Ash2 acts as an ecdysone receptor coactivator by stabilizing the histone methyltransferase Trr

Albert Carbonell\textsuperscript{a}, Alexander Mazo\textsuperscript{b}, Florenci Serras\textsuperscript{a}, and Montserrat Corominas\textsuperscript{a}

\textsuperscript{a}Departament de Genètica and Institut de Biomedicina, Universitat de Barcelona, 08028 Barcelona, Spain;  
\textsuperscript{b}Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA 19107

ABSTRACT The molting hormone ecdysone triggers chromatin changes via histone modifications that are important for gene regulation. On hormone activation, the ecdysone receptor (EcR) binds to the SET domain–containing histone H3 methyltransferase trithorax-related protein (Trr). Methylation of histone H3 at lysine 4 (H3K4me), which is associated with transcriptional activation, requires several cofactors, including Ash2. We find that ash2 mutants have severe defects in pupariation and metamorphosis due to a lack of activation of ecdysone-responsive genes. This transcriptional defect is caused by the absence of the H3K4me3 marks set by Trr in these genes. We present evidence that Ash2 interacts with Trr and is required for its stabilization. Thus we propose that Ash2 functions together with Trr as an ecdysone receptor coactivator.

INTRODUCTION

The ecdysone receptor is a nuclear hormone receptor found in invertebrates and consists of a noncovalent heterodimer of two proteins—the ecdysone receptor (EcR) and ultraspiracle (USP; Oro et al., 1990; Koelle et al., 1991; Christianson et al., 1992; Yao et al., 1992). On ecdysterone (20E) binding, the ecdysone receptor triggers all molting transitions of the larva and many of the events that occur during metamorphosis (Berger and Dubrovsky, 2005). A prerequisite for the transcriptional regulation of ecdysone-dependent genes is the nuclear localization of EcR/USP and its interaction with specific DNA sequences—the hormone response elements (Vogtli et al., 1998). Although previous reports show that ecdysone receptor binds DNA constitutively and associates with either coactivators or corepressors depending on their status of ligand binding (Dressel et al., 1999; Tsai et al., 1999; Bai et al., 2000; Beckstead et al., 2001; Sedkov et al., 2003; Gates et al., 2004; Badenhorst et al., 2005; Francis et al., 2010), a recent study shows that, in the absence of the hormone, both EcR subunits localize to the cytoplasm, and the hormone-binding nuclear receptor E75A replaces EcR/USP at common target sequences in several genes (Johnston et al., 2011).

Whereas usp encodes a single protein product (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990), EcR encodes three isoforms that differ in their N-terminal sequences: EcR-A, EcR-B1, and EcR-B2 (Talbot et al., 1993). Differential expression of these isoforms dictates the tissue specificity of ecdysone responses in developing Drosophila (Talbot et al., 1993). For example, EcR-A is predominantly expressed in imaginal cells, which contribute to adult structures, whereas EcR-B1 is predominantly expressed in larval cells, which are committed to die after larval stages.

Changes in chromatin organization and histone modifications are crucial for gene activation mediated by nuclear receptors. One such modification is methylation of lysine residues, which can carry multiple methyl groups (Eisenberg and Shilatifard, 2010; Justin et al., 2010). Lysine methylation requires complexes that contain proteins with the SET domain, a conserved sequence first recognized in three Drosophila melanogaster proteins: Su(var)3-9, E(z), and trithorax (Trx; Tschiersch et al., 1994). Histone H3 lysine 4 methylation (H3K4me) is mostly driven by type 2 histone lysine methyltransferases (KMT2; Allis et al., 2007), which contain proteins of the Set1/COMPASS in yeast (Miller et al., 2001; Roguev et al., 2001; Nagy et al., 2002), the trithorax group (TrxG), the trithorax-related (Trn), and the dSet1 proteins in flies (Sedkov et al., 2003; Smith et al., 2004; Papp and Muller, 2006; Ardehali et al., 2011; Mohan et al., 2011), and the mixed-lineage leukemia family (MLL1-5, Set1A/B) in mammals (Milne et al., 2002; Goo et al., 2003; Wysocka et al., 2003).
Hughes et al., 2004; Yokoyama et al., 2004; Lee and Skalnik, 2005; Lee et al., 2006, 2007; Ruthenburg et al., 2007). Trimethylation of H3K4 (H3K4me3) is associated with transcriptionally active regions (Dillon et al., 2005; Eisenberg and Shilatifard, 2010) and is a conserved mark of chromatin at nucleosomes immediately downstream of promoters of transcribed genes (Pokholok et al., 2005; Banski et al., 2007; Guenther et al., 2007; Schuettengruber et al., 2009; Perez-Lluch et al., 2011). Trx is the prototypical Drosophila member of the TrxG family. In line with the TrxG function, loss-of-function mutations of trx cause homeotic transformations in embryos and larvae (Ingham, 1983; Breen and Harte, 1991). TrxG and MLL proteins have been found in numerous complexes in different organisms (Shilatifard, 2008), and analysis of polytenic chromosomes in flies show that the number of sites that accumulate H3K4me3 is greater than can be attributed to these individual proteins, indicating nonredundant activities (Eisenberg and Shilatifard, 2010). Trr is a KMT2 similar to Trx (Sedkov et al., 1999). Trr mutants, however, do not display homeotic changes but instead interact with EcR, indicating that Trr functions as a coactivator of EcR by altering the chromatin structure at ecdysone-responsive promoters (Sedkov et al., 2003).

The ash2 gene, a member of the TrxG, was discovered in a screen for mutants in Drosophila with imaginal disk abnormalities (Shearn et al., 1971; Shearn and Garen, 1974). Loss-of-function mutations of this gene cause homeotic transformations and down-regulation of Hox genes (LaLeuensse and Shearn, 1995), in addition to several abnormalities, such as reduction of intervein and enhancement of vein tissues in the wing (Adanson and Shearn, 1996; Amoros et al., 2002; Angulo et al., 2004). The Ash2 protein is similar to two subunits of the COMPASS complex in yeast—Bre2 (Cps60) and Spp1 (Cps40; Roguev et al., 2001; Krogan et al., 2002; Steward et al., 2006). Biochemical and RNA interference studies have identified the mammalian orthologue of Ash2, Ash2L, to be a core subunit of KMT2 complexes that is required for H3K4me3 (Dou et al., 2006; Steward et al., 2006; Southall et al., 2009; Cao et al., 2010), and nucleosome Western blots and clonal analysis indicate that Ash2 is also necessary for H3K4me3 in Drosophila (Beltran et al., 2007).

Here we show that Ash2 is required for pupariation and metamorphosis, playing a role in the transcriptional activation of ecdysone-responsive genes by promoting H3K4me3. By investigating the relationship of Ash2 with KMT2s in several Drosophila tissues, we also provide evidence that Ash2 is involved in the stabilization of the EcR coactivator Trr.

**RESULTS**

**ash2 mutants affected ecdysone-triggered biological responses**

Flies homozygous for the ash2′ allele have reduced and abnormal imaginal disk and brain, and this is lethal in late third instar (Amoros et al., 2002; Beltran et al., 2003; Angulo et al., 2004). At this stage, wandering ash2′ mutant larvae stopped moving and became extended and stiff in an apparent attempt to pupariate. Defects associated with ecdysteroid signaling become apparent. Ninety-seven percent of animals did not evert their anterior spiracles or displayed defects in this eversion (Figure 1, A and B). Moreover, ash2′ mutant larvae did not undergo body shortening or sticking to the wall to enter into prepupa stage, phenomena that normally occur before metamorphosis; instead, they remained in larval stage for 6 extra days before dying. Homozygous mutants of the hypomorphic allele ash2′ displayed similar, although milder, phenotypes (5.2% of spiracle eversion defects). Approximately 12% of ash2′ homozygous flies developed into sterile adults, which are known to survive for 2 d (Amoros et al., 2002); however, some of them died during metamorphosis, with defects in ecdysone-triggered biological responses such as the head eversion (6%; Figure 1, C and D).

Failure in anterior spiracle eversion, a nonpupariating phenotype, and/or defects during metamorphosis are hallmarks of mutations in the loci associated with ecdysoid signaling (Li and Bender, 2000; Bashirullah et al., 2007; Francis et al., 2010). To test whether ash2 interacts with EcR, we analyzed the phenotype of double mutants. Two sets of evidence suggest that both proteins act in common processes. First, compared with ash2/ash2 flies, we obtained fewer flies carrying the EcR/M546 and ash2/ash2 alleles that were capable of completing metamorphosis (1.3% of survival, n = 78, as compared with 17.9% of survival, n = 76; Figure 1J). Heterozygous flies for either of the mutant alleles used as a control did not present any viability defects. Second, because Ash2 and EcR play a role in wing morphogenesis (D’Avino and Thummel, 2000; Amoros et al., 2002; Angulo et al., 2004), we also checked the adult wings of EcR/M546/+; ash2/ash2 flies. Although only a small number of double mutants survived, they displayed an enhancement of the ash2/ash2 wing phenotype. For example, in addition to the extra cross-vein defects, mutant wings displayed an aberrant shape not observed in either EcR/M546/+ or ash2/ash2 mutants alone (Figure 1, E–I). Taken together, these results suggest that Ash2 and EcR are required for the viability and proper progression of the larvae through pupariation and metamorphosis events.

**ASH2 is associated with EcR at the majority of ecdysone-responsive genes**

To clarify the relationship between ASH2 and EcR, we compared their distribution on polytenic chromosomes. Overlapping signals were observed in a significant fraction of binding sites for both proteins (Figure 2, A and B). In addition, we used data from recent genomic studies to compare the target genes of Ash2 and EcR. The Ash2 target genes were obtained by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) in wing imaginal disk (Perez-Lluch et al., 2011), whereas the EcR target genes were obtained by ChIP-Seq in white pupae (Roy et al., 2010). Despite the differences in tissues and developmental stages, we found that 85% (361 genes) of the EcR target genes are also Ash2 target genes (Figure 2C). This strong overlap indicates that EcR and ASH2 cooperate in regulating a significant subset of ecdysone-responsive genes. Furthermore, the comparison with target genes previously obtained by ChIP-Seq of H3K4me3 in wing disk (Perez-Lluch et al., 2011) suggests that 66% (239 genes) of these common target genes are indeed active in the third-instar wing imaginal disk. Gene Ontology (GO) analyses of the Ash2 and EcR common target genes showed that enriched categories are related to larval/pupal morphogenesis and metamorphosis (Figure 2D). This enrichment is expected for EcR function and reinforces the finding that Ash2 and EcR cooperate in pupariation and metamorphosis events.

We then analyzed the location of Ash2 and EcR in the genome by projecting the mean reads over the transcription start sites (TSS) of common target genes (Figure 2E). Although the Ash2 peak appears to be slightly displaced toward the 5’ side, both proteins, as well as H3K4me3, seem to occupy the same genomic region. This result suggests that EcR interacts with Ash2 to promote the activation of ecdysone-responsive genes.

**Ash2-dependent H3K4 trimethylation at EcR target genes is required for their transcriptional activation**

We next investigated the mRNA and protein levels of EcR in the absence of Ash2 in several tissues. Previously, in a comparative
expression analysis of wild-type and ash2 mutants in wing disks, we detected similar transcript levels of EcR (289.7 in wild-type compared with 271.8 in ash2 mutants (Beltran et al., 2007)). Consistent with these data, mutant clones of ash2 induced 60 h after egg laying (AEL) in the wing disk showed no differences in the protein levels or in the subcellular localization of the EcR isoforms, neither in the peripodial membrane (where EcR-B1 is predominantly expressed; Figure 3A) nor in the columnar epithelium (where EcR-A is predominantly expressed; Figure 3B). Moreover, we did not observe changes in the level of EcR-A in ash2 mutant disks compared with wild-type disks (Supplemental Figure S2). Thus EcR transcript and protein levels are not altered in ash2 mutants. Similarly, no significant differences in the amount of the nuclear EcR protein were detected in mutant clones and to the aberrant bristle differentiation (Figure 4, A and B). This phenotype was highly penetrant (79.4% of the clones) and was consistent with the ones observed in the ash2-mutant clones (Amoros et al., 2002; Angulo et al., 2004). Animals with trr clones normally die if clones are induced early in development (60 ± 12 h AEL) but can survive if clones are induced later (85 ± 12 h AEL). In the wing, trr clones (marked by yellow) were apparent only at the dorsoventral margin. These clones did not show the uniform polarized orientation of stout mechanosensory bristles but instead presented definite defects in bristle spacing and differentiation (Figure 4, A and B). This phenotype was highly penetrant (79.4% of the clones) and was consistent with the ones observed in the ash2-mutant clones (Amoros et al., 2002; Beltran et al., 2007). We also explored the differentiation of mutant clones in the abdomen, which allows low-viability clones to be easily recovered, and found that trr cells presented wild-type trichomes but lacked chaetes and macrochaetes in the abdominal a4 and a5 segments, respectively (Figure 4C). Thus the results from both wing and abdomen support a function for Trr in bristle development.

We next examined the genetic interaction between ash2 and trr. Similar to EcR mutants, the combination of trr with the hypomorphic
results provide strong support for a functional relationship between the two genes.

In agreement with recently published results (Mohan et al., 2011), coimmunoprecipitation experiments in embryos revealed a direct physical association of Ash2 with Trr (Figure 5A), providing additional support to the genetic finding of a functional relationship between Ash2 and Trr. These proteins were also observed to partially colocalize on salivary gland polytene chromosomes (Figure 5, B and C). The fact that additional bands were observed for Ash2 that did not overlap with Trr is consistent with the role of Ash2 as a coactivator for other histone methyltransferases.

Because Trr is recruited to the EcR-positive loci in response to ecdysone (Sedkov et al., 2003; Johnston et al., 2011), we next addressed whether Ash2 binding at the early ecdysone-induced puff 2B is also dependent on hormone treatment. Late larval and prepupal ecdysone pulses trigger a sequential induction of puffs in polytene chromosomes of salivary glands that correspond to loose chromatin structures where genes are actively transcribed. Early-responding puff 2B contains the BR-C gene, the expression of which depends on both Trr (Sedkov et al., 2003) and Ash2 (Figure 3, C and D). Ash2 recruitment in response to ecdysone was analyzed after ectopic ecdysone treatment in salivary glands, which were dissected from mid-third-instar larvae and cultured at 25°C for 2 h either in the absence or in the presence of ecdysone. We found that raising the ecdysone levels in vivo resulted in an increased staining of Ash2 and EcR (Figure 6A) and of Trr and EcR (Figure 6B) in the 2B loci that was concomitant with puff formation. These observations support the idea that ecdysone signaling regulates Ash2 binding at EcR-positive targets.

Ash2 is required for Trr but not Trx stability

To clarify the functional relationship between Ash2 and Trr, we compared Trr distribution on the polytene chromosomes of wild-type and ash2 mutant larvae. The loss of Ash2 resulted in a significant reduction in the levels of Trr protein associated with polytene chromosomes (Figure 7A). A dramatic reduction of Trr nuclear levels was also detected by immunostaining in ash2 homozygous flies (3.4% in trr; ash2, n = 60, compared with 17.9% in ash2 heterozygous mutants, n = 76; Figure 4D). Heterozygous flies for either of the mutant alleles used as controls did not present any viability defects. Wings of surviving flies also presented a more extreme phenotype, consisting of an aberrant shape, reduced wing size, partial fusion of the L2 and L3 veins, and massive intervein-to-vein transformation (Figure 4G). This phenotype was not observed for either the trr or the ash2 mutant alone (Figure 4, E and F). These results confirmed our previous

allele ash2 resulted in a severe reduction of viability of ash2 homozygous flies (3.4% in trr; ash2, n = 60, compared with 17.9% in ash2 mutants, n = 76; Figure 4D). Heterozygous flies for either of the mutant alleles used as controls did not present any viability defects. Wings of surviving flies also presented a more extreme phenotype, consisting of an aberrant shape, reduced wing size, partial fusion of the L2 and L3 veins, and massive intervein-to-vein transformation (Figure 4G). This phenotype was not observed for either the trr or the ash2 mutant alone (Figure 4, E and F).
The Ash2 contribution to H3K4me3 levels at EcR target genes is required for their transcriptional activation. (A, B) Loss of Ash2 function did not reduce EcR levels. (A) Detection of EcR-B1 in wild-type (bright green), heterozygous (green), and ash2I1 homozygous (black) cells from the peripodial membrane of wing imaginal disk. (B) Detection of EcR-A in wild-type (bright green), heterozygous (green), and ash2I1 homozygous (black) cells from columnar epithelia of wing imaginal disk. (C) Effects of an ash2 mutation on the expression of BR-C and E75A. Fat body mRNA levels of BR-C and E75A from late-wandering larva were measured by quantitative RT-PCR relative to rp49, used as a control. Error bars represent SEM. (D) ChiP analysis of wild-type and ash2I1 late-wandering larva with H3K4me3 antibody. The regions analyzed were the promoter region of E75A (of −1852 to −1751 from the TSS) and the 5′-untranslated region of BR-C (of +1079 to +1212 from the TSS). Real-time PCR results were normalized against the mock sample and are depicted as fold enrichment. Error bars represent SEM.

microarray analyses of wild-type and ash2I1 wing disks, from which we detected similar transcript levels of Trr (225.6 in wild-type compared with 219.6 in ash2I1 mutants; Beltran et al., 2007).

Given that the mammalian orthologue of Ash2 (ASH2L) has been identified as a subunit of a core complex with several KMT2s (Dou et al., 2006; Steward et al., 2006; Southall et al., 2009; Cao et al., 2010), we next examined whether Drosophila Ash2 could play a general role in stabilizing other KMT2s, such as Trx. The physical interaction of Ash2 with Trx was determined by coimmunoprecipitation and immunostaining. By analyzing their distribution on polytene chromosomes, we found that Ash2 colocalized with Trx (Figure 8B) in a significant subset of bands, although, as expected, the number of chromosomal sites that accumulated Ash2 was greater that those with Trx. We also found Ash2 associated with the N-terminal cleaved form of Trx (Figure 8A), consistent with previous findings (Milne et al., 2005; Mohan et al., 2011).

We next assessed whether removing Ash2 would affect the Trx function. In contrast to our observations on Trr, the loss of ash2 resulted in no major changes in the binding of Trx to polytene chromosomes (Figure 8C) or in the Trx levels in ash2I1-mutant clones in the wing imaginal disk (Figure 8D), indicating the specificity of Ash2 function for Trr. Given that studies on the mammalian ASH2L and MLL1 have shown that ASH2L stimulates the catalytic activity of MLL1 (Dou et al., 2006; Steward et al., 2006; Southall et al., 2009; Cao et al., 2010), we next examined the effect of removing Ash2 on the H3K4me3 levels and Trx recruitment at specific genes. For this purpose, we performed ChiP experiments using antibodies against H3K4me3 and Trx at the lcp9 gene. As expected, Trx was present in the absence of Ash2, but there was a severe drop of the H3K4me3 levels (Figure 8, E and F), suggesting that, as in mammals, Ash2 stimulates the catalytic activity of Trx. Taken together, our results confirm that Ash2 is a common partner for Trx and Trr KMT2s in Drosophila and reveal its specific role as a coactivator of EcR through stabilization of Trr.

**DISCUSSION**

In this work, we identified several ash2 phenotypes that are characteristic for defects in ecdysone signaling, suggesting a critical role for Ash2 in ecdysone responses during late larval and pupal development. Two classes of genes are known to produce mutant phenotypes that resemble those seen in ash2 mutant animals: those required for ecdysone biosynthesis or release (Garen et al.,
morphogenesis is induced by an Ash2/Trr complex functioning as an EcR coactivator complex. In line with this, it has been reported that an ecdysone regulatory pathway controls wing morphogenesis and integrin expression during Drosophila metamorphosis (D’Avino and Thummel, 2000).

Our data confirm the role of Ash2 in promoting H3K4 trimethylation, as well as its function as a cofactor of the Drosophila Trx and Trr KMT2 proteins, supporting conservation between human and fly SET complexes. Our finding that Ash2 is a common partner of the Drosophila SET complexes is fully consistent with the recent characterization of the DSET1 complex, which contains Ash2 (Ardehali et al., 2011), and of the Drosophila COMPASS complexes, which contain Ash2 and are similar in their subunit composition to their mammalian counterparts (Mohan et al., 2011). Both studies also showed that dSet1 is responsible for the majority of H3K4 dimethylation and trimethylation. In agreement with these and other reports (Dou et al., 2006; Steward et al., 2006), we observed a reduction of H3K4me3 and H3K4me2, but not of H3K4me1, upon depletion of ASH2 (Supplemental Figure S3), reinforcing a role for ASH2 as a general cofactor of this type of histone methyltransferase.

Nevertheless, our results demonstrate some specificity of the Ash2/Trr-induced H3K4me3 marking at ecdysone-responsive genes. With regard to the function of Ash2 in different SET complexes, it has been shown in Saccharomyces cerevisiae that inactivation of the Ash2 relative Bre2 did not affect the integrity of the complex but significantly impaired the catalytic activity of Set1 (Schneider et al., 2005; Dehe et al., 2006). Data from mammals revealed that this function is conserved, as ASH2L also stimulated the KMT2 activity of MLL1 (Dou et al., 2006; Steward et al., 2006; Southall et al., 2009) and is involved directly with RbBPS in the catalytic reaction by its ability to interact with H3 and S-adenosyl-l-[methyl-3H]methionine (Cao et al., 2010). Given the highly conserved core configuration among the MLL/SET1 family of KMT2s and the global effects of ASH2L on H3K4 methylation, it has been proposed that this function could be a common feature for the regulation of other MLL/SET1 family members (Dou et al., 2006; Cao et al., 2010). Our results indicate that, in addition to its role in facilitating H3K4 trimethylation, Ash2 could play complex-specific roles. The depletion of Trr protein levels observed in ash2 mutants points to a destabilization of Trr in the absence of Ash2, since only a mild reduction was observed at the RNA level that does not support transcriptional control. Several scenarios can explain this depletion. First, Ash2 may be required to stabilize Trr to chromatin upon EcR signaling and could subsequently contribute to its catalytic activity. Second, Ash2 could directly or indirectly control Trr synthesis or stability before binding to chromatin. There are several examples of chromatin-bound proteins that are degraded when released from chromatin (Li and DePamphilis, 2002; Sharma et al., 2011). Although the proteasome pathway seems to play a role in the degradation of these, other mechanisms are also likely to be involved (Sharma et al., 2011). Further experiments are necessary to address whether the mechanism involving Ash2-dependent Trr stabilization involves proteosomal degradation, but it is important to point out that Trr, similar to Trx,
In contrast, Trx is present at promoters and polycomb responsive elements from both transcriptionally active and inactive genes (Papp and Muller, 2006; Schuettengruber et al., 2009; Schwartz et al., 2010). Consistently, it has been demonstrated that recruitment of Trr, but not of Trx, is affected by the absence of EcR (Johnston et al., 2011). In addition, a recent report showed that the protein Cara Mitad (CMI) associates with TRR and EcR-USP, is required for proper trimethylation of H3K4, and needs to bind to chromatin for hormone-stimulated transcription (Chauhan et al., 2012). CMI/Lpt had already been identified as a component of the Trr complex that contains Ash2 (Mohan et al., 2011). Taken together, these results point to the singularity and specificity of Trr-containing complexes in involved in EcR responses.

It has been suggested that H3K4me3 may act by recruiting factors that generate a particular architecture at promoters that is critical for optimal transcription (Ardehali et al., 2011). In fact, the functional implications of this histone modification is determined by several effector proteins that bind to the trimethylated H3K4 marks; possible outcomes include regulation of transcription initiation, chromatin remodeling, and modulation of splicing efficiency (Sims et al., 2007; Vermeulen et al., 2007). Moreover, Ash2 and its associated H3K4me3 play a role in transcriptional pausing control (Perez-Lluch et al., 2011). In addition to the mechanisms by which H3K4me3 influence transcription, we can infer that Ash2 regulates the majority of ecdysone-responsive genes by its ability to stabilize Trr. Although additional research is necessary to describe the exact coverage of Trr over ecdysone-inducible genes, the hypothesis that the Ash2 modulation of Trr stabilization has a regulatory role is supported by the colocalization of Ash2 and Trr on polytene chromosomes that we report here, as well as with the previously described overlap of the Trr and EcR polytene chromosome-binding sites (Sedkov et al., 2003).
To examine the interactions between ash2 and trr, we crossed males w; ash21124.11/TM6C with females y w trr1/FM6GFP; +; ash21124.11/TM6C and determined the number of y w trr1/w; +; ash21124.11 females that reached the adult stage. Heterozygous flies for either ash21124.11 or trr1 were used as controls.

**Developmental staging of larvae**

To assess the expression of ecdysone-responsive genes and the presence of H3K4me3, we staged wild-type and homozygous ash21 larvae on bromophenol blue-containing media at 25°C and then collected at clear gut stage (~4 h before pupariation) as described (Andres and Thummel, 1994).

**Genetic mosaics**

Clones mutant for all TrxG genes analyzed in wing imaginal disks from third-instar larvae were obtained by mitotic recombination using the FLP/FRT technique (Xu and Rubin, 1993). Larvae of the appropriate genotypes were cultured at 25°C and timed in hours AEL. Heat shock was carried out for 45 min at 37°C (60 ± 12 h AEL) to induce clone formation.

For ash21 mutant clones, y w; FRT82Bash21/TM6C flies were crossed with y w hsflp; FRT82BubiGFP/TM6B.

Clones mutant for trr1 analyzed in adult structures were obtained by mitotic recombination using the FLP/FRT technique by crossing y w trr1/10DFRT18A/FM7C flies with y w hsflp; FRT18A/FM6; hsflp/TM6. Larvae were cultured at 25°C, and heat shock was performed for 45 min at 37°C (85 ± 12 h AEL).

**Antibodies**

The following primary antibodies were used: anti-Trx N-terminal antibody N1 (Kuzin et al., 1994), anti-Trx (Sedkov et al., 2003), anti-Ecr-B1 (DSHB, AD4.4), anti–Ecr-A (DSHB, 15G1a), anti-hemagglutinin (HA; Roche, Indianapolis, IN), anti-V5 (Sigma-Aldrich, St. Louis, MO), anti-H3K4me3 (Millipore, Billerica, MA), anti-H3K4me2 (Upstate, Millipore), anti-H3K4me1 (Diagenode, Denville, NJ), anti-H1, anti-H3 (Abcam, Cambridge, MA), anti-Ash2Nt, and anti-Ash2Ct.

Characterization of the polyclonal Ash2 antibodies generated in our laboratory is shown in Supplemental Figure S1. Specifically, to produce the anti-Ash2Nt antibody, an N-terminal cDNA of 117 base pairs corresponding to the second exon of ash2 was inserted into a pGEX-2TK expression vector (Invitrogen, Carlsbad, CA) to produce a glutathione S-transferase (GST) fusion protein. The GST-tagged protein was purified from bacterial extract using glutathione–Sepharose 4B (GE Healthcare, Piscataway, NJ). GST was cleaved off by incubating with thrombin, and the peptide was purified by high-performance liquid chromatography (AKTA purifier; GE Healthcare) and verified by matrix-assisted laser desorption/ionization–time-of-flight. The solution of purified peptide was injected into rabbits to generate polyclonal antibodies. To produce the anti-Ash2Ct...
then collected, diluted 1:4 with RIPA buffer (140 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate, and protease inhibitors), and incubated with 2 μl of serum antibody on a rotating wheel overnight at 4ºC. Complexes were immunoprecipitated with 35 μl of protein–Sepharose A affinity matrix. Samples were run on 8% SDS–PAGE gels and then transferred to polyvinylidene fluoride membranes. The following antibodies were used for Western blots: rabbit anti-Trx (1:1500), rabbit anti-Trr (1:1500), and goat anti-rabbit peroxidase (1:3000).

Immunohistochemistry

Wing imaginal disk staining. Immunohistochemistry was performed according to standard protocols. Primary antibodies used were mouse anti–EcR-B1 (1:50), mouse anti–EcR-A (1:50), rabbit anti-H3K4me3 (1:200), rabbit anti-Trr (1:500), and rabbit anti-Trx (1:200).

Polytene chromosome staining. Salivary glands of wild-type and UAS-ash2 transgenic flies were dissected in Gohen buffer, fixed for 2 min, and transferred to a solution with acetic acid and formaldehyde for 3 min before squashing to spread the polytene chromosomes. Staining was performed by incubation overnight at 4°C with the

antibody, we inserted a C-terminal cDNA of 383 base pairs corresponding to the fifth and sixth exons of ash2 into a pPROEX-HTa expression vector (Invitrogen) to produce a fusion protein with 6× histidine (His) residues. A His-tagged protein from bacterial extract was purified using HIS-Select Nickel Affinity Gel (Sigma-Aldrich). The purified peptide solution was injected into rats to generate polyclonal antibodies.

Coimmunoprecipitation and Western blot assays

For coimmunoprecipitation experiments from embryo extracts, 0- to 16-h-old Drosophila embryos were decorionated, rinsed extensively with 0.1% Triton X-100, washed two times with 10 mM Tris-HCl (pH 7.5) and then once with nuclear extraction buffer (10% saccharose, 10 mM Tris-HCl, pH 8, 1 mM CaCl₂, 0.1 mM phenylmethylsulfonyl fluoride), homogenized, and centrifuged for 5 min at 5000 rpm. The pellet was resuspended in 1 ml of TS buffer (15 mM Tris-HCl, pH 7.5, 60 mM KCl, 2 mM EDTA, and 1 mM dithiothreitol), and centrifuged for 5 min at 5000 rpm. The pellet was resuspended in lysis buffer (100 mM NaCl, 0.1% NP40, 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.9, and a protease inhibitor cocktail tablet) and centrifuged at 13,000 rpm; the supernatant was

FIGURE 8: Ash2 associates with Trx and facilitates its catalytic activity. (A) Ash2 coimmunoprecipitated with Trx. Anti-Ash2 immunoprecipitations were performed with nuclear extracts from embryos. The input lane shows 10% of the total extract volume used for coimmunoprecipitation. (B) Ash2 colocalized with Trx on polytene chromosomes from third-instar larvae. The distribution of Ash2-HA was compared with that of Trx on a representative region of a wild-type polytene chromosome corresponding to the 2L arm. (C, D) Loss of Ash2 function did not affect Trx levels. (C) Distribution of Trx on polytene chromosomes from wild-type (left) and ash2I1 (right) third-instar larvae. (D) Trx detected in wild-type (bright green), heterozygous (green), and ash2I1 homozygous (black) wing disk cells. (E, F) Trx and H3K4me3 levels at the TSS region of lcp9, a common target gene of Trx and Ash2. ChIP analysis of wild-type (gray) and ash2I1 (black) late-wandering larva using Trx (E) and H3K4me3 (F) antibodies. Real-time PCR results were normalized against the mock sample and are depicted as fold enrichment. Error bars represent SEM.

Preparations were incubated with fluorescein isothiocyanate–or rhodamine red–conjugated secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) and then mounted with phosphate-buffered saline/glycerol/4%, 6-diamidino-2-phenylindole to stain the DNA.

**Ec dysone treatment**

For in vivo ec dysone treatment, larvae were synchronized at the sec ond- to third-instar molt and collected 24 h later at mid-L3 stage. Salivary glands were removed and placed into Robb medium. Each gland was divided into two parts, one treated with ec dysone, and the other used as a control. For ec dysone treatment, 20 μM 20-OH ec dysone (Sigma-Aldrich) was added to the medium, and the lobes of the glands were incubated at 25°C for 2 h. After incubation, ec dysone and control salivary glands were used to prepare polytenic chromosome squashes.

**Chromatin immunoprecipitation**

For qPCR ChiPs, 40 wild-type and ash2I third-instar larvae were fixed. Real-time PCRs were normalized against the mock sample (background control without antibody) and depicted as fold enrich ment above the mock.

The larva pool was suspended in 700 μl of sonication buffer and then sonicated in a Branson sonifier. Conditions were established to obtain chromatin fragment lengths of 200–1000 base pairs. Chromatin was centrifuged for 10 min at top speed at 4°C, and the supernatant was recovered. For the input, 10 μl of chromatin were de-cross-linked and purified. Immunoprecipitations were carried out in RIPA buffer. To preclear, 35 μl of 50% (vol/vol) protein A–Sepharose CL4B was added to each immunoprecipitation, and they were incubated for 1.5 h at 4°C on a rotating wheel. Protein A was removed by centrifugation at 3000 rpm for 2 min. A suitable amount of antibody (2 μl for Trx protein and 2 μg for H3K4me3) was added to each chromatin aliquot, and they were incubated on a rotating wheel overnight at 4°C. As a negative control, an aliquot was immunoprecipitated without antibody. Immunocomplexes were recovered by adding 35 μl of 50% (vol/vol) protein A–Sepharose (previously blocked in RIPA or IP/1% bovine serum albumen for 2 h at 4°C) to the sample and incubating with rocking for 3 h at 4°C. Protein A was washed five times for 10 min each time in 1 ml of RIPA buffer or IP buffer, once in 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxyxolhol, 1 mM Na-EDTA, and 10 mM Tris-HCl (pH 8.0), and twice in TE (1 mM Na-EDTA, 10 mM Tris-HCl, pH 8.0). Protein A was resuspended in 100 μl of TE, and DNase-free RNase was added and incubated for 30 min at 37°C. To purify the immunoprecipitated DNA, samples were adjusted to 0.5% SDS and 500 μl proteinase K and incubated overnight at 65°C. Immunoprecipitated chromatin was purified with Qiaquin (Valencia, CA) PCR purification columns, following the manufacturer’s instructions. The primers are shown in Table 1.

**RNA extraction, RT-PCR, and real-time PCR**

RNA was prepared from wing imaginal disks and fat bodies of w; ash2I and Canton S late-third-instar larvae using RNeasy Mini Kit (Qiagen) for RNA extraction from wing imaginal disks and TRizol reagent (Invitrogen) for RNA extraction from fat bodies, according to the manufacturers’ instructions. Quality was assessed in all samples using the Eukaryote Total RNA Nano Assay on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Total RNA (1 μg) was used for cDNA synthesis. Reverse transcription was performed using random hexamers and AMV reverse transcriptase (Roche). Real-time PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA), using SYBR Green and standard Applied Biosystems settings. Reactions were run in triplicate in at least two independent experiments. Expression data were normalized to the control genes sly (for wing imaginal disk expression data) or rp49 (for fat body expression data). The primers are shown in Table 2.

**Bioinformatics analysis**

We considered the GO enrichments identified by DAVID (Huang da et al., 2009) in FAT-filtered biological process and molecular function categories and in the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/) pathway.

To produce a graphical distribution for the reads from each sample around the TSS, we calculated the weighted number of reads on each position from 2000 base pairs upstream to 2000 base pairs downstream of the TSS of all genes (according to RefSeq, www.ncbi.nlm.nih.gov/RefSeq/).

**AC KNOWLEDGMENTS**

We thank E. Blanco for kindly providing bioinformatics support, S. Pérez-Lluch for insightful suggestions, A. Mateo for technical support, and J. Muller for Drosophila stocks. We also thank the Confo cal Unit of the Universitat de Barcelona Core Facilities (Barcelona, Spain). This project was funded by grants BMC2006-07334, BFU2009-09781, and CSD2007-00008 from the Ministerio de Cien cia e Innovacion, Spain. A.C. was supported by an APIF fellowship from Universitat de Barcelona.

<table>
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<th>Primer</th>
<th>PRIMER 1</th>
<th>Primer 2</th>
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<tbody>
<tr>
<td>BR-C (+1079 to +1212; isoform M)</td>
<td>FW_5’-TTGACATTTAAAAATCTGATT-3’</td>
<td>RV_5’-AAGTTGTCATTTGTGTTT-3’</td>
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<tr>
<td>E75A (-1852 to −1751)</td>
<td>FW_5’-ACGAGATAACAATTGGGCTTGGA-3’</td>
<td>RV_5’-TGACGGCGAGTAACTCCGTGAA-3’</td>
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<tr>
<td>lcp9 (+143 to +215)</td>
<td>FW_5’-TTGATAGAAGCGGACCTCCA-3’</td>
<td>RV_5’-CGCGAATATGGTTGGATAG-3’</td>
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Coordinates are given relative to the TSS of each target gene.

**TABLE 1: Primers for chromatin immunoprecipitation.**

**TABLE 2: Primers for qPCR.**
REFERENCES


