Polytene chromosomes were prepared as described previously (Sullivan et al., 2000) and several different fixation and staining procedures were tested with similar results. In order to judge the amount of protein staining in samples from different genetic backgrounds, polytene chromosomes were prepared from three different larvae; at least five nuclei per preparation were analysed. In order to analyse the staining in the linear range, each chromosome spread was imaged with five different exposure times. Images acquired with the same settings were then compared as raw data.

### BrdU labelling and detection

Imaginal discs were dissected in PBS from wandering third instar larvae and incubated in 1× PBS containing 150 μg/ml 5'-bromo-2-deoxyuridine (BrdU; Sigma). After appropriate times, discs were fixed in 5% PFA in 1× PBS for 30 minutes at room temperature. DNA was denatured for 30 minutes in freshly prepared 3 M HCl. The samples were rinsed three times with 0.3% Triton X-100 in 1× PBS. The tissues were then incubated for 1 hour at room temperature in blocking buffer (0.1% Triton X-100, 5% NGS in 1× PBS) on a rotating wheel. Primary antibodies, prepared in blocking buffer, were added and incubated overnight at 4°C. BrdU was detected using a mouse monoclonal anti-BrdU antibody (1:100; clone IU-4; Accurate Chemicals) followed by a donkey anti-mouse IgG conjugated with Alexa 488 (1:300; Invitrogen). The experiments were repeated three times and a total of 30 eye discs were evaluated for each experiment, about ten per BrdU pulse.

### Immunostaining of larval imaginal discs and salivary glands

Imaginal discs were dissected in PBS from wandering third instar larvae and discs were processed as described (<http://www.epigenome-noe.net/WWW/researchtools/protocol.php?protid=5>). However, in order to

preserve the best morphology and to increase reproducibility of the staining, samples were fixed without detergent in 3.7% PFA in 1× PBS for 30 minutes at room temperature. Removal of PFA and permeabilisation of tissues was carried out by rinsing three times for 10 minutes each in 0.1% Triton X-100 in 1× PBS. The tissues were then incubated for 1 hour at room temperature in blocking buffer (0.1% Triton X-100, 5% NGS in 1× PBS) on a rotating wheel. Tissues were incubated with primary antibodies (diluted in blocking buffer) overnight at room temperature. Following four 15 minute washes in 0.1% Triton X-100 in 1× PBS, secondary antibodies were applied as described for embryo immunostaining. The antibody against activated Caspase 3 (1:100; Cell Signaling Technology; catalogue number 9661) was detected using donkey anti-rabbit Alexa 488 (1:250; Invitrogen). In order to achieve good sensitivity and to preserve nuclear morphology, we prepared the fixative with particular attention. PFA was dissolved in 1× PBS to give a final concentration of 3.7%. In order to allow the PFA to dissolve, the solution was heated to a maximum of 60°C. When completely dissolved, the solution was chilled on ice, filtered and the pH was adjusted to 7-7.2. Aliquots were frozen and used only once after thawing.

Discs were mounted onto slides using Vectashield mounting medium (Vector Laboratories). Images were acquired and processed as described for embryo immunostaining.

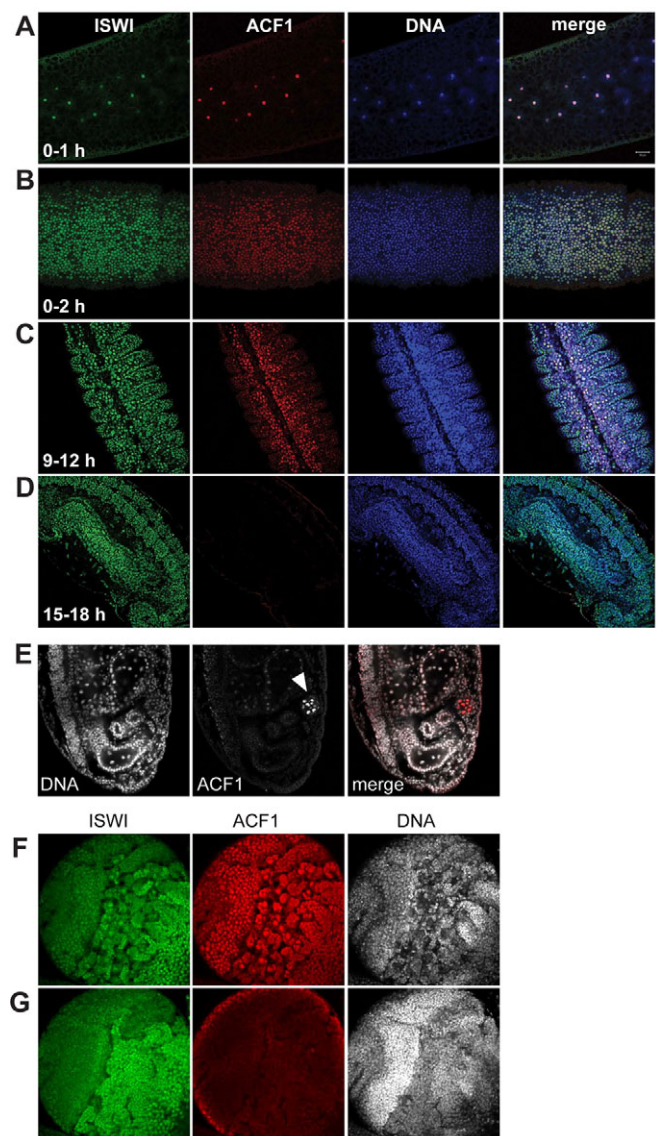
#### Extract preparation and quantitative western blotting

Embryo extracts were prepared as described previously (Eberharter et al., 2001). Larval extracts were obtained from five female third instar larvae, that had been frozen in liquid nitrogen and ground while still frozen. The samples were dissolved in 100 µl Laemmli buffer and denatured for 10 minutes at 98°C. Different amounts were electrophoresed and transferred onto Immobilon P membrane (Millipore). Proteins were detected using secondary antibodies conjugated with IRDye 800 and quantified using the Odyssey System (LI-COR, Bad Homburg, Germany) according to the manufacturer's instructions. Antibody dilutions used were: rat anti-ACF1, 1:100; rabbit anti-ISWI, 1:5000; rabbit anti-H2Av, 1:2000; and mouse anti-HP1 (C1A9), 1:3000 (provided by S. Elgin).

## RESULTS

### ACF1, the defining subunit of CHRAC and ACF, is developmentally regulated

Detection of ACF1 on western blots normalised for total protein suggested that it was present at much lower levels at non-embryonic developmental stages than in embryos (Ito et al., 1999), in a similar manner to its interaction partner ISWI (Elfring et al., 1994). However, several groups subsequently showed that ISWI was also quite abundant in all tissues at later developmental stages (Badenhorst et al., 2002; Badenhorst et al., 2005; Corona et al., 2007; Deuring et al., 2000; Hanai et al., 2008). In order to explore whether this was also true for ACF1 and to monitor its distribution at the single-cell level, we raised monoclonal antibodies and used them for both western blotting and whole-mount immunofluorescence microscopy (IFM). Detection of ACF1 at later developmental stages required an optimised, non-standard IFM protocol (see Materials and methods), whereas ISWI was visualised using a variety of different procedures. The antibodies were highly specific for ACF1 as they did not detect any protein in *Acf1*-null embryos or nuclear extracts derived thereof (see Fig. S1 in the supplementary material). Whereas ISWI was easily detectable at all embryonic stages and in all larval tissues (Fig. 1; see Fig. S2 in the supplementary material), ACF1 was found to be abundant in all nuclei during only the first 6 hours of development (Fig. 1A,B and data not shown). By 12 hours after egg laying (AEL), the ACF1 signal became restricted to fewer sets of cells, presumably including the neuronal precursors along the ventral furrow (Odden et al., 2002) (Fig. 1C). At later stages, when the majority of cells had already committed to a given cell fate, the



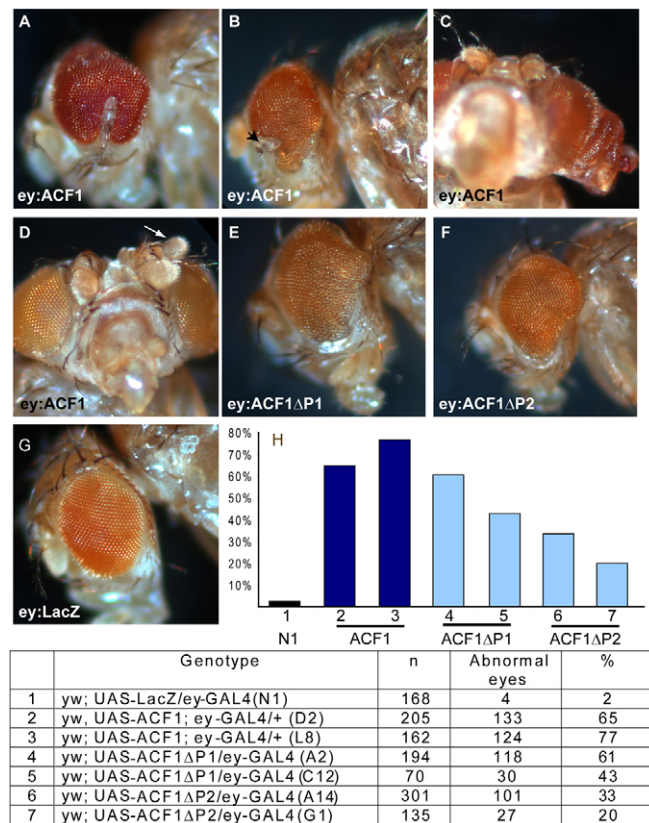
**Fig. 1. ACF1 expression is developmentally regulated.** Whole-mount immunofluorescence microscopy (IFM) was performed on different developmental stages of *Drosophila*. ACF1 is very abundant at early embryonic stages (A,B), but its expression is progressively restricted to subsets of cells (C) and generally reduced by 18 hours after egg laying (AEL) (D). The embryonic age is indicated (hours AEL). Samples were stained for ISWI (green), ACF1 (red) and DNA was counterstained with TO-PRO-3 (blue). Scale bar: 20 µm. (E) Arrowhead indicates the gonadal anlagen in a 15- to 18-hour-old embryo. DNA is in white, ACF1 is in red. (F,G) Sections of the larval optic lobe of the brain, where F is the top section and G is a 15 µm inner section. The outer layer contains the majority of neural precursors, while the inner part contains mostly differentiated neurons. ISWI (green) is detected in all cells, whereas ACF1 (red) is particularly abundant in neural precursor cells. DNA is shown in white.

ACF1 signal was below the detection limit (Fig. 1D). However, the pole cells (the primordial germ cells) retained a relatively strong signal for ACF1 throughout embryogenesis and were clearly distinguishable in the gonadal anlagen (Fig. 1E and data not shown). In larval tissues, we found that ACF1 levels were generally much reduced in salivary glands and imaginal discs, but

were clearly above the background levels defined by the *Acf1* null flies (see Figs S1 and S2 in the supplementary material). ACF1 could not be detected by western blotting or by RT-PCR at later developmental stages (not shown), in agreement with previous observations (Ito et al., 1999). A notable exception, detected only using IFM, was abundant staining in the larval brain (Fig. 1F,G; see Fig. S2A,C in the supplementary material), which, at this stage, contains many neural stem cells and progenitors specifically located at the surface of the optic lobe (Fig. 1F) (Yasugi et al., 2008). A common denominator of the cells found at this stage is that they are not terminally differentiated and undergo tightly regulated cell divisions that are necessary to acquire their final cell identity (Maurange et al., 2008). Interestingly, ACF1, but not ISWI, is reduced in differentiated neurons (Fig. 1G) located in the inner layers of the optic lobe. In summary, IFM analysis reveals a developmental and cell type-specific expression of ACF1 that had previously been overlooked. A common denominator of the cells that retain high levels of ACF1 expression is that they appear not to be fully differentiated. The fact that we observed cell type-specific exceptions for ACF1 expression that point to interesting biological functions, highlights the importance of immunological detection at the single cell level as a complement to global RT-PCR or western blotting analyses.

### Ectopic expression of ACF1 perturbs chromatin and nuclear programmes

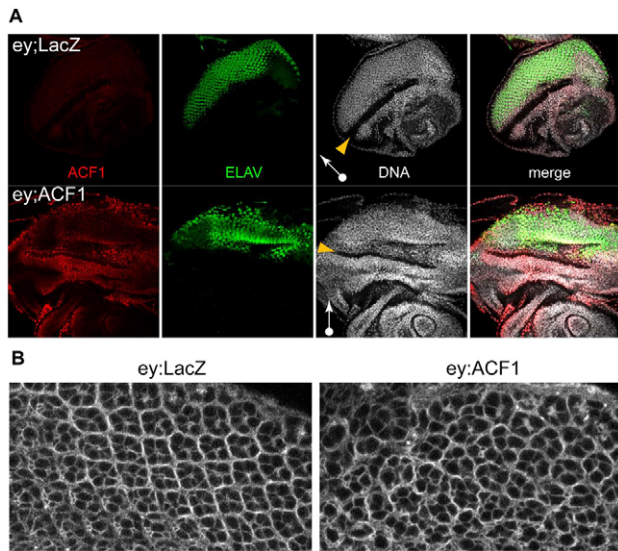
The developmental ACF1 expression profile suggests cell type-specific rather than general functions for ACF1-containing remodelling factors, which are apparently obsolete in most differentiated cells. To explore whether the presence of ACF1 in those cells would matter, we generated transgenic flies expressing the ACF1 cDNA from a minimal promoter driven by UAS<sub>Gal</sub> sequences inserted in different chromosomes. Initial attempts to express ACF1 globally under the direct control of the  $\alpha$ -tubulin promoter were unsuccessful (data not shown), suggesting that constitutive expression of ACF1 is lethal. We used several Gal4 driver lines to monitor the effect of ACF1 expression in defined cells, but focused our study on the eyeless-Gal4 driver line (*ey-Gal4*), as eye phenotypes are easily scored. The expression of ACF1 yielded gross abnormalities in the adult fly eyes, ranging from areas that lacked ommatidia (Fig. 2A), the outgrowth of antenna-like structures from the eye field (Fig. 2B), oversized or deformed eyes (Fig. 2C) and antenna duplication (Fig. 2D). These phenotypes were highly penetrant regardless of the transgene insertion site, as they were observed in 65–77% of the adult flies analysed (Fig. 2H). These eye aberrations were specific to the ectopic expression of ACF1 as they were observed neither in the controls (Fig. 2G) nor upon depletion of the trace amounts of ACF1 in eye imaginal disc by RNAi using the same *ey-Gal4* driver (not shown). In order to ascertain whether the observed effects were due to increased ACF levels rather than to the competition of ACF1 with other ISWI-associated factors, we expressed ACF1 mutants that had either of the two PHD finger domains deleted (ACF1 $\Delta$ PHD1, ACF1 $\Delta$ PHD2). In earlier work we had shown that deletion of the C-terminal PHD2 does not alter the potential of ACF1 to interact with ISWI, but significantly reduces the ability of ACF1 to bind histones and hence the nucleosome sliding activity of ACF (Eberharter et al., 2004). In addition, deletion of PHD1 had a milder effect on remodelling in vitro and the deletion of PHD2 closely mirrors an inactivating mutation. In accordance with these biochemical observations, overexpression of ACF1-bearing PHD deletions led to much milder phenotypes (Fig. 2E,F show the most



**Fig. 2. Ectopic expression of ACF1 leads to defects in cell fate.** ACF1 expression was driven by the eyeless promoter through the UAS-GAL4 inducible system. Homozygous *ey-GAL4* males were mated to virgin females bearing the UAS-ACF1-flag transgene on different chromosomes and scored for adult eye phenotype. UAS-*lacZ* virgin females crossed to *ey:Gal4* males served as controls. All crosses were carried out at 25°C. The adult flies displayed abnormal eye morphology with various types of defects: (A) missing ommatidia, (B) antenna-like structures (arrow), (C) excess ommatidia and (D) ectopic antenna (arrow). (E,F) Expression of ACF1 derivatives with deletions of either the PHD1 or PHD2 domain (ACF1 $\Delta$ P1 and ACF1 $\Delta$ P2, respectively) have much milder phenotypes. (G) None of the phenotypes were observed when eyeless-Gal4 induced  $\beta$ -galactosidase (*ey:lacZ*) as a control gene. (H) The percentage of abnormal eyes observed for lines 1–7. For each genotype two independent lines with insertions on different chromosomes were scored. N1 indicates the line carrying the UAS- $\beta$ Gal gene serving as a control (Zink and Paro, 1995). Dark blue bars indicate severe phenotypes (A–D), light blue bars indicate milder phenotypes (E,F). n, absolute numbers of eyes scored.

severe phenotypes observed). Particularly for ACF1 $\Delta$ PHD2, we also observed significantly lower penetrance in two independent insertion lines (Fig. 2H). The strength and penetrance of the phenotypes thus correlated well with the in vitro activities of the corresponding complexes, in strong support of the interpretation that the phenotypes were due to ACF rather than to unbalancing of other ISWI-containing complexes.

The structures of the composite adult eye result from a differentiation process that occurs in the eye-antenna imaginal discs of third instar larvae. There, the differentiation of determined cells into photoreceptor cells occurs in concert with the migration of the morphogenetic furrow (MF), an anatomical structure specifically described for the eye disc. In the MF, a highly synchronous cell

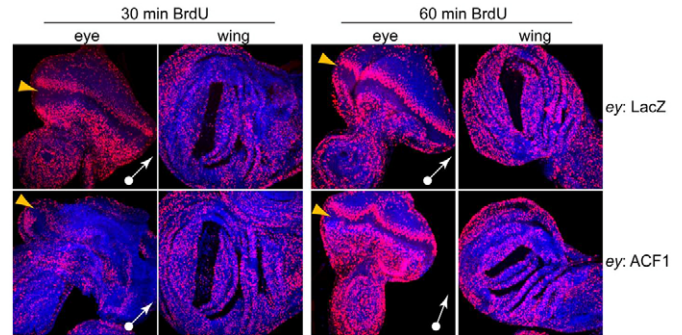


**Fig. 3. Ectopic expression of ACF1 in eye imaginal discs affects photoreceptor differentiation.** (A) Whole-mount IFM of eye-antenna imaginal discs. Expression of ACF1 (red) and of the differentiation marker ELAV (green) is shown in the control line (*ey:lacZ*) and upon ACF1 overexpression (*ey:ACF1*). Physiologically, ACF1 is barely detectable in eye imaginal discs (*ey:lacZ*). When ACF1 is induced in the eye imaginal disc (*ey:ACF1*), the cells that are positive for the neural marker ELAV (green) lose the regular organisation typical of the photoreceptors. The yellow arrowhead indicates the morphogenetic furrow (MF). The white arrows indicate the orientation of the eye-antenna disc: the head of the arrow shows the direction of the eye portion and the tail indicates the antennal region. (B) Magnified images of differentiated photoreceptors behind the MF, which show their disorganised arrangements when stained for actin (phalloidin). The regular arrangement in the control (left) contrasts with various defects upon ACF1 expression (right).

cycle coordinates differentiation of precursor cells into the regular clusters of photoreceptor cells (Baker, 2007). Cells along the MF are synchronised by arrest in the G1 phase. Upon signalling, they are released into S phase in a coordinated manner, divide synchronously and terminally differentiate (Hauck et al., 1999). The neuronal differentiation marker ELAV can be used to monitor the progression of the MF and the regularity of the photoreceptor cell clusters (Duong et al., 2008). Uncoupling the tight coordination between the cell cycle and signalling leads to eye phenotypes of the type we observed.

To gain information about the molecular nature underlying the phenotypes, we specifically expressed ACF1 anterior to the MF using the *ey-Gal4* driver or in the post-mitotic cells behind the MF with the *glass-Gal4* driver. By monitoring ELAV staining in the ACF1 D2 line we found that 16 out of 35 eye imaginal discs showed obvious morphological abnormalities and irregularities in photoreceptor cell arrangement upon expression of ACF1 with the *ey-Gal4* driver (Fig. 3A). Within error, this corresponds well to the frequency of abnormal adult eyes scored in this line (Fig. 2). The morphological and spatial irregularities in the photoreceptor arrays are easily observed by actin staining (Fig. 3B). By comparison, only four out of 35 control discs were scored as irregular.

Induction of ACF1 expression in embryos (in which endogenous ACF is naturally abundant), using the engrailed- and daughterless-*Gal4* drivers, resulted in no abnormalities in adult fly structures



**Fig. 4. ACF1 perturbs S-phase synchronisation at the morphogenetic furrow.** BrdU incorporation (red; DNA stain in blue) was monitored in whole-mount imaginal discs (eye-antenna and wing) of larvae expressing ACF1 (*ey:ACF1*) or an irrelevant protein (*ey:lacZ*) as a control. Synchronously replicating cells appear as a sharp stripe of BrdU-positive cells at the posterior border of the MF (orange arrowheads). BrdU labelling was for 30 or 60 minutes. Wing discs from the same animals served as a control for homogeneity of BrdU staining as ACF1 is not expressed there and BrdU incorporation is comparable in *ey:ACF1* and *ey:lacZ* larvae. The white arrows indicate the orientation of the eye-antenna disc, the head of the arrow represents the eye portion and the tail indicates the antennal region.

(data not shown). Furthermore, when ACF1 was expressed in the post-mitotic photoreceptor cells posterior to the MF with the *glass-Gal4* driver, we did not observe abnormalities, even if the crosses were set up at 28°C in order to boost ACF1 expression (not shown). This suggested that only cells at the MF were sensitive to unscheduled ACF1 expression. This supports the theory that ACF may act as a chromatin assembly factor coupled with DNA replication and not in a replication-independent manner (Collins et al., 2002; Fyodorov et al., 2004), and therefore implies that our transgene fulfils functions similar to its endogenous counterpart. As synchrony of S phase is a prerequisite for the coordination of photoreceptor differentiation, and reduced levels of ACF1 have previously been found to affect DNA replication in fly embryos and other species (Collins et al., 2002; Fyodorov et al., 2004; Vincent et al., 2008), we hypothesised that the defects in eye morphogenesis upon ectopic expression of ACF1 might be explained by an altered cell cycle. Therefore, we monitored S-phase progression by BrdU incorporation in imaginal discs of larvae expressing *ey:ACF1*. By allowing the incorporation of BrdU during a 30 minute pulse, the synchronous replication in control eye discs appears as a defined stripe of BrdU-positive cells at the posterior border of the MF (Fig. 4). Upon ACF1 expression, BrdU incorporation was reduced and synchronous DNA synthesis (indicated by a stripe of nuclei positive for BrdU incorporation) was not observable in about half of the eye discs (Fig. 4). As a control, the wing discs from the same animal incorporated BrdU to a similar extent in controls and ACF1 expressors. Similar results were obtained with a 10 minute BrdU pulse (see Fig. S3 in the supplementary material). To test whether this defect was due to a cell cycle block or just to a delay, we allowed BrdU incorporation for 60 minutes (Fig. 4). In this case, all cells immediately posterior to the MF were labelled even in the presence of ACF1. We tentatively conclude that ACF1 expression leads to a delay and asynchrony of S phase onset along the MF.

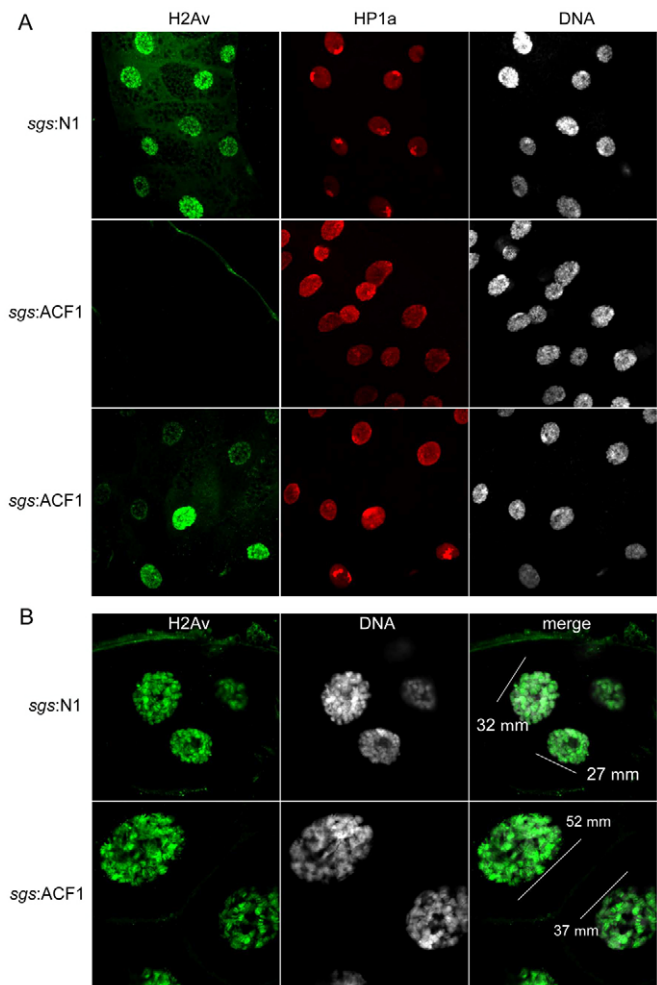
Defects in eye morphology could also result from perturbation of apoptotic events, which normally occur during eye differentiation. Monitoring the apoptotic marker activated Caspase

3 in eye discs, we did not observe profound differences in the distribution of apoptotic cells when comparing the controls with the *ey:ACF1* discs (see Fig. S4 in the supplementary material). However, in some *ey:ACF1* discs, but never in the controls, we noticed apoptotic cells that were aligned parallel to the MF, where the *eyeless* driver is mainly expressed. Thus, the *ey:ACF1* expression phenotypes might result from a combination of delay and asynchrony of S phase and unscheduled apoptosis.

So far, the known function of CHRAC/ACF *in vivo* is the assembly of chromatin fibres with regular nucleosome spacing, a prerequisite to the formation of higher order chromatin structures (Fyodorov et al., 2004). In order to relate the observed phenotypes to chromatin organisation, we analysed the consequences of ACF1 expression on chromatin structure. For this purpose, we drove the expression of ACF1 in salivary glands using the *sgs3-Gal4* driver line. This tissue is more homogeneous than the eye disc and the larger size of the polytene chromosomes allows easier identification of defects in chromatin structure that could be seen as alterations in banding pattern, in nuclear size or in distribution of markers for chromatin organisation (i.e. hetero- versus euchromatin). As an indicator of proper nuclear organisation, we monitored the distribution of H2Av and HP1a. These heterochromatin constituents mark early and late steps, respectively, in the process of heterochromatin assembly (Swaminathan et al., 2005). The expression of ACF1 in salivary glands produced obvious alterations in the distribution of both markers, although in different ways. HP1a, which is normally enriched at the chromocenter where constitutive heterochromatin is located (Fig. 5A), was found evenly distributed in the majority of the nuclei. The effects on H2Av incorporation displayed a certain degree of variability. In some ACF1-expressing glands H2Av signals were below the level of detection (Fig. 5A, middle row) and in other specimens we observed variable levels of H2Av (Fig. 5A, bottom row). Interestingly, only in the nuclei with a higher content of H2Av was an enrichment of HP1a at the chromocenter still observable. Previously, Corces and colleagues showed that a correct incorporation of H2Av was a prerequisite for HP1a loading onto heterochromatin (Swaminathan et al., 2005). Our data now suggest that inappropriate expression of ACF1 interferes with heterochromatin formation. Moreover, although a certain degree of variability was observed, the nuclei where ACF1 was expressed appeared to be generally larger than in the controls (Fig. 5B). This suggests that ectopic expression of ACF1 leads to a global derangement of chromatin organisation. Consistent with these observations, it was generally harder to obtain good morphology and integrity of polytene chromosome spreads from such glands, indicating that their chromosomes might be more sensitive to manipulation. Based on these rather global effects of ACF1 expression on chromatin organisation we consider that the replication asynchrony, unscheduled apoptosis and the subsequent deranged patterning of photoreceptor clusters in eye discs might be due to faulty chromatin organisation.

### ACF1 is an upstream factor of preblastoderm epigenome diversification

The results of ectopic expression is consistent with the earlier characterisation of the *Acf1* mutant phenotype by Fyodorov and Kadonaga, who suggested a role for ACF1 in chromatin assembly and transcriptional silencing at the level of higher order chromatin structure (Fyodorov et al., 2004). Because of the developmentally restricted ACF1 expression, we wished to analyse the homozygous *Acf1* flies further [the *Acf1*<sup>2</sup> allele corresponds to a null mutation



**Fig. 5. ACF1 disrupts chromatin organisation in larval salivary glands.** (A) Whole-mount salivary gland staining for H2Av (green) and HP1 (red) upon expression of *sgs:ACF1* (middle and bottom row) or *sgs:lacZ* (*sgs:N1*, top row). ACF1 expression perturbs the normal incorporation of H2Av into chromatin and provokes the loss of HP1a binding to the chromocenter. (B) Magnification of salivary-gland nuclei showing defects in banding pattern and size when ACF1 is ectopically expressed. The largest diameter measurements of nuclei are indicated. H2Av in green, DNA in white.

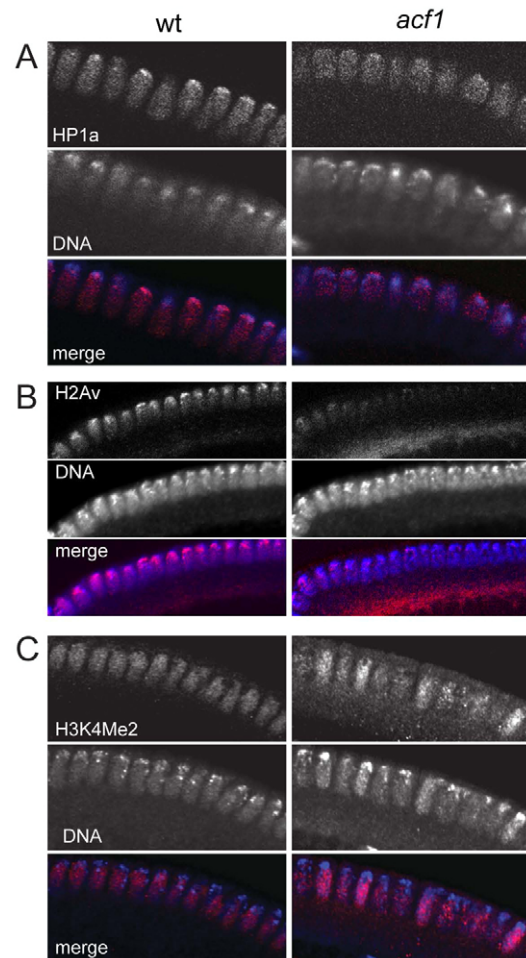
[Fyodorov et al., 2004]). Homozygous *Acf1* flies display ‘semi-lethality’: only about 30% of the expected number of flies is produced (Fyodorov et al., 2004). These ‘escapers’ are fertile and show a mild phenotype (delay in development and ‘sloppy chromatin’), suggesting that they managed to avoid the lethal aspects of ACF1 ablation by unknown compensatory measures (Fyodorov et al., 2004). Interestingly, this process of compensation continues gradually as flies are propagated as *Acf1* homozygotes. After six generations, the phenotype is lost entirely, but can be recovered by outcrossing the chromosome carrying the deletion for the *Acf1* gene before generating homozygous flies. This effect is seen with either *Acf1* allele, *Acf1*[1] or *Acf1*[2] (Fyodorov et al., 2004). For our analysis of the mutant phenotype, we exclusively used third to fifth generation homozygous *Acf1*[2] flies, which showed a relatively stable phenotype in terms of developmental delay and lethality as described by Fyodorov et al. (Fyodorov et al.,

2004). Loss and regain of the phenotype, which may be due to strong selection among polygenic modifiers or to epigenetic mechanisms, are highly reproducible.

To learn more about the role of ACF1 in chromatin organisation, we again monitored pericentric heterochromatin as its integrity can be easily visualised. A tentative pathway of heterochromatin formation in cleavage stage nuclei has been proposed, involving incorporation of the histone H2A variant H2Av, demethylation of histone H3 at lysine 4 (H3K4), methylation of H3K9 and subsequent HP1a binding (Rudolph et al., 2007; Swaminathan et al., 2005). As ACF1 is very abundant on the naïve cleavage chromatin, we tested whether heterochromatin assembly was affected at the blastoderm in homozygous *Acf1* embryos. Heterochromatin first becomes visible during the blastoderm stage, when nuclei line up at the embryo surface. At this stage, chromosomes are organised in the typical Rab1 conformation, which is characterised by clustering of heterochromatic chromocenters at the apical poles, and can serve as an index for appropriate organisation into regions of euchromatin and heterochromatin (Foe and Alberts, 1983).

We directly monitored heterochromatin markers and found that, in *Acf1* embryos, HP1 levels (Fig. 6A) appeared to be reduced at the pericentric heterochromatin. In order to evaluate HP1a levels, we determined the total amount of HP1a using quantitative western blotting and found it to be reduced by about 25% in *Acf1* embryos relative to wild type (see Fig. S5 in the supplementary material). Remarkably, incorporation of the histone variant H2Av, which is placed upstream of histone H3K9 methylation and HP1a binding in the pathway towards heterochromatin formation (Swaminathan et al., 2005), was also defective. In a large number of embryos, the majority of H2Av remained cytoplasmic and the small nuclear fraction did not colocalise with HP1a (Fig. 6B). To rule out the possibility that these defects in the establishment of heterochromatin were due to secondary mutations associated with the *Acf1[2]* allele, we also performed this analysis on the second *Acf1*-null allele, *Acf1[1]*. Indeed the incomplete and variegated incorporation of H2Av into heterochromatin was confirmed for both *Acf1[2]* and *Acf1[1]* alleles, in a parallel analysis (see Fig. S8 in the supplementary material). The similar behaviour of these two alleles had already shown by Fyodorov et al. for other aspects (Fyodorov et al., 2004). We therefore concentrated our further analysis on the *Acf1[2]* allele as it lost the semi-lethal phenotype more slowly than did the *Acf1[1]* allele.

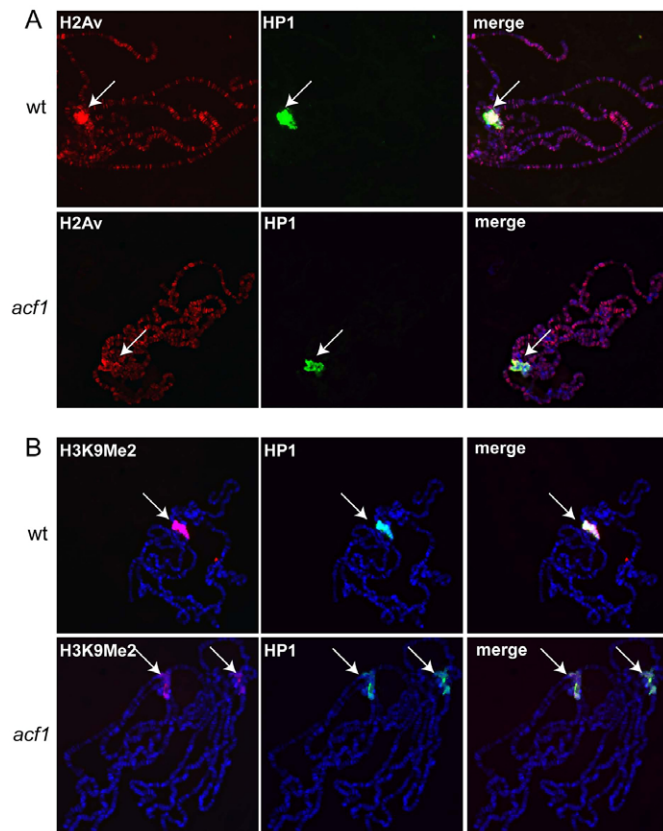
One of the earliest events in heterochromatin establishment is the removal of H3K4me2 from the naïve chromatin in order to confer competence to heterochromatin formation (Rudolph et al., 2007). In wild-type embryos, H3K4me2 is uniformly distributed along the chromosome arms and excluded from heterochromatin. Analysis of *Acf1[2]* embryos revealed a large variability of H3K4me2 signals ranging from overall greater or lesser staining than wild type (not shown) or variability between neighbouring cells within the same embryo (Fig. 6C). We also attempted to monitor the variability of the nuclear organisation caused by ACF1 depletion using DNA staining and evaluation of the Rab1 configuration as indicators of proper nuclear architecture (see Fig. S6 in the supplementary material). The polar organisation of chromatin in blastoderm nuclei was still observed in many nuclei; however, *Acf1* embryos consistently showed the highest degree of variability of the Rab1 organisation (Fig. 6; see Fig. S6 in the supplementary material). Given the instability of *Acf1* phenotypes, obviously also at the molecular level, the phenotypes were



**Fig. 6. ACF1 is required for faithful establishment of heterochromatin at the cellular blastoderm.** Heterochromatin becomes visible during the blastoderm stage when chromosomes are organised in the typical Rab1 conformation where clustering of the heterochromatic chromocenters can serve as an index for appropriate nuclear organisation (see Fig. S6 in the supplementary material). In *Acf1* flies, the localisation of heterochromatic markers is impaired at the earliest time point that can be detected. (A–C) Wild-type and *Acf1* embryos (cycle 12–14) were stained for HP1a (A), H2Av (B) and H3K4me2 (C) (red in merged images) and DNA was stained by TO-PRO3 (blue in merged images).

variegated, although analysis of flies between the third and fifth generations demonstrated that the same trends could always be observed.

Taken together, these observations document a requirement for ACF1 function in early development for epigenome diversification into euchromatin and heterochromatin to occur. This requirement might be explained if ACF1 were a heterochromatin component itself. However, this appears not to be the case as the localisation of ACF1 is largely complementary to the heterochromatin markers H3K9Me2 and H2Av during early embryogenesis (see Fig. S7 in the supplementary material). In summary, the fact that ACF1 depletion affects even the earliest known events in heterochromatin formation indicates a role for CHRAC/ACF as a key factor involved in preparing the chromatin fibre for subsequent epigenetic patterning such as heterochromatin assembly.



**Fig. 7. An embryonic deficiency of ACF1 affects heterochromatin in larvae.** (A,B) Immunostaining of salivary polytene chromosomes of wild-type (wt) and *Acf1* larvae (*Acf1*). Chromosomes were stained with antibodies detecting HP1a (green) and either the histone 2A variant H2Av (A) or H3K9me2 (B) (red). DNA was stained with DAPI (blue). Arrows indicate the chromocenter.

### Defects of CHRAC/ACF function during preblastoderm chromatin maturation cannot be rescued in later developmental stages

ACF1 expression is restricted to early embryogenesis and undifferentiated cells. However, the phenotypes associated with ACF1 depletion are also observable in differentiated tissues. For example, *Acf1* qualifies as a suppressor of position effect variegation in adult fly eyes (Fyodorov et al., 2004 and data not shown), yet it is not expressed there. These observations suggest that the consequences of impaired ACF1 function during embryogenesis reach far beyond the period of ACF1 expression. To further support this hypothesis, we monitored heterochromatin in the polytene chromosomes of larval salivary glands, where ACF1 is not expressed physiologically. We found that the levels of H2Av, H3K9me2 and HP1a at pericentric heterochromatin were significantly reduced in *Acf1* larvae (Fig. 7; see Fig. S5D,E in the supplementary material). Interestingly, only the heterochromatin localisation of H2Av but not its localisation to the euchromatic chromosomal arms was affected (Fig. 7A, arrows). Apparently, the defect in heterochromatin establishment due to lack of ACF1 during embryogenesis could not be rescued by any other chromatin remodelling or assembly factor in later developmental stages.

### DISCUSSION

The expression profile of ACF1 points to highly specific functions for CHRAC/ACF during development and in selected cell types. A common feature of the cells that express high levels of ACF1 is that they are not fully differentiated and, accordingly, their epigenomes are not terminally fixed. This is obvious for preblastoderm embryos, in which ACF1 is highly abundant. The nuclei in these embryos are largely unstructured, e.g. there is no morphological distinction between euchromatin and heterochromatin. After several rounds of most rapid nuclear divisions chromatin structure and function are diversified upon cellularisation. The abundance of ACF1 in preblastoderm embryos prompted us to monitor the consequences of the *Acf1* mutation for the earliest known events associated with the initial establishment of chromatin structure. We conclude that ACF1-containing factors are among the earliest factors to affect chromatin organisation during embryogenesis. Although we mainly looked at heterochromatin formation, which can be readily monitored at all developmental stages, we consider that CHRAC/ACF function may also be a prerequisite for the formation of other types of chromatin structure. Notably, the earliest known marker for euchromatin, H3K4me2 (Rudolph et al., 2007), was highly variegated in *Acf1* blastoderm embryos. Similarly, polycomb-mediated silencing in the adult fly eye was compromised by the *Acf1* deficiency (Fyodorov et al., 2004). CHRAC/ACF may function to safeguard the integrity of the chromatin fibre as a prerequisite for the assembly of additional layers of structure, as suggested by Kadonaga and colleagues, who observed that embryonic chromatin in the absence of ACF1 displays irregular nucleosome spacing (Fyodorov et al., 2004).

The assembly of regular nucleosomal arrays is a fundamental process, so it is surprising that the expression of CHRAC/ACF was restricted to particular cell types during development. Clearly, other remodelling factors can ensure the integrity of nucleosome fibres in cells where CHRAC/ACF are naturally absent (Hanai et al., 2008). Their compensatory action may explain the variegated nature of the *Acf1* phenotype. However, the fact that about 70% of all fresh homozygous *Acf1* flies died documents the singular importance of CHRAC/ACF. Furthermore, defects in heterochromatin structure arising in *Acf1* flies during embryogenesis were propagated until adulthood and could not be cured by those factors that organise chromatin in larval and adult stages. ACF1-containing remodellers, therefore, are the first remodelling factors known that are specifically involved in the transition from unstructured chromatin towards diversified chromatin states and for the primary establishment of higher order chromatin structures during early embryogenesis. However, they are not involved in maintaining such states later in development. The absence of ACF1 in larval tissues explains why *Acf1* flies did not show gross morphological aberrations of polytene chromosomes of the type observed when ISWI or NURF301 are depleted (Badenhorst et al., 2002; Deuring et al., 2000).

We speculate that high levels of CHRAC/ACF are a hallmark of poorly structured, plastic chromatin that awaits epigenetic patterning, as can be observed during *Drosophila* embryogenesis. It is plausible that ectopic (over-)expression of ACF1 in differentiated tissues (e.g. the salivary gland) leads to reversal of epigenetic structures to a more undifferentiated state. The effects of ACF1 expression are likely to be directly due to increased CHRAC/ACF levels (as opposed to reduced levels of other ISWI-containing remodelling factors due to competition for ISWI) for three reasons. First, expression of ACF1 in salivary glands does not



lead to a 'bloated X' phenotype in male larvae, which is a hallmark of ISWI or NURF301 deficiency (Badenhorst et al., 2002; Deuring et al., 2000). Second, nuclei of the early cleavage phase, which contain high levels of CHRAC/ACF, do not contain H2Av, but do display a uniform distribution of HP1a and are larger than nuclei after cellularisation and zygotic activation (Ner et al., 2001). This is similar to our observations of ectopic expression of ACF1 in salivary glands nuclei. Finally, expression of deleted versions of ACF1, which can still interact with ISWI yet strongly diminish ACF activity, does not produce similar phenotypes (Fig. 2); rather, the severity and the penetrance of the defects closely parallel the impaired biochemical properties of ACF1 deletions. In other words, the phenotypes correlate with ACF1 function, but not with its potential to titrate ISWI.

The morphological aberrations in adult fly eyes upon ACF1 ectopic expression in cells where the factor is normally virtually undetectable, led us to appreciate the effect of CHRAC/ACF on replication timing in the eye disc. Expression of ACF1 in the eye disc resulted in a general delay and temporal asynchrony of DNA synthesis at the MF, which we assume to be responsible for the striking eye phenotypes in *ey:ACF1* flies. The data are in line with observations of Fyodorov et al. (Fyodorov et al., 2004), who found that *Acf1* deficiency is associated with an accelerated S phase in embryos and larvae. Although direct effects of ACF1-containing remodelling complexes on replication cannot be excluded, we favour the idea that the alterations of nuclear programmes (timing of S phase, apoptosis, differentiation) are consequences of altered chromatin states that occur if ACF1 is deleted from cells where it is normally present, or if ACF1 is expressed in cells where it is normally absent. In flies, as in other species, heterochromatin and euchromatin differ in replication timing (Ahmad and Henikoff, 2001). A lack of faithful definition of euchromatin and heterochromatin domains as observed in *Acf1* embryos and in ACF1-expressing salivary glands is likely to compromise execution of precise DNA synthesis programmes.

Whatever the function of CHRAC/ACF in early embryos, this function is destructive in later tissues. CHRAC/ACF might contribute to defining a 'hyperdynamic' type of chromatin (Meshorer and Misteli, 2006) that characterises the cleavage states and hence interferes with ordered chromatin at later stages. Alternatively, CHRAC/ACF might serve to maintain regular nucleosomal fibres even in the context of those most disruptive replication events that characterise preblastoderm nuclei and cause inappropriate chromatin reorganisation if they occur out of context.

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#### Competing interests statement

The authors declare no competing financial interests.

#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.048405/-DC1>.

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