CAF-1 promotes Notch signaling through epigenetic control of target gene expression during Drosophila development

Zhongsheng Yu1,2, Honggang Wu1,2, Hanqing Chen1,2, Ruoqi Wang1, Xuehong Liang1, Jiyong Liu1, Changqing Li1, Wu-Min Deng3,* and Renjie Jiao1,*

SUMMARY
The histone chaperone CAF-1 is known for its role in DNA replication-coupled histone deposition. However, loss of function causes lethality only in higher multicellular organisms such as mice and flies, but not in unicellular organisms such as yeasts, suggesting that CAF-1 has other important functions than histone deposition during animal development. Emerging evidence indicates that CAF-1 also has a role in higher order chromatin organization and heterochromatin-mediated gene expression; it remains unclear whether CAF-1 has a role in specific signaling cascades to promote gene expression during development. Here, we report that knockdown of one of the subunits of Drosophila CAF-1, dCAF-1-p105 (Caf1-105), results in phenotypes that resemble those of, and are augmented synergistically by, mutations of Notch positive regulatory pathway components. Depletion of dCAF-1-p105 leads to abrogation of cut expression and to downregulation of other Notch target genes in wing imaginal discs. dCAF-1-p105 is associated with Suppressor of Hairless [Su(H)] and regulates its binding to the enhancer region of E(spl)m. The association of dCAF-1-p105 with Su(H) on chromatin establishes an active local chromatin status for transcription by maintaining a high level of histone H4 acetylation. In response to induced Notch activation, dCAF-1 associates with the Notch intracellular domain to activate the expression of Notch target genes in cultured S2 cells, manifesting the role of dCAF-1 in Notch signaling. Together, our results reveal a novel epigenetic function of dCAF-1 in promoting Notch pathway activity that regulates normal Drosophila development.

KEY WORDS: Drosophila, CAF-1, Notch, Epigenetic regulation, H4 acetylation

INTRODUCTION
Chromatin assembly factor 1 (CAF-1) is a highly conserved three-subunit histone chaperone in eukaryotes (Ridgway and Almouzni, 2000), which has been shown to facilitate chromatin assembly by depositing histone H3 and H4 onto newly synthesized DNA (Tyler et al., 2001; Groth et al., 2007). Increasing evidence suggests that CAF-1 has important functions in other cellular and developmental processes in addition to DNA replication (Ridgway and Almouzni, 2000; Groth et al., 2007; Quivy et al., 2008; Huang et al., 2010; Autran et al., 2011; Nakano et al., 2011). For example, it is involved in the restoration of chromatin structure after DNA repair (Groth et al., 2007; Chen et al., 2008; Li et al., 2008); CAF-1 also participates in the establishment of epigenetic information during heterochromatin formation (Song et al., 2007; Quivy et al., 2008; Huang et al., 2010); recent studies in Arabidopsis and C. elegans suggest that CAF-1 is involved in the regulation of gene expression as well as asymmetric cell division (Autran et al., 2011; Nakano et al., 2011). However, the molecular mechanisms by which CAF-1 regulates transcription and whether this function is coupled to specific signaling pathways that are essential for animal development remain unclear.

In metazoans, the highly conserved Notch signaling pathway plays essential roles in the control of cell proliferation and cell fate specification during animal development (Artavanis-Tsakonas and Muskhatchev, 2010). Defects in the Notch pathway are associated with various types of human disorders, such as T-cell leukemia and several breast cancers (Ranganathan et al., 2011). In Drosophila, the core components of the Notch pathway consist of ligands Delta/Serrate (Dl/Ser), receptor Notch and the DNA-binding transcription factor Suppressor of Hairless [Su(H)] (Bray, 2006). The binding of Dl/Ser situated on one cell to Notch situated on the neighboring cell results in two proteolytic cleavages of Notch, which mediate the release of Notch intracellular domain (NINV) into the nucleus to activate Notch target gene transcription with the assistance of Su(H) and co-activator Mastermind (Mam) (Bray, 2006; Artavanis-Tsakonas and Muskhatchev, 2010). Despite the relative simplicity of primary Notch signaling, the presence of a large number of fine-tuning regulators at different levels, from the outside of the cell membrane to the nucleus, dramatically increases the complexity of Notch pathway outputs and its cellular responses, thus controlling a wide range of developmental processes (Neumann and Cohen, 1998; Acar et al., 2008; Hautbergue et al., 2009; Kim et al., 2009; Xie et al., 2012). At the nuclear level, recent findings suggest that chromatin-associated regulatory mechanisms are important for proper Notch target gene expression (Bray et al., 2005; Kugler and Nagel, 2007; Moschkin et al., 2009; Duan et al., 2011; Mulligan et al., 2011; Domantowska and Schüpbach, 2012; Endo et al., 2012). Transcriptional silencing complexes have been identified by proteomic methods in the absence of Notch activation (Moschkin et al., 2009; Mulligan et al., 2011). In flies, LAF (LID-associated factor) and RLAF (RPD3-LID-associated factor) silencing complexes cooperate with histone chaperones Asf1 and Nap1 to mediate epigenetic silencing at the Notch target Enhancer of Split [E(spl)] cluster (Moschkin et al., 2009). In both mammals and flies, an SIRT1-LSD1 co-repressor complex represses Notch target genes in cultured cells (Mulligan et al., 2011). Epigenetic regulators and chromatin modifiers that execute positive regulatory roles in Notch signaling are now being identified (Bray, 2006). Specifically, in...
mammalian cells, the histone acetyl transferases (HATs) PCAF (KAT2B) and GCN5 (KAT2A) have been shown biochemically to function in RBP-J [the mammalian homolog of Su(H)]-mediated transactivation by NCoR (Kurooka and Honjo, 2000). In flies, the RING-finger E3 ubiquitin ligase Br1 and chromatin remodelling complex NURF have been shown to be involved in the epigenetic regulation of Notch target gene expression (Bray et al., 2005; Kugler and Nagel, 2010). Recently, an RNAi screen in cultured *Drosophila* cells revealed that a Notch pathway transcriptional reporter is sensitive to chromatin-modifying enzymes and remodelers (Mourikis et al., 2010). However, the precise mechanism of how Notch signaling is epigenetically regulated during development remains unclear.

In this report, we describe a novel function of *Drosophila* chromatin assembly factor 1 (dCAF-1) in regulating the expression of Notch target genes. dCAF-1 genetically interacted with the Notch pathway in both the eye and wing. The expression of two Notch target genes, cut and wg, was largely abolished in dCAF-1-p105 flies. dCAF-1-p105 (Caf1-105 – FlyBase) mutant cells of the wing disc. Biochemical and chromatin immunoprecipitation experiments revealed that dCAF-1-p105 regulates the binding of Su(H) to the enhancer region of *E(spl)mj* to establish a local active chromatin structure by maintaining a high level of histone H4 acetylation. Our results show that dCAF-1 functions specifically to regulate the Notch signaling pathway, promoting its target gene expression through epigenetic regulation, during *Drosophila* development.

**MATERIALS AND METHODS**

**Fly strains and genetics**

The dCAF-1-p105 mutant line (p105<sup>56</sup>) was generated by a standard P-element jump-out strategy (Song et al., 2007) (Fig. 2A). For clonal analyses, the mutation of p105<sup>56</sup> was recombined with FRT42D on the left arm of the second chromosome. To produce the UAS-HA-dCAF-1-p105 transgenic flies, a modified pUAST-HA vector was used: an ATG start codon and HA tag sequence were inserted at the EcoRI site of the pUAST construct. PCR products of dCAF-1-p105 were amplified using 5'-AAGTGCAGATCCCGAGATTCTC-3' and 5'-GTTAAGTCCTACCTGCTCGTCAC-3' specific oligonucleotide primers. The UAS-dCAF-1-p105 (UAS-dCAF-1-p105<sup>56</sup>) lines 12892R-1 (II) and 12892R-3 (III) were obtained from the National Institute of Genetics, Japan; the UAS-dCAF-1-p105 RNAi line of v26456 was obtained from the FlyBase. The UAS-dCAF-1-p105 RNAi line of dCAF-1-p105<sup>56</sup> was recombined with FRT42D on the left arm of the second chromosome. To produce the pUAST-HA-dCAF-1-p105 construct of correct DNA sequence was used for microinjection following standard protocols (Xu et al., 2009). Transgenic lines were verified by their ability to rescue the p105<sup>56</sup> mutants.

**RNA isolation and quantification**

Total RNA from adults was isolated using Trizol (Invitrogen) and further cleaned with TURBO DNase (Ambion). Then, 5 µg total RNA was used for reverse transcription with the SuperScript III reverse transcriptase kit (Invitrogen) according to the standard protocol (Liu et al., 2011). Quantitative PCR (qPCR) was performed on an MJ Research CHROMO4 real-time detector machine and the results were analyzed with Opticon Monitor 2 software. All qPCR values are the mean of three independent experiments after normalization and Student’s t-tests were used to evaluate significance.

**Chromatin immunoprecipitation (ChIP) assay**

Second instar larvae were harvested and fixed in 1% paraformaldehyde in PBS at 37°C for 15 minutes with vortex. The cross-linked chromatin was resuspended in IP lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 0.1% SDS) in the presence of protease inhibitors [1 mM PMSF; protease inhibitor cocktail (Calbiochem)]. For co-IP assays, extracts were incubated with specific antibodies and protein A/G agarose beads for 4 hours (Promega) as previously described (Huang et al., 2011). Primers (forward and reverse, 5'-3') were: dCAF-1-p105, gatcataatacgactaactagg-gcggtcagcacaattgtgatc agtgtagaagggcgaggaagctg and gatctatactactagagt-aactcaactgctagagt-aactcaactgctaga (lowercase letters indicate T7 promoter sequences). For a 6-well plate, cells in 2 ml medium in each well were treated with 15 µg dsRNA for 4 days prior to plasmid transfection or CuSO<sub>4</sub> induction.

**Immunohistochemistry and antibodies**

Wandering third instar larvae of the correct genotype were collected and dissected in cold PBS. Wing imaginal discs were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature. After four washes of 0.3% PBST (PBS containing 0.3% Triton X-100) and 15 minutes blocking in 10% goat serum in 0.3% PBST, wing discs were incubated with the following primary antibodies: mouse anti-Cut, mouse anti-Wg and mouse anti-β-Gal [1:50, Developmental Studies Hybridoma Bank (DSHB)]; rabbit anti-dCAF-1-p108 (1:100, Abcam); rabbit anti-dCAF-1-p105 (1:100, Abcam); for antigen production, a fragment of *Drosophila* CAF-1-p105 (amino acids 281-539) was cloned into the pGEX vector; expression, purification and subsequent immunization of the GST fusion protein were carried out by Antibiotics; and rabbit anti-dCAF-1-p55 [1:500, a gift of Jessica Tyler (Tyler et al., 2003)]. Fluorescent secondary antibodies were used for signal detection. Images were captured with a Leica SP5 confocal microscope as previously described (Chen et al., 2010).

**S2 cell culture, transfection and RNA interference (RNAi) assays**

*Drosophila* S2 cells were cultured at room temperature in Hyclone serum-free insect cell culture media (Roche). Transfection of S2 cells was performed using FuGENE HD transfection kit reagents (Roche) following the manufacturer’s instruction. Constructs used for transfections are described in supplementary material Table S2. The S2 cells were normally harvested 48 hours after transfection for further experiments. Full-length Notch expression was induced by 500 μM CuSO<sub>4</sub> for 24 hours after pMT-Notch transfection (Feohan et al., 1990). Double-stranded (ds) RNA was prepared with the RibomAX large-scale RNA production system-T7 kit (Promega) as previously described (Huang et al., 2011). Primers (forward and reverse, 5'-3') were: dCAF-1-p105, gatcataatacgactaactagg-gcggtcagcacaattgtgatc agtgtagaagggcgaggaagctg and gatctatactactagagt-aactcaactgctagagt-aactcaactgctaga (lowercase letters indicate T7 promoter sequences). For a 6-well plate, cells in 2 ml medium in each well were treated with 15 µg dsRNA for 4 days prior to plasmid transfection or CuSO<sub>4</sub> induction.

**Immunoprecipitation (co-IP) assay**

Total protein extracts from embryos or S2 cells were prepared in IP lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 0.1% SDS) in the presence of protease inhibitors [1 mM PMSF; protease inhibitor cocktail (Calbiochem)]. For co-IP assays, extracts were incubated with specific antibodies and protein A/G agarose beads (Abmart) at 4°C overnight before washes and elution. Immunoprecipitates were boiled in 2×SDS loading buffer for elution from the beads. Goat anti-Su(H) (1:100, Santa Cruz), rabbit anti-Flag (1:100, Sigma) and mouse anti-HA (1:100, Abcam) antibodies were used for co-IP experiments; mouse anti-HA (1:1000, Abcam), mouse anti-NICD (1:1000, DSHB), and rabbit anti-Myc (1:2000, Sigma) were used for western blots.

**Immunofluorescence**

Immunofluorescent images were obtained using a TCS-LP laser scanning confocal microscope as previously described (Chen et al., 2010). Fluorescent secondary antibodies were used for signal detection. Images were captured with a Leica SP5 confocal microscope as previously described (Chen et al., 2010).

**qPCR**

Total RNA from animals of the correct genotype was isolated using Trizol reagent (Invitrogen) and further cleaned with TURBO DNase (Ambion). Then, 5 µg total RNA was used for reverse transcription with the SuperScript III reverse transcriptase kit (Invitrogen) according to the standard protocol (Liu et al., 2011). Quantitative PCR (qPCR) was performed on an MJ Research CHROMO4 real-time detector machine and the results were analyzed with Opticon Monitor 2 software. All qPCR values are the mean of three independent experiments after normalization and Student’s t-tests were used to evaluate significance.

**Gene-specific primers used for qPCR are listed in supplementary material Table S3.**

**Chromatin immunoprecipitation (ChIP) assay**

Second instar larvae were harvested and fixed in 1% paraformaldehyde in PBS at 37°C for 15 minutes with vortex. The cross-linked chromatin was resuspended in IP lysis buffer (see co-IP assay) followed by sonication to obtain DNA fragments of ~500-1000 bp. Anti-acetylated H4 (Upstate Biotechnology), anti-dCAF-1-p105 (Anticeps), anti-H3 (Abcam) or anti-Su(H) (Santa Cruz) together with protein A/G agarose beads were incubated with sonicated lysates at 4°C overnight. Following elution (Huang et al., 2010), cross-linking of the chromatin samples was reversed at 65°C for 6 hours. Subsequently, genomic DNA was extracted and used as PCR template for quantitative analyses. Primers used to detect the *E(spl)mj* enhancer and *hh* enhancer are listed in supplementary material Table S3. The PCR values in all ChIP experiments are the mean of three independent experiments after normalization and Student’s t-tests were used to evaluate significance.
Depletion of dCAF-1-p105 leads to developmental defects that resemble the effects of Notch signaling downregulation in the Drosophila eye and wing

In a search for signaling pathways that are regulated by CAF-1 in Drosophila, we performed a small-scale candidate screen using dCAF-1-p105 RNAi (dCAF-1-p105IR) flies, in which the RNAi is specifically expressed in the eye under the control of eyeless-Gal4 (ey-Gal4). dCAF-1-p105IR flies exhibited a mild small-eye phenotype (Fig. 1A,C versus 1E, Ctrl). Two lines of evidence suggest that this represents a specific loss-of-function phenotype of dCAF-1-p105: (1) the small-eye phenotype was rescued by expression of UAS-HA-dCAF-1-p105 under the control of the same Gal4 driver (supplementary material Table S1); (2) in dCAF-1-p105IR flies, the transcription of dCAF-1-p105 was specifically downregulated (supplementary material Fig. S1).

Next, we examined how the alteration of particular signaling pathways that are known to control cell proliferation and/or patterning during Drosophila eye development affected this small-eye phenotype. The pathways examined included the Hedgehog (Hh), Hippo, Epidermal growth factor receptor (Egfr), Decapentaplegic (Dpp) and Notch (N) signaling pathways (Fig. 1E). The small-eye phenotype was significantly enhanced in heterozygous mutant backgrounds of different Notch positive regulatory pathway components. The enhancement was judged by: (1) the fraction of flies with small eyes increased from 33.9% to 100% as compared with the control (ey>dCAF-1-p105IR); (2) the eyes were also generally smaller than those of the control flies (Fig. 1C-E, N, H, Dl, mam and eyg represent flies of ey>dCAF-1-p105IR in the background of one copy of the mutation for each indicated gene). By contrast, the heterozygous mutation of Hairless (H), a negative regulator of Notch signaling, fully suppressed the small-eye phenotype (compare H and Ctrl in Fig. 1E). Mutations of components of other signaling pathways (e.g. smo, Egfr, yki and tkv) did not appear to affect the small-eye phenotype of ey>dCAF-1-p105IR flies (Fig. 1E; supplementary material Table S1). These results suggest that dCAF-1-p105 genetically interacts with the Notch pathway to control Drosophila eye development.

Next, we extended our investigation to determine whether dCAF-1-p105 also interacts with the Notch pathway during wing development, where Notch plays important roles in vein patterning and wing margin specification (Artavanis-Tsakonas and...
Muskavitch, 2010). Expression of dCAF-p-105{sup}IR{sup} in the wing under the control of scalloped-Gal4 (sd-Gal4) led to a notched wing phenotype (Fig. 1F) resembling that of Notch loss-of-function mutations (Fig. 1K). These wings typically exhibit notches at the wing margin of the anterior/posterior (A/P) boundary and mildly thickened veins at longitudinal vein (LV) 3 and 5 (Fig. 1K; supplementary material Fig. S1B,D). When the N{sup}{sup}I heterozygous mutation was introduced into the sd>dCAF-1-p105{sup}IR{sup} flies, the entire wing margin failed to develop, constituting a much stronger notched wing phenotype than dCAF-1-p105{sup}IR{sup} alone (Fig. 1G,F). In addition, LV3 and LV5 were much more thickened than those of heterozygous N{sup}{sup}I wings (Fig. 1G,K), suggesting a synergistic effect between dCAF-1-p105{sup}IR{sup} and N{sup}{sup}I. To show directly the genetic interaction between dCAF-1-p105 and Notch, we examined whether loss of one copy of the dCAF-1-p105 gene affected the N{sup}{sup}I phenotype. One copy of p105{sup}{sup}IR{sup} enhanced the notched wing phenotype of N{sup}{sup}I, with increased penetrance from 11.8% to 17.9% (supplementary material Fig. S3A,B). These results suggest that dCAF-1-p105 synergistically interacts with the Notch pathway to regulate normal tissue development not only in the eye but also in the wing.

dCAF-1-p105 is required for proper expression of the Notch target genes cut and wg
To exclude any potential off-target artifacts in the RNAi experiments, and to ascertain that dCAF-1-p105 is required for the output of Notch signal transduction, we generated a null allele of dCAF-1-p105 through P-element-mediated imprecise excision. Imprecise excision of the EP{sup}G2212{sup} insertion resulted in a 2480 bp deletion, which covers most of the dCAF-1-p105 coding region and part of the adjacent gene CG11777, yielding a predicted double mutant of dCAF-1-p105 and CG11777 designated p105{sup}{sup}IR{sup} (Fig. 2A,B). Further RT-PCR analysis showed that dCAF-1-p105 mRNA was completely abolished in p105{sup}{sup}IR{sup} homozygous mutants (Fig. 2C). The majority of the p105{sup}{sup}IR{sup} mutant larvae did not survive longer than 48 hours after egg deposition (AED) and exhibited an apparent developmental delay and small body size when compared with wild-type larvae of the same age (Fig. 2D). A few escapes of the p105{sup}{sup}IR{sup} mutants lived longer than 72 hours, but never survived to the pupal stage (Fig. 2D). The p105{sup}{sup}IR{sup} phenotypes, including lethality, were completely rescued by ubiquitous expression of the UAS-HA-dCAF-1-p105 transgene under the control of daughterless-Gal4 (da-Gal4) (Fig. 2E; supplementary material Fig. S2A), suggesting that p105{sup}{sup}IR{sup} is a null allele for dCAF-1-p105 and that the adjacent CG11777 is a non-essential gene.

To test the effect of dCAF-1-p105 null mutation on Notch signaling, we performed clonal analyses to monitor developmental defects and to detect the expression of Notch target genes in the absence of dCAF-1-p105. p105{sup}{sup}IR{sup} mutant clones were generated in the Minute background to overcome the growth disadvantage of the mutant cells. After clonal induction by heat shock, flies carrying p105{sup}{sup}IR{sup} clones showed a notched wing phenotype (Fig. 3B), whereas flies bearing mock clones showed no defects in the wing (Fig. 3A). The Notch-like phenotype in p105{sup}{sup}IR{sup} mosaic flies suggests that Notch signaling is compromised in the absence of dCAF-1-105. To confirm this, we examined the expression of two well-characterized Notch target genes, cut and wg, in p105{sup}{sup}IR{sup} mosaic wing imaginal discs. In wild-type wing discs and discs with mock clones, Cut and Wg were both expressed along the dorsal/ventral (D/V) boundary (Fig. 3C,D). In p105{sup}{sup}IR{sup} mutant clones, their expression was significantly reduced to almost below the detection level (Fig. 3E-F{sup}′{sup}), indicating significant downregulation of Notch signaling in the absence of dCAF-1-105. To verify the specificity of the effects of dCAF-1-p105 on Notch signaling, we examined the activity of Hh signaling in p105{sup}{sup}IR{sup} mosaic wing discs. Consistent with the genetic interaction results (Fig. 1), in p105{sup}{sup}IR{sup} mutant clones, the expression of two Hh signaling readouts, hh-lacZ and dpp-lacZ, was at about the same level as that in the wild-type region (supplementary material Fig. S3A,B). These results suggest that dCAF-1-p105 specifically regulates the output of Notch signaling in the wing disc.

Next, we investigated the molecular mechanism(s) underlying how dCAF-1-p105 functions in Notch signaling. To test whether dCAF-1-p105 functions at the transcriptional level to regulate Notch target gene expression, we examined the transcription of two well-defined reporter genes of the Notch signaling pathway:
cut and the E(spl) complex component gene E(spl)mβ (Jack et al., 1991; Neumann and Cohen, 1996; Xie et al., 2012). The cut-lacZ (Jack et al., 1991) and E(spl)mβ-lacZ (Xie et al., 2012) transgenic reporters were used for this purpose. When dCAF-1-p105 was knocked down at the posterior region of the wing disc, cut-lacZ and E(spl)mβ-lacZ expression was significantly downregulated specifically in the posterior region (Fig. 4B,B′,D,D′). These results suggest that dCAF-1-p105 functions as a positive regulator of the Notch signaling pathway by promoting its target gene transcription.

dCAF-1-p105 associates with Su(H) to regulate its binding to the enhancer region of E(spl)mβ
Su(H) is the core transcription factor regulating Notch target gene expression (Bray, 2006). To determine whether dCAF-1-p105 physically associates with Su(H) in vivo, we expressed a UAS-HA-dCAF-1-p105 transgene under the control of da-Gal4 in developing Drosophila embryos, in which the total amount (non-tagged plus HA-tagged) of dCAF-1-p105 was 2.39-fold that in wild-type animals (supplementary material Fig. S2A). From the extracts of such embryos, HA-tagged dCAF-1-p105 was co-

Fig. 3. dCAF-1-p105 is required for the proper expression of Notch target genes cut and wg. (A,B) Induction of dCAF-1-p105 (p10556) mutant clones leads to a wing notch, whereas induction of mock clones leads to wild-type wings. Mock clones and p10556 mutant clones were induced at the second instar larval stage at 38°C for 1 hour. The genotype of flies with mock clones is hs-Flp/4; FRT42D, Minute, ubi-GFP/FRT42D. The genotype of flies with p10556 mutant clones is hs-Flp/4; FRT42D, Minute, ubi-GFP/FRT42D, p10556. (C-F) In dCAF-1-p105 mutant clones, the expression of Cut (E,E′, GFP-negative area, arrowheads) and Wg (F,F′, GFP-negative area, arrowheads) is abolished in a cell-autonomous manner, whereas in the mock clones the expression of both Cut (C,C′) and Wg (D,D′) is unaffected. Wing discs were dissected for immunostaining 3 days after clonal induction.

Fig. 4. Notch target genes are regulated by dCAF-1-p105 at the transcription level. (A-B′) The expression of cut-lacZ is downregulated upon depletion of dCAF-1-p105. In control discs (A,A′) cut-lacZ is expressed normally along the D/V boundary (dashed line) in both the anterior and posterior regions. Knocking down dCAF-1-p105 under the control of en-Gal4 (B,B′) substantially reduces cut-lacZ expression in the posterior region. Genotypes: (A,A′) UAS-Dicer2/+; en-Gal4, UAS-GFP/+; cut-lacZ/+; (B,B′) UAS-Dicer2/+; en-Gal4, UAS-GFP/UAS-dCAF-1-p10556, cut-lacZ/+. UAS-Dicer2 was used to enhance RNAi efficiency. (C-D′) The expression of E(spl)mβ-lacZ is downregulated in the dCAF-1-p105-depleted area. In control discs (C,C′) E(spl)mβ-lacZ is expressed normally in the entire wing disc. Knocking down dCAF-1-p105 under the control of en-Gal4 (D,D′) substantially reduces E(spl)mβ-lacZ expression. The genotype of Ctrl is UAS-Dicer2/+; en-Gal4, UAS-GFP/E(spl)mβ-lacZ, and en>dCAF-1-p10556 is UAS-Dicer2/+; en-Gal4, UAS-GFP/E(spl)mβ-lacZ, UAS-dCAF-1-p10556.
immunoprecipitated with an anti-Su(H) antibody (Fig. 5A), but not with the control antibody (Fig. 5). More importantly, in the extracts of wild-type embryos the association of Su(H) with endogenous dCAF-1-p105 was also detected (supplementary material Fig. S2B), suggesting that dCAF-1-p105 is physically associated with Su(H) in *Drosophila*.

Next, we asked whether dCAF-1-p105 is specifically associated with the chromatin at the Su(H)-binding enhancer region of one of the Notch target genes, *E(spl)mβ*. Using a ChIP assay, we detected a significant increase in dCAF-1-p105-associated *E(spl)mβ* enhancer DNA as compared with the control IgG (Fig. 5B), suggesting an enrichment of dCAF-1-p105 at the *E(spl)mβ* enhancer region. To assess the biological effects of this enrichment in Notch signaling, we examined the expression of *E(spl)mβ* in the presence and absence of dCAF-1-p105. The expression level of *E(spl)mβ* was consistently and significantly reduced in the second instar larvae of dCAF-1-p105 mutants compared with the wild type, whereas expression of target genes of the Hh pathway, such as *hh*, was unaffected (Fig. 5C; supplementary material Fig. S3C).

Following Notch induction, the occupancy of Su(H) at Notch target genes has been reported to be increased (Krejcí and Bray, 2007). We hypothesized that the local chromatin structure, as regulated by dCAF-1-p105, affects Su(H) occupancy of its target DNA sequence. To test this hypothesis, we assessed the binding efficiency of Su(H) at the *E(spl)mβ* enhancer region in a ChIP assay. Compared with the wild-type control, in *p105* mutant larvae exhibited a significant increase in the abundance of Su(H) at the *E(spl)mβ* enhancer region, as judged by the decreased amount of chromatin immunoprecipitated by the Su(H) antibody (Fig. 5D). These data suggest that dCAF-1-p105 regulates Notch target gene expression, probably by controlling the accessibility of Su(H) to its target DNA sequence in the enhancer regions of Notch target genes.

**dCAF-1-p105 maintains the acetylation level of histone H4 at the enhancer region of *E(spl)mβ***

To elucidate the precise mechanism of how dCAF-1-p105 promotes Notch target gene transcription, we examined the possibility that dCAF-1-p105 plays a role in providing a chromatin platform that is permissive for transcription by regulating histone modifications. Because histone H4 acetylation is associated with active promoters of Notch target genes (Krejcí and Bray, 2007), we monitored the level of active histone H4 acetylation in the *E(spl)mβ* enhancer region in both wild type and *dCAF-1-p105* mutants. Using acetylated H4 (H4ac)-specific antibodies that recognize H4K5ac, H4K8ac, H4K12ac and H4K16ac in a ChIP assay, we showed that, in wild-type flies, the amount of H4ac-associated *E(spl)mβ* enhancer DNA was significantly higher than that associated with control IgG, suggesting that H4ac was enriched at the enhancer region of *E(spl)mβ* (Fig. 5E). Interestingly, the amount of immunoprecipitated DNA from the *E(spl)mβ* enhancer region by anti-H4ac was significantly lower in the *dCAF-1-p105* mutant than in wild type (Fig. 5E), suggesting a decrease in H4ac associated with the absence of dCAF-1-p105. To exclude the possibility that the reduction of H4ac in *dCAF-1-p105* mutant larvae was caused by a decrease in overall histone abundance or nucleosome density at the *E(spl)mβ* enhancer region, an antibody that recognizes total histone H3 was used in the ChIP assay for normalization (Fig. 5F). Compared with the control, the normalized H4ac level in the *E(spl)mβ* enhancer region also appeared significantly reduced in *dCAF-1-p105* mutant larvae compared with that in wild-type animals (Fig. 5F). By
contrast, loss of dCAF-1-p105 did not affect the H4ac level in the enhancer region of the hh gene (supplementary material Fig. S3D). Collectively, these results suggest that dCAF-1-p105 regulates the epigenetic modification mark of H4 acetylation in the enhancer region of the Notch target gene E(spl)mβ in order to maintain an active chromatin status.

dCAF-1-p105 is required for Notch target gene expression in S2 cells following Notch signal induction

To determine whether dCAF-1-p105 can promote Notch target gene transcription when Notch signaling is artificially induced, we made use of a transient Notch activation system in cultured S2 cells by transfecting a Notch-expressing plasmid, pMT-Notch (Krejcí and Bray, 2007). In this assay, the overexpressed full-length Notch can be processed to an active form of NICD that physically interacts with Su(H), mimicking the in vivo Notch activation process (supplementary material Fig. S3A). A significant upregulation of the E(spl) complex genes E(spl)m3 and E(spl)m7 (Krejcí and Bray, 2007; Endo et al., 2012) was detected when Notch signaling was induced (supplementary material Fig. S4B,C). In this transient Notch activation system, assuming that dCAF-1-p105 is required for regulating Notch target gene expression, the induced expression should be compromised by a perturbation of dCAF-1-p105. As expected, the introduction of interfering dCAF-1-p105 dsRNAs (dsp105), but not dsGFP, into the Notch-activating S2 cells led to a significant reduction in expression of the primary endogenous Notch target genes E(spl)m3 and E(spl)m7 (Fig. 6A,B). No significant difference in transcription was detected for the control gene Gapdh (Fig. 6C). These results suggest that dCAF-1-p105 is also required for artificially induced Notch signaling in cultured S2 cells.

The NICD-Su(H)-Mam ternary complex has been reported in many organisms to regulate Notch target gene activation (Bray, 2006), and we considered whether dCAF-1 physically interacts with NICD to promote Notch activation. To test this, a construct that expresses Myc-tagged dCAF-1-p105 was co-transfected with HA-tagged NICD into S2 cells (Fig. 6E). In the co-IP assay, dCAF-1-p105 was detected in association with NICD (Fig. 6G). Interestingly, in addition to dCAF-1-p105, the other two subunits of dCAF-1 were also co-immunoprecipitated with NICD (Fig. 6F-H). Together, these results suggest that dCAF-1 might physically interact with NICD in the form of a complex in Drosophila cells, consequently promoting Notch target gene expression.

An integral dCAF-1 complex, rather than a subunit, is required for proper Notch signaling during Drosophila development

dCAF-1 has been identified biochemically as a heterotrimeric complex consisting of dCAF-1-p180, dCAF-1-p105 and dCAF-1-p55 (Caf1-180, Caf1-105 and Caf1 – FlyBase) (Tyler et al., 2001), which are evolutionarily conserved throughout the eukaryotes.
(Ridgway and Almouzni, 2000; Loyola and Almouzni, 2004; Huang and Jiao, 2012). Our co-IP results suggested that all three subunits of dCAF-1 were associated with N\textsuperscript{ICD} during Notch activation in cultured S2 cells (Fig. 6F-H). We next asked whether these three subunits of dCAF-1 function as a complex in Notch signaling in vivo. First, we examined the effects of their individual depletion by RNAi on cut expression (the effectiveness and specificity of the RNAi were validated by testing independent RNAi lines, RT-PCR and rescue experiments; data not shown). Consistent with what was observed in the p105\textsuperscript{36} mutant clones (Fig. 3E), knockdown of dCAF-1-p105 at the posterior region of the wing disc led to a significant decrease in cut expression (Fig. 7E,E'), whereas adult flies that developed from these larvae exhibited the notched wing phenotype (Fig. 7B). Similarly, en-Gal4-driven knockdown of either dCAF-1-p180 or dCAF-1-p55 also resulted in reduced Cut expression at the DV boundary of the posterior wing disc and the notched wing phenotype (Fig. 7A,C-D',F,F'), suggesting that the three subunits of dCAF-1 are all required for the activation of the Notch signaling pathway.

Given that all three subunits of dCAF-1 are associated with N\textsuperscript{ICD} (Fig. 6F-H), we hypothesized that dCAF-1 regulates Notch signaling as an integral protein complex. One possibility in maintaining a functional protein complex is that the subunits of this complex mutually stabilize each other. Indeed, knockdown of dCAF-1-p105 at the posterior region of the wing disc led to downregulation of dCAF-1-p105 cell-autonomously (Fig. 7G,J). The reverse situation was also true; when dCAF-1-p180 was knocked down, a significant downregulation of the dCAF-1-p105 level was detected (Fig. 7H,J). However, the smallest subunit, dCAF-1-p55, was evidently unaffected by knockdown of either dCAF-1-p105 or dCAF-1-p180 (Fig. 7K,L). Additionally, when dCAF-1-p55 was knocked down in the posterior region of the wing discs, neither dCAF-1-p180 nor dCAF-1-p105 was affected (supplementary material Fig. S5). When dCAF-1-p180 was knocked down by the ubiquitously expressed da-Gal4, dCAF-1-p105 transcription was apparently unaltered (supplementary material Fig. S5). Similarly, when dCAF-1-p105 was knocked down, the transcription level of dCAF-1-p180 remained unchanged (supplementary material Fig. S6), indicating that dCAF-1-p105 and dCAF-1-p180 mutually stabilize each other at the protein level, rather than the loss of one affecting the expression of the other. These results suggest that the function of dCAF-1 in regulating Notch signaling during Drosophila development is dependent on its integrity as a protein complex.

**DISCUSSION**

Signaling cascades are essential in instructing cell proliferation and differentiation during animal development and are often manifested by turning on or shutting off the expression of a group of genes. The specificity of different signaling pathways is usually determined by activating specific transcription factors that bind to the enhancers of their target genes. However, in eukaryotic cells the full transcription of a particular target gene is also dependent on the recruitment of chromatin remodelers and histone modifiers, in addition to transcription factors, in order to ensure a local chromatin environment that is permissive for the access of a complete set of regulatory proteins. Although CAF-1 was initially identified as a histone chaperone for DNA synthesis-coupled chromatin assembly (Smith and Stillman, 1989; Das et al., 2009), it is becoming increasingly evident that CAF-1 has functions in the regulation of other cellular and developmental processes, such as heterochromatin formation and asymmetric cell division (Quivy et al., 2008; Huang et al., 2010; Nakano et al., 2011). Recent studies in C. elegans and Arabidopsis imply that CAF-1 is involved in gene activation (Autran et al., 2011; Nakano et al., 2011); however, the precise mechanisms by which CAF-1 mediates gene activation and whether CAF-1 specifically regulates gene expression in association with a particular developmental pathway remain unclear. Here, we report
a novel function of dCAF-1 in epigenetically regulating the Notch signaling pathway. Our results show that, by interacting with Su(H) and NICD, dCAF-1 is enriched in the enhancer regions of Notch target genes, maintaining the local H4 acetylation level to provide an appropriate chromatin niche for gene activation.

Our results also show that depletion of dCAF-1-p105 affects the binding efficiency of Su(H) to its target DNA sequence (Fig. 4D). Therefore, the role of dCAF-1-p105 in regulating Notch target gene expression may be interpreted as follows: in response to Notch binding efficiency of Su(H) to its target DNA sequence (Fig. 4D), the recruited dCAF-1-p105 alters local histone H4 acetylation (Fig. 4E,F), establishing a chromatin structure that is more accessible to Su(H) binding. The mutual recruitment of dCAF-1-p105 and Su(H) eventually promotes the establishment of a microenvironment (chromatin niche) for the expression of Notch target genes. We have shown that, in the absence of dCAF-1-p105, the binding of Su(H) to the enhancer of Notch target genes is compromised (Fig. 4D); however, owing to technical difficulties we could not perform a reverse ChIP experiment to examine how dCAF-1-p105 binding to chromatin is affected in the absence of Su(H) because these mutants die at a very early stage. Our proposal that the binding affinity of Su(H) for the enhancer region of Notch genes depends not solely on the DNA sequence but also on an appropriate local chromatin structure is supported by findings from other laboratories (Krejcí and Bray, 2007; Endo et al., 2012). In cultured Drosophila cells, an increased occupancy of Su(H) and histone modifications (H4ac and H3K4me3) at Notch targets are found to be in proportion to Notch induction (Krejcí and Bray, 2007). Endo and colleagues reported that Hamlet acts at the chromatin level through altering histone modifications to change the accessibility of Su(H) at Notch target loci (Endo et al., 2012).

In Drosophila, LAF and RLAF silencing complexes cooperate with the histone chaperones Asf1 and Nap1, respectively, to mediate epigenetic silencing (Moshkin et al., 2009). Asf1 delivers histone H3/H4 heterodimers to CAF-1 during de novo DNA synthesis (Ridgway and Almouzni, 2000), but it is not known whether Asf1 is also a histone deliverer for CAF-1 during transcriptional silencing. Our findings do not support the notion that CAF-1 and Asf1 function in the same pathway to mediate gene silencing in Drosophila, at least not in the case of regulating Notch target gene expression. Instead of silencing Notch target genes, dCAF-1-p105 interacts with positive Notch pathway components during Drosophila tissue development, leading to the activation of Notch target gene expression. Our results appear to be consistent with the finding that the binding of Asf1 to LAF and CAF-1 is mutually exclusive (Moshkin et al., 2009), suggesting that Asf1 and CAF-1 might function synergistically in DNA synthesis, but distinctively in the process of regulating gene expression during developmental signaling.

CAF-1 is a conserved three-subunit protein complex in metazoans (Ridgway and Almouzni, 2000). Previous studies in mammals reported that loss of CAF-1-p60 (CHAF1B) results in phenotypes that are indistinguishable from that of loss of CAF-1-p55 (CHAF1A) in the chromatin assembly process during DNA replication and DNA repair (Ye et al., 2003; Polo et al., 2006). Expression of a dominant-negative form of CAF-1-p105, CAF-1-p150C, in human U2OS cells leads to a reduction of endogenous CAF-1-p150, suggesting that CAF-1-p150 is degraded when not assembled with CAF-1-p60 (Ye et al., 2003). These data indicate that the subunits of CAF-1 are stable only in the form of a complex during their functions in DNA synthesis-coupled chromatin assembly. However, whether the subunits of CAF-1 function independently during their roles in regulating gene expression is not known. Here, we show that downregulation of dCAF-1-p105 leads to a reduction in the protein level of dCAF-1-p180 and vice versa, but not of dCAF-1-p55. Given that depletion of any one of the three subunits of dCAF-1 leads to similar Notch-like phenotypes, and that all three subunits are associated with NICD in S2 cells, our data suggest that dCAF-1 is likely to function as a complex, rather than as individual subunits, during Notch signaling.

In conclusion, our study demonstrates that dCAF-1 plays an essential role during tissue development through regulating the Notch pathway by an epigenetic mechanism. For the first time, we have linked the function of CAF-1 to a specific developmental signaling pathway during Drosophila development. Our study provides clues as to why loss of function of CAF-1 leads to lethality only in higher multicellular organisms, such as Drosophila, but not in unicellular organisms, such as yeast; it is the role of CAF-1 in regulating developmental pathways, rather than its role in DNA replication, that leads to lethality upon its loss in multicellular animals.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
R.J., W.-M.D. and Z.Y. conceived and designed the experiments. Z.Y., H.W., H.C. and R.W. performed the experiments. R.J., W.-M.D., Z.Y., H.W., H.C., C.L. and J.L. analyzed the data: X.L. contributed reagents/materials/analysis tools. Z.Y., W.-M.D. and R.J. wrote the paper.

Supplementary material
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