CoREST acts as a positive regulator of Notch signaling in the follicle cells of *Drosophila melanogaster*

Elena Domanitskaya and Trudi Schüpbach*

Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Washington Road, Princeton, NJ, 08544-1014, USA

*Author for correspondence (schupbac@princeton.edu)

Accepted 27 July 2011
Journal of Cell Science 125, 399–410

doi: 10.1242/jcs.089797

Summary

The Notch signaling pathway plays important roles in a variety of developmental events. The context-dependent activities of positive and negative modulators dramatically increase the diversity of cellular responses to Notch signaling. In a screen for mutations affecting the *Drosophila melanogaster* follicular epithelium, we isolated a mutation in *CoREST* that disrupts the Notch-dependent mitotic-to-endocycle switch of follicle cells at stage 6 of oogenesis. We show that *Drosophila* CoREST positively regulates Notch signaling, acting downstream of the proteolytic cleavage of Notch but upstream of Hindsight activity; the Hindsight gene is a Notch target that coordinates responses in the follicle cells. We show that CoREST genetically interacts with components of the Notch repressor complex, Hairless, C-terminal Binding Protein and Groucho. In addition, we demonstrate that levels of H3K27me3 and H4K16 acetylation are dramatically increased in *CoREST* mutant follicle cells. Our data indicate that CoREST acts as a positive modulator of the Notch pathway in the follicular epithelium as well as in wing tissue, and suggests a previously unidentified role for CoREST in the regulation of Notch signaling. Given its high degree of conservation among species, CoREST probably also functions as a regulator of Notch-dependent cellular events in other organisms.

Key words: CoREST, Notch, Oogenesis, *Drosophila melanogaster*

Introduction

The highly conserved Notch signaling pathway plays a crucial role in a broad array of developmental events, including the maintenance of stem cells, cell fate specification, control of proliferation and apoptosis (Borggrefe and Oswald, 2009; Artavanis-Tsakonas and Muskavitch, 2010). Misregulation of the Notch pathway is associated with a number of diseases, including different types of cancer (Koch and Radtke, 2010). The binding of the transmembrane ligands DSL (Delta, Serrate, LAG-2) to the extracellular domain of Notch, exposed on a neighboring cell, activates the signaling cascade by triggering a sequence of proteolytic cleavages of Notch protein. Extracellular cleavage (S2) leads to the formation of an intermediate membrane-bound C-terminal fragment of Notch, called NEXT. This event is followed by an intramembranous cleavage (S3) by the γ-secretase complex (Struhl and Greenwald, 1999; Struhl and Greenwald, 2001). The intracellular domain of Notch (NICD) then translocates to the nucleus and binds to a transcription factor of the CSL family [CBF-1, Su(H), LAG-1], converting it from a transcriptional repressor to an activator. In the canonical Notch pathway, Su(H) directly activates Notch target genes in response to signaling (Bailey and Posakony, 1995; Furukawa et al., 1995; Lecourtois and Schweisguth, 1995). Despite the relative simplicity of the Notch transduction pathway, the presence of a large number of proteins that positively or negatively influence Notch signaling dramatically increases the complexity of the Notch pathway and its cellular responses (Panin and Irvine, 1998; Schweisguth, 2004; Borggrefe and Oswald, 2009). For instance, extracellular modulators, such as Fringe (Panin et al., 1997), alter ligand-specific Notch activation, whereas cytoplasmic modulators, such as Numb (Guo et al., 1996), restrict signal transduction. Nuclear modulators, for instance Mastermind (Wu et al., 2000), influence the transcriptional activity of the NICD-containing complex. In addition, there is increasing evidence of the importance of the epigenetic regulation of Notch targets, which can cause differential cellular responses upon Notch activation (Borggrefe and Oswald, 2009).

Drosophila melanogaster serves as an excellent model system to dissect the regulation of the Notch pathway. The *Drosophila* genome contains only a single Notch protein and two ligands [Delta (Dl) and Serrate (Ser)]. The Notch pathway is involved in several aspects of *Drosophila* development. The role of Notch in lateral inhibition during neurogenesis has been extensively studied; it restricts neural cell fates in the embryo, and leads to restriction of sensory-organ formation and induction of boundary formation in the wing discs (Micchelli and Blair, 1999; Portin, 2002; Cau and Blader, 2009). Notch activity is also required for many aspects of oogenesis, such as the establishment of egg chamber polarity, polar cell formation, control of follicle cell (FC) proliferation, differentiation, cell fate specification and morphogenesis (Deng et al., 2001; Grammont and Irvine, 2001; Lopez-Schier and St Johnston, 2001; Horne-Badovinac and Bilder, 2005; Grammont, 2007; Shyu et al., 2009; Klusza and Deng, 2011; Vachias et al., 2010). The *Drosophila* FCs are somatically derived epithelial cells that form a monolayer covering the germline cells during oogenesis. FCs divide mitotically from stage 2 to stage 6 of oogenesis, followed by the switch from the mitotic cycle to the endocycle (the M/E transition). Endocycles take place from stage 7 to stage 10A of oogenesis and include three rounds of DNA duplication without subsequent cell division (Klusza and Deng,

Journal of Cell Science

2011). The M/E switch is triggered upon Notch pathway activation. Dl produced in the germline binds to its receptor Notch, expressed in the FCs, and induces activation of the canonical Notch signaling pathway (Deng et al., 2001; Lopez-Schier and St Johnston, 2001; Lopez-Schier and St Johnston, 2002). Removal of Dl from germline cells, or of Notch from FCs, maintains follicle cells in the mitotic cycle throughout oogenesis. NICD complexed with Su(H) activates transcription of downstream target genes required for the M/E switch, such as Hindsight (Hnt) (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Hnt then mediates the Notch-dependent downregulation of Cut, String (Stg) and Hedgehog (Hh) signaling in the FCs, thus promoting the M/E switch (Sun and Deng, 2007).

We have identified the transcriptional cofactor Corepressor for element-1-silencing transcription factor (CoREST) as a positive modulator of Notch signaling in the FCs and during wing development. We show that CoREST is required for the promotion of the M/E switch during oogenesis. CoREST acts downstream of NICD release but upstream of Hnt activity, and it is a previously unidentified modulator of the Notch pathway. The genetic interactions between CoREST and Hairless (H), CtBP and Groucho (Gro), members of the Notch repressor complex, suggest that CoREST might influence the activity of either Notch transcriptional repressor or activator complexes. In addition, we found that CoREST specifically affects tri-methylation of lysine 27 of histone 3 (H3K27) and acetylation of H4K16 in FCs, because these chromatin modifications show elevated levels in the *CoREST* mutant cells. These findings point to a possible role of CoREST in regulation of the activity of the Notch repressor–activator complexes and/or epigenetic regulation of the components of the repressor–activator complexes or of factors involved in the transduction of the signaling or directly of target genes of the Notch signaling pathway.

Results

GF60 mutant follicle cells fail to switch from the mitotic cycle to the endocycle

The FCs of *Drosophila* divide mitotically from stage 2 to stage 6 of oogenesis, and then switch from the mitotic cycle to the endocycle, in the so called M/E transition (Klusza and Deng, 2011). In a genetic mosaic screen designed to identify genes involved in FC patterning, differentiation and morphogenesis (Denef et al., 2008; Yan et al., 2009), the *GF60* allele was isolated. *GF60* homozygous mutant posterior follicle cells (PFCs) are smaller and have much smaller nuclei than their neighboring wild-type cells, which is an indicator of a failure in the M/E switch (Fig. 1A; supplementary material Fig. S1). The apical–basal polarity of the mutant cells, as tested by immunostaining for aPKC, dlg, DE-Cad and Arm, was, however, not affected (data not shown). To confirm that *GF60* mutant FCs are blocked in the mitotic cycle, we stained the mosaic egg chambers for the mitotic markers phosphorylated histone H3 (PH3) and cyclin B (CycB). Although in wild-type egg chambers PH3 and CycB are only detected up to stage 6, in *GF60* homozygous mutant clones both of these mitotic markers were still present after stage 6 (Fig. 1B,C). Interestingly, this proliferation phenotype was more pronounced in the PFCs, and less frequently observed in the lateral or anterior follicle epithelial clones. Thus, in $CoREST^{GF60}$ homozygous mutant FCs, PH3 expression after stage 7 was detected in 74% of PFC clones ($n=65$), 24% of lateral ($n=46$) and 18% of anterior clones ($n=55$). We therefore concluded that the gene product disrupted in *GF60* mutant cells is involved in the control of the M/E transition in FCs, and that its effect is largely restricted to PFCs.

The *GF60* mutant allele encodes a truncated CoREST protein that lacks any functional domains

We mapped the lethal *GF60* mutation to the gene encoding CoREST, using a combination of meiotic recombination with visible recessive markers, P-element insertions and single nucleotide polymorphism (SNP) mapping approaches. The *Drosophila* genome contains only one gene encoding CoREST (Fig. 2A), whereas three *CoREST* homologs are found in mammalian genomes (Dallman et al., 2004). The $CoREST^{GF60}$ allele carries a guanine to adenine point mutation in the first nucleotide of the third intron (Fig. 2A), leading to a non-functional splice donor site. The $CoREST^{GF60}$ mutant retains the third intron, as we confirmed by RT-PCR analysis (data not shown). The presence of an in-frame stop codon at the end of the third intron results in a truncated CoREST protein lacking known functional

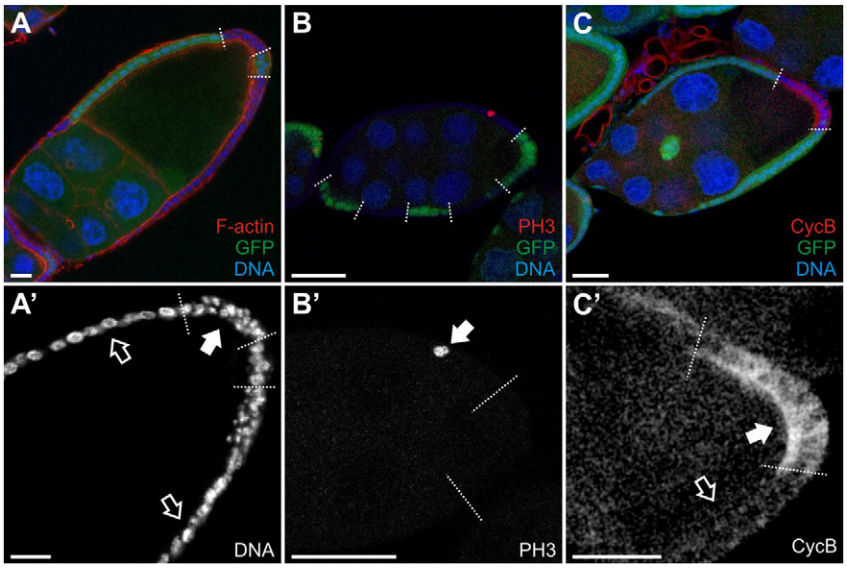

Fig. 1. *GF60* function is required for the M/E switch. Egg chambers at stage 10 (**A**) and 8 (**B**,**C**) of oogenesis containing *GF60* mutant follicle cell clones, stained with Hoechst (A,A′,B,C) to label DNA, F-actin (A), PH3 (B,B′) and CycB (C,C′). Mutant cells are identifiable by the absence of GFP (green), the borders between mutant clones and neighboring wild-type cells are marked by dotted lines. Scale bars: 25 µm. (A,A′) *GF60* mutant follicle cell nuclei (filled arrow in A′) are smaller and more crowded than neighboring wild-type cell nuclei (open arrows in A′). (B,B′) PH3-positive cells (filled arrow in B′) are observed in the *GF60* mutant PFCs after stage 7, indicating continued mitotic division. (C,C′) CycB is continuously expressed in the *GF60* mutant PFCs (filled arrow in C′) but is not observed in neighboring wild-type cells (open arrow in C′) in an egg chamber at stage 9.

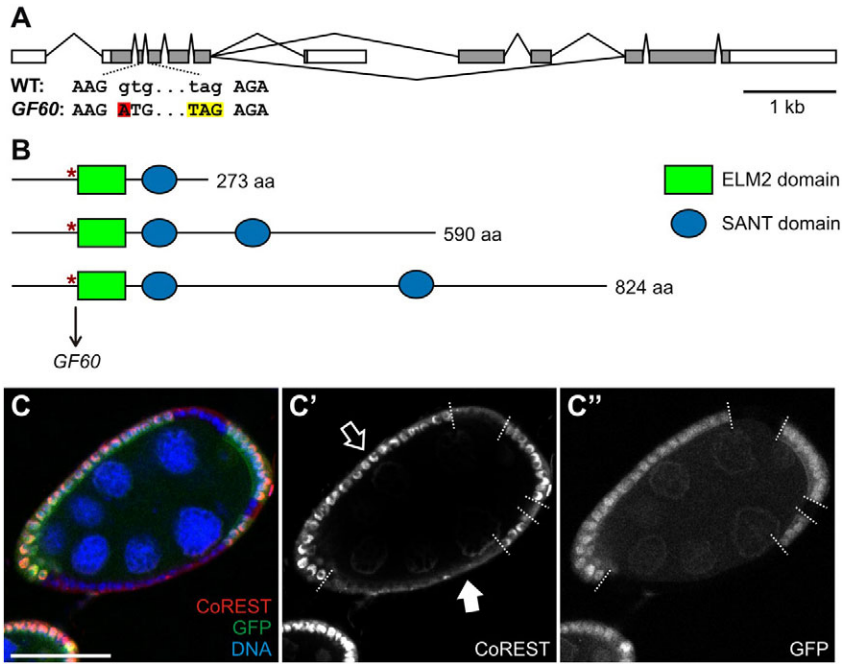

Fig. 2. The defect in the M/E switch in *GF60* is caused by a mutation in *CoREST*. (**A**) Schematic representation of the intron–exon structure of *CoREST*. White rectangles represent the 5′ and 3′ UTR, grey rectangles represent translated regions. The third intron is mutated in the *GF60* allele leading to the substitution of A for G (highlighted in red), which abolishes its splicing (data not shown). The stop codon in the third intron is highlighted in yellow. (**B**) Schematic diagrams illustrating three *Drosophila* CoREST splice variants [adopted from Dallman et al. (Dallman et al., 2004)]. ELM2 domains are depicted as green rectangles, SANT domains are represented by blue ovals. The short, medium and long isoforms of CoREST are 273, 590 and 824 aa, respectively. The *GF60* mutation leads to the truncation of CoREST before the ELM2 domains, and is indicated by a red star. (**C**) Stage 7 egg chamber containing $CoREST^{GF60}$ homozygous mutant clones stained with anti-CoREST antibodies (red in C, white in C′) and Hoechst (blue in C) to label DNA. Mutant clones are identifiable by the absence of GFP (green in C, white in C′′), and the borders between mutant clones and neighboring wild-type cells are marked by dotted lines (C′,C′′). CoREST is present in wild-type follicle epithelial cells (open arrow in C′), and absent from mutant clones (filled arrow in C′). Egg chambers are oriented with the posterior to the right. Scale bar: 50 μm.

domains (Fig. 2B) and it affects all three splice variants identified for *Drosophila* CoREST (Dallman et al., 2004).

Using an antibody that recognizes a sequence after the second SANT domain of CoREST present in two of the three splice variants (Dallman et al., 2004), we identified bands corresponding to the 97 kDa and 130 kDa isoforms on western blots from lysates of wild-type egg chambers (data not shown). This finding indicates that at least two splice variants of CoREST are produced in the ovaries. Furthermore, we found that CoREST is ubiquitously expressed in nuclei of wild-type FCs as well as in germline cells at all stages of oogenesis (supplementary material Fig. S2), whereas no CoREST expression was detected in $CoREST^{GF60}$ mutant FCs (Fig. 2C). To confirm that the discovered mutation in the *CoREST* gene is indeed responsible for the phenotype observed in the *GF60* mutant, we expressed an HA-tagged CoREST cDNA in *GF60* mutant FCs, and rescued the mutant phenotype, restoring the PH3 staining after stage 6 of oogenesis and reverting other mutant phenotypes associated with the *GF60* mutation, which are described below (supplementary material Fig. S3). Notably, misexpression of the *CoREST* cDNA did not lead to a premature M/E switch.

Notch signaling is disrupted in *CoREST* mutant PFCs

The M/E switch in *Drosophila* FCs is under the control of the Notch pathway (Deng et al., 2001; Lopez-Schier and St Johnston, 2001; Sun and Deng, 2005; Sun and Deng, 2007). Germline cells express Dl (Lopez-Schier and St Johnston, 2001), which activates Notch signaling in FCs at stages 6–7 of oogenesis, resulting in the expression of the zinc-finger transcription factor Hnt, which in turn promotes the M/E switch through suppression of *cut* expression (Sun and Deng, 2007). To determine whether the failure of the M/E switch is a consequence of a disrupted Notch signaling in $CoREST^{GF60}$ mutant cells, we stained mosaic egg chambers with antibodies specific for Hnt and Cut. Hnt is upregulated upon Notch pathway activation at stages 6–7 in the entire follicle epithelium (Fig. 3A) (Sun and Deng, 2007). In $CoREST^{GF60}$ mutant PFCs, Hnt expression was not detected during mid-oogenesis, whereas in $CoREST^{GF60}$ mutant lateral and anterior clones Hnt expression appeared comparable to that in the neighboring wild-type cells (Fig. 3B). Cut is expressed in FCs until stages 6–7 of oogenesis, but is afterwards repressed in all FCs, except the polar and stalk cells, through the activity of Hnt (Fig. 3D). The downregulation of Cut in FCs is necessary to induce the M/E switch (Sun and Deng, 2005; Sun and Deng, 2007). In $CoREST^{GF60}$ homozygous mutant PFCs, Cut was not downregulated during mid-oogenesis, whereas Cut repression appeared mostly normal in anterior and lateral mutant clones (Fig. 3E). To determine whether CoREST affects expression of other Notch targets and not only the expression of *Hnt*, we used an E(Spl)-mβ-CD2 transgenic line (de Celis et al., 1998) to assay Notch activity in $CoREST^{GF60}$ mutant clones. Upon Notch activation, CD2 staining was detected in all follicle cells from

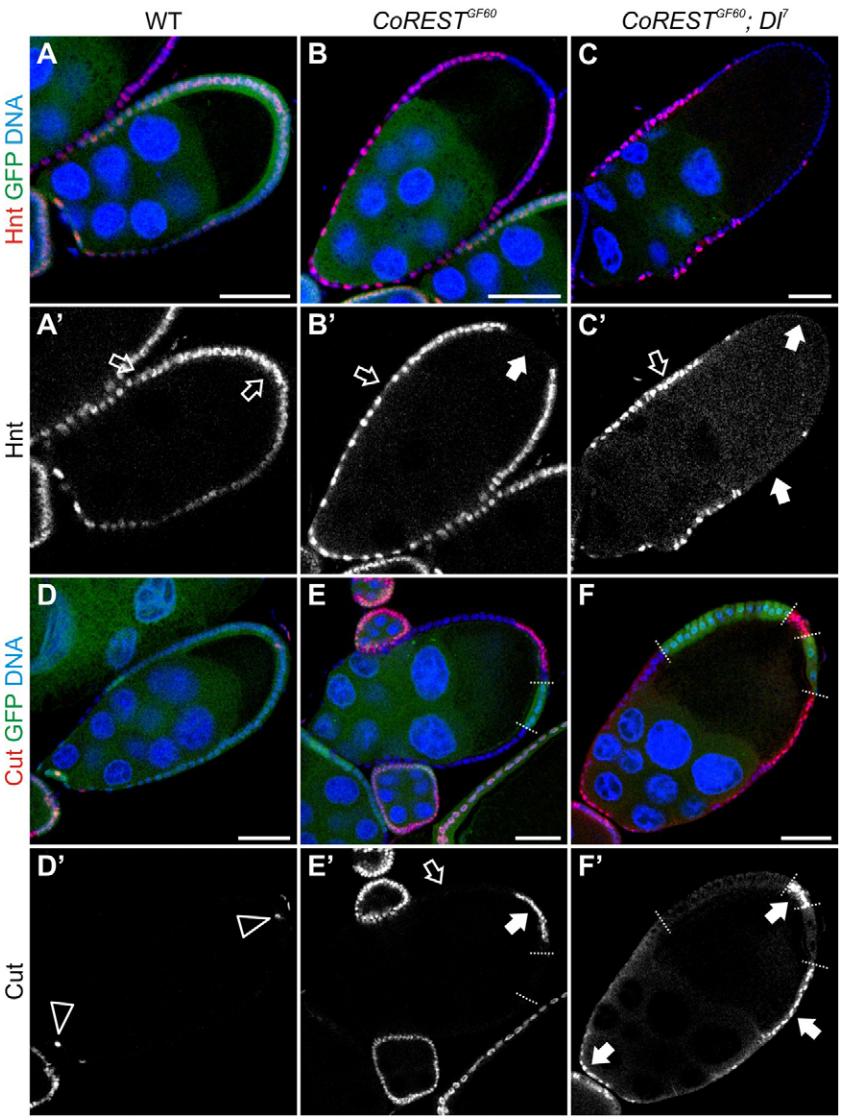

Fig. 3. CoREST function is required for Notch signaling in the follicle epithelial cells. Egg chambers stained with Hoechst (**A–F**) to label nuclei, and with anti-Hnt (A–C) and anti-Cut (D–F) antibodies. *CoREST*^{GF60} homozygous mutant clones are identifiable by the absence of GFP. The borders between mutant clones and neighboring wild-type cells are marked by dotted lines (E,F,E′,F′). Egg chambers are oriented with the posterior to the right. Scale bars: 50 μm. (A) In the wild-type FCs Hnt is upregulated after stage 7 of oogenesis (open arrows, A′). (B) Egg chamber containing whole follicle-cell clone. Hnt fails to be upregulated after stage 7 in *CoREST*^{GF60} homozygous mutant PFCs (filled arrow, B′), whereas Hnt is normally upregulated after stage 7 in *CoREST*^{GF60} homozygous mutant lateral and anterior FCs (open arrow, B′). (C) Absence of Hnt expression is observed in *CoREST*^{GF60} homozygous mutant PFCs and lateral FCs in the *Dl*^{7} heterozygous background (filled arrows, C′), due to the increased penetrance of the mutant phenotype. Hnt expression is indicated by the open arrow in C′. (D) Notch activation leads to the downregulation of Cut expression after stage 7 in entire follicle epithelium except for anterior and posterior polar cells (open arrowheads, D′). (E) Cut is continuously present after stage 7 in the *CoREST*^{GF60} homozygous mutant PFCs (filled arrow, E′), but it is correctly downregulated after stage 7 in *CoREST*^{GF60} homozygous mutant lateral and anterior FCs (open arrow, E′). (F) Cut expression is present in the *CoREST*^{GF60} homozygous mutant posterior, lateral and anterior follicle cells in the *Dl*^{7} heterozygous background (filled arrows, F′), which indicates an increased penetrance of the mutant phenotype.

stage 7 to stage 10A (Sun et al., 2008), but it was not present in *CoREST*^{GF60} mutant PFCs (supplementary material Fig. S4A). These data indicate that Notch signaling is disrupted in *CoREST*^{GF60} mutant PFCs. In addition to the failure of the M/E switch, prolonged Cut expression maintains follicle cells in an immature state, as indicated by the extended presence of FasIII (Lopez-Schier and St Johnston, 2001; Sun and Deng, 2005) and Eya (Bai and Montell, 2002; Sun and Deng, 2005) after stages 6–7 (supplementary material Fig. S4B,C). Thus, we conclude that CoREST controls the response to Notch signaling, and acts predominantly in the PFCs.

Reduction of Dl leads to higher penetrance of the CoREST phenotype in the follicle cells

The observation that *CoREST*^{GF60} mainly disrupts Notch activity in PFCs suggests either that CoREST functions specifically in those cells, or alternatively that Notch signaling in PFCs is particularly sensitive to the loss of CoREST. Therefore, we tested whether *CoREST*^{GF60} could produce a mutant phenotype in lateral and anterior cells of the follicle epithelium by creating mutant clones in a *Dl*^{7} heterozygous background, which is more sensitive to Notch pathway disruption (Guo et al., 1999). In the control background, no Hnt expression was detected after stage 7 in 67% of *CoREST*^{GF60} homozygous mutant PFCs (n=51), whereas absence of Hnt was only observed in 12% of lateral (n=43) and 5% of anterior clones (n=14; Fig. 3B). Generation of *CoREST*^{GF60} homozygous mutant clones in the *Dl*^{7} heterozygous background increased the penetrance of this phenotype in posterior clones to 97%, (n=37), in lateral clones to 40% (n=35) and in anterior clones to 20%, (n=15; Fig. 3C). Similarly, in the control background, in *CoREST*^{GF60} homozygous mutant FCs, the presence of Cut expression after stage 7 was detected primarily in the posterior mutant clones (97.5% of PFC clones were Cut positive; n=193), and less frequent in lateral (46%, n=187) and anterior clones (20%, n=110; Fig. 3E). *CoREST*^{GF60} homozygous mutant clones in the *Dl*^{7} heterozygous background exhibited an enhanced phenotype with 100% of posterior mutant clones (n=60), 79% of lateral (n=67) and 80% of anterior clones (n=20) with Cut-positive cells (Fig. 3F). Thus, the penetrance of the Notch phenotype was

dramatically increased when the dose of Dl was reduced by half, indicating that the regulation of Notch signaling by CoREST is not solely restricted to the PFCs. Rather, PFCs are more sensitive to the loss of CoREST than lateral and anterior FCs.

CoREST regulates Notch signaling downstream of the Notch proteolytic cleavage but upstream of Hnt

Notch, upon binding to Dl, undergoes a sequence of proteolytic cleavages. Extracellular cleavage (S2) leads to the formation of the intermediate membrane-bound C-terminal fragment of Notch, called NEXT. This event is followed by the second intramembranous cleavage (S3) resulting in the release of the Notch intracellular domain NICD (Lai, 2004; Schweisguth, 2004). To test whether any of these Notch fragments can rescue the *CoREST*^{GF60} mutant phenotype, we overexpressed full-length Notch (NFL), NEXT and NICD constructs in *CoREST*^{GF60} homozygous mutant clones using the MARCM system (Lee and Luo, 1999). As a readout of Notch signaling, we analyzed the expression of Cut in the PFCs after stage 7 of oogenesis. Cut expression was detected in 99% of NFL- (n=63; Fig. 4A), in 94% of NEXT- (n=63; Fig. 4B) and in 90% of NICD-overexpressing (n=137; Fig. 4C) *CoREST*^{GF60} mutant PFC clones. These results indicate that neither NFL, NEXT or NICD could rescue the *CoREST* mutant phenotype, which positions the effect of CoREST on Notch signaling downstream of NICD release.

The absence of Hnt expression in *CoREST*^{GF60} mutant PFCs during mid-oogenesis suggests that CoREST is acting upstream or at the level of Hnt transcriptional regulation. The prolonged expression of Cut in the *CoREST*^{GF60} clones is expected to result from the lack of its repression by Hnt. To confirm this relationship, we misexpressed Hnt in mutant clones using the MARCM system (Lee and Luo, 1999), and examined whether it was possible to rescue the *CoREST*^{GF60} mutant phenotype. The misexpression of Hnt in *CoREST*^{GF60} clones (Fig. 4D) resulted in Cut downregulation before stage 6 (Fig. 4E). The follicle cell nuclei in the clones were substantially larger than those of the neighboring wild-type cells even before stage 6, indicating a premature M/E switch (Fig. 4E″). Therefore, we were able to rescue the *CoREST*^{GF60} mutant phenotype (Cut expression, M/E transition) by Hnt misexpression. Together with the inability of

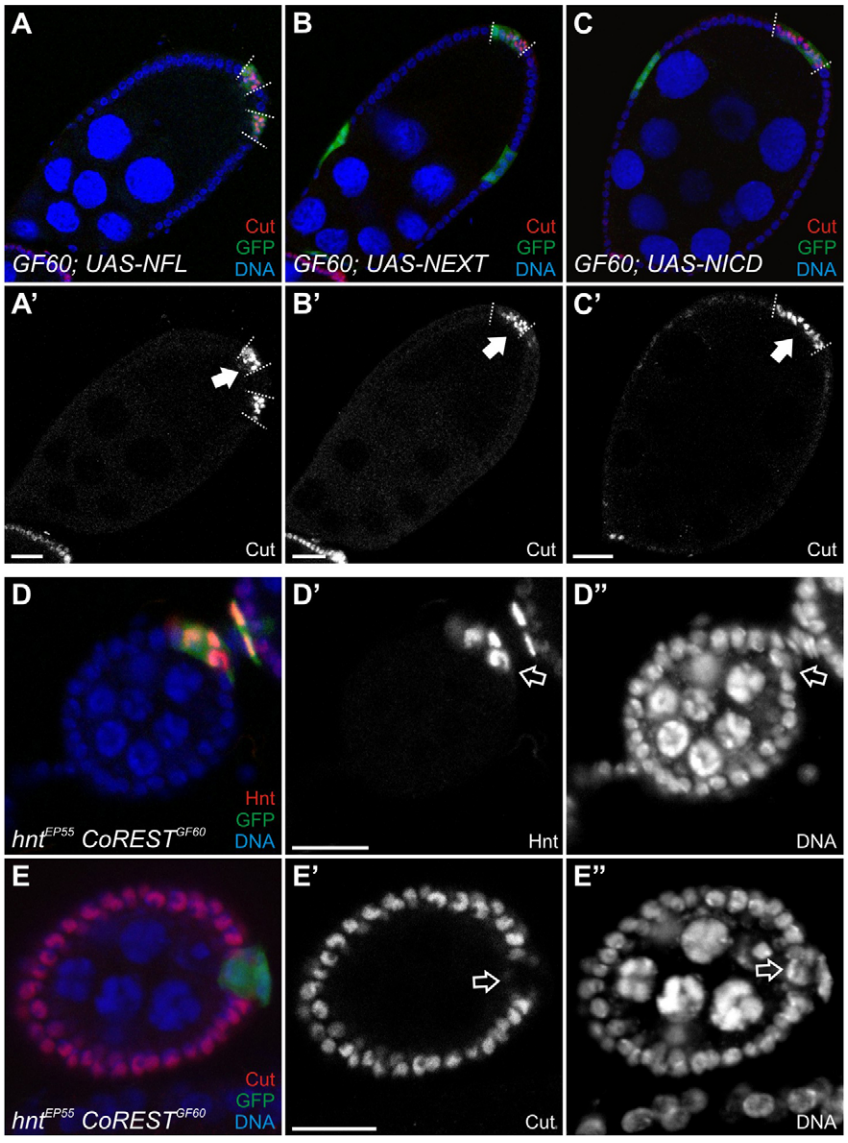

Fig. 4. CoREST acts downstream of Notch proteolytic cleavage and upstream of Hnt in the regulation of Notch signaling and the M/E switch. *CoREST*^{GF60} homozygous mutant clones positively marked by GFP; the borders between mutant clones and neighboring wild-type cells are marked by dotted lines. Egg chambers are oriented with the posterior to the right. Scale bars: 20 μm. (**A**–**C**′) Egg chambers, at stage 8 to stage 9, containing *CoREST*^{GF60} homozygous mutant clones with simultaneous overexpression of NFL (A,A′), NEXT (B,B′) or NICD (C,C′), and stained with an antibody against Cut (red in A–C, white in A′–C′). In *CoREST*^{GF60} homozygous mutant clones with simultaneous expression of either of the Notch cleavage products, Cut was continuously expressed in PFCs (filled arrows in A′,B′,C′). (**D**,**E**′′) Egg chambers, at stage 4 to stage 5, containing *CoREST*^{GF60} homozygous mutant clones with simultaneous Hnt misexpression, stained either with anti-Hnt antibodies (red in D, white in D′) or anti-Cut antibodies (red in E, white in E′), and with Hoechst to indicate the nuclei (blue in D and E and white in D′′and E′′). The *hnt*^{EP55} allele contains the UAS sequence inserted 585 bp upstream of the *hnt* gene and is used for Hnt misexpression. Misexpression of Hnt in *CoREST*^{GF60} homozygous mutant clones (open arrow in D′) is sufficient to downregulate Cut before stage 6 (open arrow in E′) and to drive follicle cells to enter endocycle prematurely (open arrow in E′′).

Notch fragments to rescue the mutant phenotype, these results indicate that CoREST is involved in the regulation of Notch signaling downstream of NICD release and upstream of Hnt.

Reduction of H, CtBP and Gro dosage leads to a mild suppression of the *CoREST* mutant phenotype

In the absence of Notch activation, expression of Notch target genes is repressed by the transcription factor Su(H) acting in a complex with H. H recruits two corepressors, CtBP and Gro, which are required for H-mediated repression (Nagel et al., 2005). The translocation of NICD to the nucleus displaces these corepressors from Su(H), converting it from a transcriptional repressor to a transcriptional activator. Subsequently, the transcriptional coactivator protein Mastermind (Mam) binds to NICD-Su(H) forming a coactivator complex (Fryer et al., 2002; Kovall, 2007). Because CoREST is required for the Notch pathway downstream of NICD release and upstream of Hnt in the FCs, it could be directly involved in the regulation of *hnt* expression. It might act through either negative regulation of repressor complex members, such as H, CtBP or Gro, or by positive regulation of activator complex members, such as Mam, thus modulating their ability to transcriptionally regulate *hnt* expression. To test for genetic interactions with these regulatory proteins, we examined whether we could modulate the penetrance of the $CoREST^{GF60}$ mutant phenotype by changing the dosage of H, CtBP or Gro. We generated $CoREST^{GF60}$ homozygous mutant follicle epithelial clones in H^{1} (Maier et al., 1992), $CtBP^{P1590}$ (Poortinga et al., 1998) or gro^{e47} (Orian et al., 2007) heterozygous backgrounds. In the $CoREST^{GF60}$ homozygous mutant clones generated in the control background, Cut was detected after stage 7 of oogenesis mainly in the posterior mutant clones (97.5%, $n=193$; Fig. 3E), and less frequently in lateral (46%; $n=187$) and anterior clones (20%, $n=110$; Fig. 3E). The reduction of H dosage in the $CoREST^{GF60}$ homozygous mutant cells significantly ($P<0.001$) suppressed the *CoREST* mutant phenotype: 51% of PFC clones ($n=73$), 6% of the lateral ($n=64$) and 0% of the anterior clones ($n=52$) were Cut positive after stage 7 of oogenesis (Fig. 5A,D). Similarly, the reduction of CtBP dosage in the $CoREST^{GF60}$ homozygous mutant cells slightly but statistically significantly ($P<0.001$) suppressed the *CoREST* mutant phenotype: 73% of PFC clones ($n=224$), 19% of the lateral ($n=206$) and 3% of the anterior clones ($n=135$) were Cut positive after stage 7 of oogenesis (Fig. 5B,D). In comparison with the $CoREST^{GF60}$ mutant clones in the control background, $CoREST^{GF60}$ homozygous mutant clones in the gro^{e47} heterozygous background (Fig. 5C,D) exhibited a similar penetrance of the *CoREST* mutant phenotype in the PFCs, with 90% of Cut-positive PFC clones ($n=73$). However, the penetrance of the *CoREST* mutant phenotype was weakly but statistically significantly reduced for the $CoREST^{GF60}$ homozygous mutant lateral follicle cell clones (27%, $n=55$; $P<0.001$) and anterior follicle cell clones (5%, $n=38$; $P<0.001$) in the gro^{e47} heterozygous background (Fig. 5D). Notably, we observed a stronger suppression of the *CoREST* mutant phenotype in the H^{1} and $CtBP^{P1590}$ heterozygous backgrounds in comparison with the gro^{e47} heterozygous background. In summary, we were able to modulate the strength of the $CoREST^{GF60}$ mutant phenotype by changing the dosage of components of the Notch repressor complex, H, CtBP or Gro, indicating that H-CtBP-Gro and CoREST play antagonistic roles in Notch signaling. This is consistent with the conclusion that CoREST acts as a positive modulator of the Notch pathway in the FCs. The genetic interactions between *H* and *CoREST*, *CtBP* and *CoREST*, and *gro* and *CoREST* further suggest that CoREST might be involved in the regulation of the Notch repressor–activator complex.

CoREST does not act in concert with Chn in Notch signaling regulation in FCs

CoREST was first identified in humans as a primary cofactor for RE1 silencing transcription factor (REST; also known as neural-restrictive silencing factor; NRSF) (Andres et al., 1999), and has been implicated in transcriptional and epigenetic regulation of neural cell fate decisions (Qureshi et al., 2010). Interestingly, no REST homolog has been identified in *Drosophila* (Bruce et al., 2004). However, *Drosophila* Charlatan (Chn), which encodes a C2H2-type zinc-finger protein, has several structural and functional similarities to human REST and can associate with CoREST in cultured S2 cells (Tsuda et al., 2006). Chn was shown to repress Dl expression and play a role in the initiation of eye development (Tsuda et al., 2006). To determine whether CoREST plays a Chn-dependent role in the Notch pathway regulation in FCs, we generated chn^{ECJ1} (Escudero et al., 2005) homozygous mutant FC clones, and looked at the expression patterns of the Notch downstream genes, *hnt* and *cut*. We observed wild-type expression of Hnt and Cut in *chn* mutant cells (supplementary material Fig. S5), and therefore concluded that CoREST plays a Chn-independent role in Notch signaling regulation in FCs.

CoREST is a chromatin regulatory protein that affects H3K27 tri-methylation and H4K16 acetylation in the follicle cells

The CoREST transcriptional complex was shown to be involved in regulation of neuronal gene expression in mammals where it acts to modify the acetylation and methylation status of histones through recruitment of histone-modifying enzymes (Ballas et al., 2001; Lunyak et al., 2002; Lakowski et al., 2006). In a two-hybrid interaction screen, *Drosophila* CoREST was shown to interact with Su(VAR)3-3, the *Drosophila* homolog of LSD1 (lysine-specific histone demethylase 1), and with Rpd3, a *Drosophila* class-I HDAC (Dallman et al., 2004). LSD1 is able to remove activating mono- and dimethyl groups on lysine 4 of histone 3 (H3K4) (Shi et al., 2004), and HDAC1 removes activating acetyl groups from lysine residues on histones, leading to transcriptional repression.

To determine whether CoREST is involved in the epigenetic regulation of gene expression in FCs, for instance, of components of Notch repressor–activator complexes or of Notch target genes, we analyzed histone modifications in mosaic egg chambers. Using specific antibodies recognizing different histone modifications, we noticed that the state of histones is dynamic in FCs and changes at different stages of oogenesis. Thus, we observed strong staining for H3K4me2, H3K9me3, H3K27me3, H3K9, H4K8, H4K12 and H4K16 acetylation until stages 6–7. All these signals weakened after the M/E transition (data not shown). To determine whether any histone modifications are affected in $CoREST^{GF60}$ mutant cells specifically as a result of the absence of the functional CoREST protein, and not as a secondary effect of a block in the mitotic cycle, we used hnt^{FG47} mutant follicle cell clones as a control. Abnormal levels of histone modifications observed in

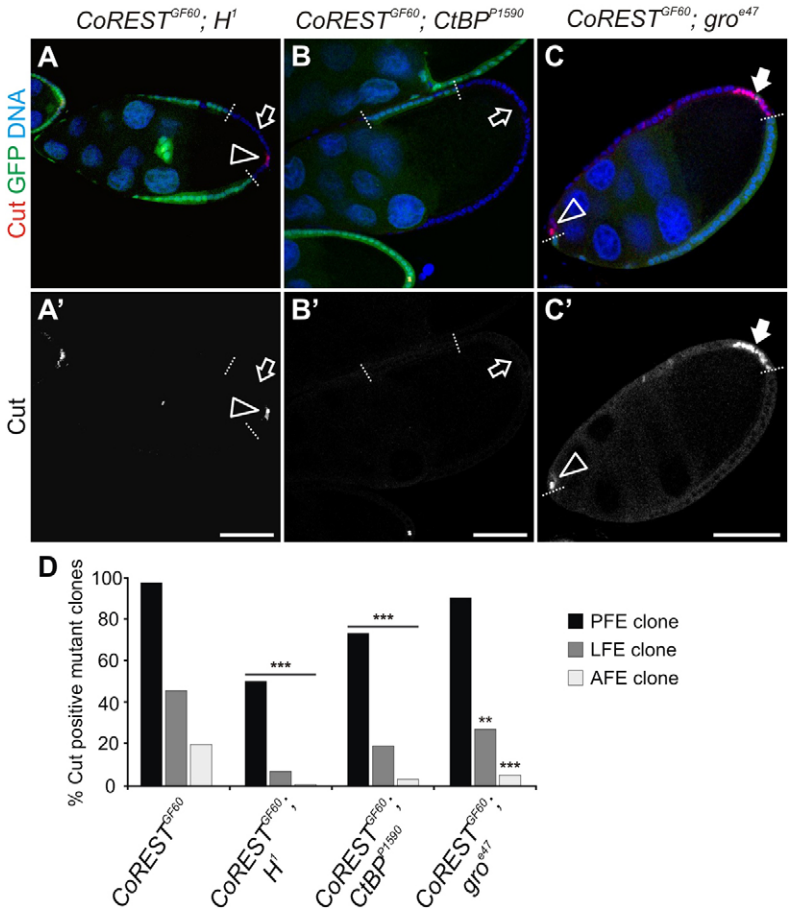

Fig. 5. Notch mutant phenotype in *CoREST*^{GF60} mutants is suppressed in *H*^{1}, *CtBP*^{P150} and *gro*^{e47} heterozygous backgrounds. Egg chambers containing *GF60* mutant follicle cell clones stained with Hoechst (**A–C**) to label DNA, and anti-Cut antibody (A–C, A′–C′). Mutant cells are identifiable by the absence of GFP (green), the borders between mutant clones and neighboring wild-type cells are marked by dotted lines. Egg chambers are oriented with the posterior to the right. Scale bars: 50 µm. (A,B) In egg chambers containing *CoREST*^{GF60} homozygous mutant follicle cell clones in the *H*^{1} heterozygous background (A) or in the *CtBP*^{P150} heterozygous background (B), the Notch phenotype was strongly suppressed as indicated by complete Cut downregulation in the mutant clones after stage 7 (open arrow in A,A′,B,B′), except for the polar cells (open arrowhead in A,A′). (C) In egg chambers containing *CoREST*^{GF60} homozygous mutant clones in the *gro*^{e47} heterozygous background, Cut was continuously expressed in PFCs after stage 7 (filled arrow in C′), and the strength of this phenotype was similar to the phenotype in *CoREST*^{GF60} homozygous mutant FC clones in the wild-type background. Cut staining in the polar cells is indicated by open arrowhead (C,C′). (**D**) Percentage of the egg chambers with Cut-positive cells in the *CoREST*^{GF60} homozygous mutant posterior (black), lateral (dark gray) and anterior (light gray) FC clones, in the control, *H*^{1}, *CtBP*^{P150} or *Gro*^{e47} heterozygous backgrounds. Percentage of egg chambers with Cut-positive cells in *CoREST*^{GF60} homozygous mutant clones in wild-type background: 97.5% of PFC clones (n=193), 46% of lateral clones (n=218), 20% of anterior clones (n=110); in *CoREST*^{GF60} homozygous mutant clones in the *H*^{1} heterozygous background: 51% of PFC clones (n=73), 6% of lateral clones (n=64), 0% of anterior clones (n=52); in *CoREST*^{GF60} homozygous mutant clones in the *CtBP*^{P150} heterozygous background: 73% of PFC clones (n=224), 19% of lateral clones (n=206), 3% of anterior clones (n=135); in *CoREST*^{GF60} homozygous mutant clones in the *gro*^{e47} heterozygous background: 90% PFC clones (n=73), 27% lateral clones (n=55), 5% anterior clones (n=38). The χ^2 test was applied for statistical analysis. ***P<0.001 and **P<0.01 for the comparison between *CoREST*^{GF60} homozygous mutant clones in the wild-type background and all other groups.

CoREST^{GF60} mutant FCs, but not in *hnt*^{FG47} mutant cells, after stages 6–7 of oogenesis were considered as CoREST specific. Interestingly, we found an increased level of staining for H3K27me3 and acetylated H4K16 in *CoREST*^{GF60} mutant FCs after stage 7 of oogenesis, compared with their wild-type neighboring cells or *hnt*^{FG47} mutant clones (Fig. 6). Furthermore, this phenotype was observed irrespective of clone position and was not restricted to overproliferating *CoREST*^{GF60} mutant PFCs. In addition, we identified abnormally high levels of H3K4me2, H3K9, H4K8 and H4K12 acetylation in both *CoREST*^{GF60} and *hnt*^{FG47} mutant clones; this phenotype was always coupled to overproliferation of the FCs (data not shown). Therefore, CoREST activity specifically affects the downregulation of H3K27me3 and H4K16 acetylation, whereas other chromatin modifications remain at high levels as a secondary consequence of the failure to promote the M/E switch.

CoREST affects Notch signaling in wings

To determine whether CoREST also regulates Notch signaling in tissues other than the follicular epithelium, we crossed the *CoREST*^{GF60} allele to several alleles of *Notch* (*N*) and *Dl*, and examined whether any dominant dosage-sensitive interactions could be detected in the wings of transheterozygous adult flies, because the wing area is very sensitive to defects in Notch

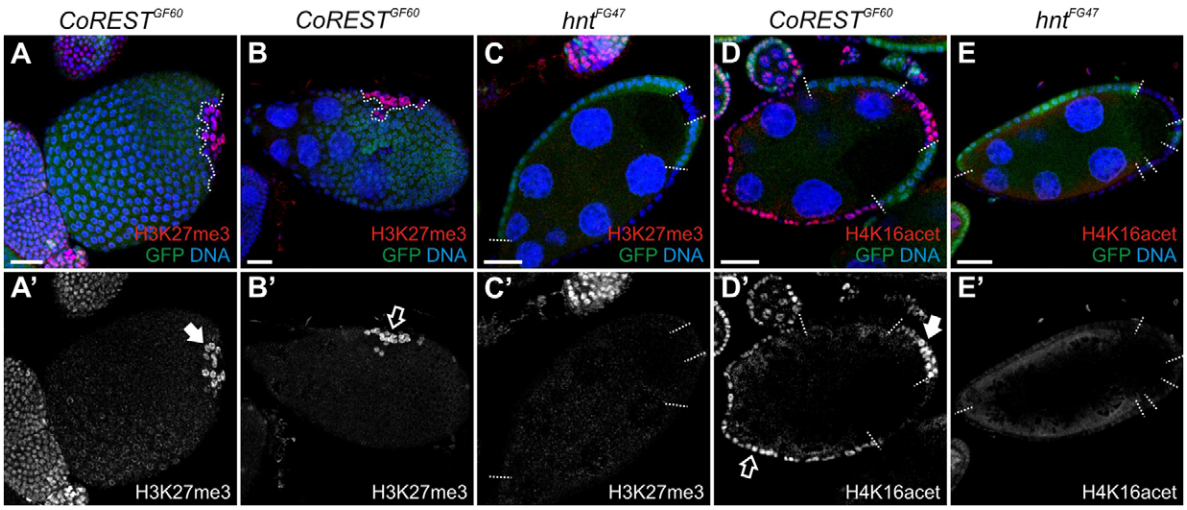

Fig. 6. Loss of CoREST alters global levels of chromatin methylation and acetylation. Egg chambers stained with Hoechst to label nuclei (blue in A–E), and antibodies specific for H3K27me3 (red in A–C, white in A′′–C′′), and H4K16 acetylation (red in D and E, white in D′′ and E′′). *CoREST*^{GF60} homozygous mutant clones (**A**,**B**,**D**) and *hnt*^{FG47} homozygous mutant clones (**C**,**E**) are identifiable by the absence of GFP. The borders between mutant clones and neighboring wild-type cells are marked by dotted lines. Egg chambers are oriented with the posterior to the right. Scale bars: 20 μm. (A,B) A higher level of H3K27me3 staining was observed in *CoREST*^{GF60} homozygous mutant FC clones, independent of clone position (filled arrow indicates posterior clone in A′, open arrow indicates lateral clone in B′) in stage 7 and older egg chambers. (C) The level of H3K27me3 staining in *hnt*^{FG47} homozygous mutant follicle epithelial was comparable to that in wild-type neighboring cells. (D) The level of H4K16 acetylation was higher in *CoREST*^{GF60} homozygous mutant follicle cell clones independent of clone position (filled arrow indicates a posterior clone in D′, and open arrow indicates an anterior-lateral clone in D′) in the stage 7 and older egg chambers. (C) The level of H4K16 acetylation was comparable in *hnt*^{FG47} homozygous mutant follicle epithelial cells with their wild-type neighboring cells.

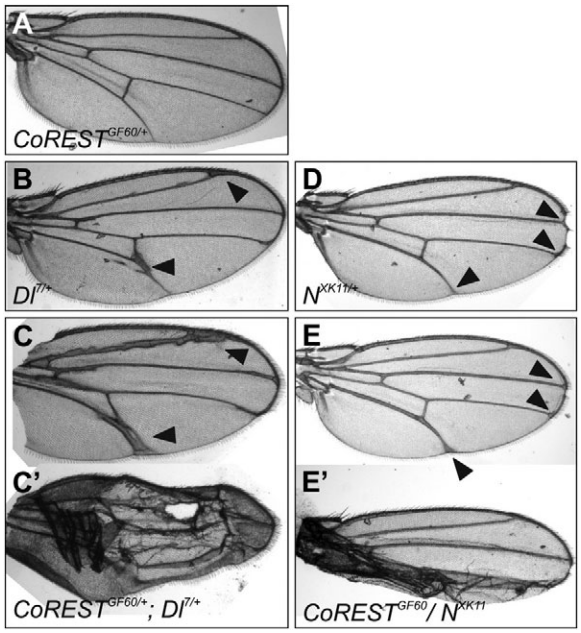

Fig. 7. CoREST genetically interacts with Notch and Delta in wing development. The *CoREST*^{GF60} mutant can modify the phenotype of Delta and Notch alleles in transheterozygotes. (**A**) A wing from a female heterozygous for *CoREST*^{GF60} shows wild-type wing morphology. (**B**) *Dl*$^{7/+}$. (**C**) *CoREST*$^{GF60/+}$; *Dl*$^{7/+}$. The *CoREST*^{GF60} mutation enhances the phenotype of the wing vein thickening (filled arrowheads) and the delta between L4 and L5. (**D**) *N*$^{XK11/+}$. (**E**) *CoREST*^{GF60}/*N*^{XK11}. The *CoREST*^{GF60} mutation also enhances the thickening of deltas (filled arrowheads). In addition, wing blistering is present in some instances (C′,E′).

signaling (Guo et al., 1999). Initially, we tested the ability of the heterozygous *CoREST*^{GF60} mutant to interact with the *Dl*^{7} allele, which, as we showed previously, dramatically increases the penetrance of *CoREST* mutant phenotype in the FCs (Fig. 3C,F). The *Dl*^{7} allele is characterized by thickened wing veins and small deltas located near cross-veins (Fig. 7B), and we found that a heterozygous *CoREST*^{GF60} allele enhanced this phenotype. In transheterozygotes between *Dl*^{7} and *CoREST*^{GF60} mutants, we observed a greater extent of thickening and deltas at the wing veins and even in some cases a severe blistering of the wings (Fig. 7C,C′). Similarly, we investigated whether the *CoREST*^{GF60} mutant interacts with Notch alleles, *N*^{FU42} and *N*^{XK11} (Wieschaus et al., 1984; Hoppe and Greenspan, 1986). Both of these Notch alleles produce the dominant notching of the wing blade (Fig. 7D, and data not shown), and we found that transheterozygotes of *Notch* and *CoREST* exhibited slightly thicker wing veins and deltas (Fig. 7E). We also observed a few cases of wing blistering (Fig. 7E′). The occasional wing blistering observed in this study indicates a cell adhesion defect (Zusman et al., 1990; Zusman et al., 1993; Zhang et al., 2010), and this is probably a Notch-independent phenotype. Overall our findings indicate that CoREST acts as a positive modulator of Notch signaling not just in the FCs, but also in wing tissue, indicating a common role for CoREST in the regulation of Notch signaling.

Discussion

Initially, CoREST was identified in humans as a corepressor with REST (RE1 silencing transcription factor) in mediating repression of the proneuronal genes, and thus as an important factor in the establishment of non-neural cell specificity (Andres et al., 1999; Lunyak et al., 2002). Subsequently, CoREST was identified in a variety of vertebrate and invertebrate species, and was shown to

play a functionally conserved role in neurogenesis (Andres et al., 1999; Tontsch et al., 2001; de la Calle-Mustienes et al., 2002; Jarriault and Greenwald, 2002; Dallman et al., 2004). Recent studies show that CoREST regulates a very broad range of genes by both REST-dependent and REST-independent means, including genes encoding members of key neural developmental signaling pathways, such as BMP, SHH, Notch, RA, FGF, EGF and WNT (Abrajano et al., 2009a; Abrajano et al., 2010; Qureshi et al., 2010). Analysis of CoREST downstream target genes and their developmental expression profiles suggested that the liberation of CoREST from gene promoters is associated with both gene repression and activation depending on the cell context (Abrajano et al., 2009a; Abrajano et al., 2009b; Abrajano et al., 2010). In the work reported here, we isolated a lethal allele of *Drosophila CoREST*, and analyzed the contribution of CoREST to the development of FCs, a process that involves cell proliferation and differentiation. We demonstrated that CoREST is implicated in the regulation of Notch signaling, and acts as a positive modulator of the Notch pathway in *Drosophila* FCs.

CoREST acts as a positive modulator of Notch signaling in the follicle cells

In this study, we identified a previously unidentified function of CoREST in the Notch-mediated regulation of the M/E switch during stage 6 of oogenesis. We showed that the loss of CoREST activity in FCs primarily disrupts the Notch signaling pathway. We further demonstrated that CoREST regulates the Notch pathway downstream of NICD release and upstream of Hnt. The misexpression of Hnt in the *CoREST* mutant clones rescues the failure in the M/E switch. Furthermore, the role of CoREST in Notch pathway regulation is not restricted to FCs: CoREST also interacts with Notch during wing development. Interestingly, CoREST was identified as a negative modulator of Notch signaling in *Caenorhabditis elegans* in a genetic screen for suppressors of the developmental defects in *sel-12* presenilin mutants (Eimer et al., 2002; Jarriault and Greenwald, 2002; Lakowski et al., 2006). Presenilin is a component of the γ-secretase complex that performs the S3 cleavage of Notch (Struhl and Greenwald, 1999; Struhl and Greenwald, 2001; Lai, 2004; Steiner et al., 2008). Mutations in *spr-1*, the *C. elegans* homolog of *CoREST*, suppress the developmental defects observed in *sel-12* animals by derepressing the transcription of the other functionally redundant presenilin gene, *hop-1* (Jarriault and Greenwald, 2002; Lakowski et al., 2006). Therefore, CoREST acts as a negative regulator of the γ-secretase complex in *C. elegans*, and hence proteolytic cleavage of Notch and release of NICD. By contrast, *Drosophila* CoREST does not affect the processing of the Notch receptor in the follicle cells, and instead acts as a positive modulator of the Notch pathway functioning downstream of NICD release.

Transcriptional and epigenetic functions of CoREST

CoREST plays transcriptional and epigenetic regulatory roles: it can promote gene activation in addition to repression, as well as being able to modify the epigenetic status of target gene loci distinct from its effects on transcription (Qureshi et al., 2010). Below we discuss several possible scenarios of how CoREST could be involved in the regulation of Notch signaling, based on the previous knowledge about CoREST and considering our data.

We showed that *hnt*, the downstream target gene of Notch signaling in FCs, fails to be properly upregulated upon Notch activation in the *CoREST* mutant cells. CoREST might therefore act as a transcriptional repressor for an unknown factor, which is in turn involved in the transcriptional repression of *hnt*. Alternatively, CoREST could be directly involved in the transcriptional regulation of *hnt* and act as an activator. *hnt* was shown to be a putative direct target of Notch signaling in DmD8 cells (Krejci et al., 2009) from the analysis of genes for which mRNA levels increase within 30 minutes of Notch activation, and which contain regions occupied by Su(H). If *hnt* is a direct target of Notch in FCs, its transcription would be regulated by the balance between Notch repressor and activator complexes, and CoREST might be involved in the regulation of stability or activity of either of these. Interestingly, CoREST was shown to interact with CtBP1 in mammals (Kuppuswamy et al., 2008), and to bind to the SIRT1–LSD1–CtBP1 complex, which is required for the repression of certain Notch target genes (Mulligan et al., 2011). Thus, *Drosophila* CoREST might similarly directly bind to the repressor complex containing CtBP and modify its activity or destabilize it. However, CoREST could be involved in the transcriptional regulation of the components of Notch repressor or activator complexes. In this scenario, in *CoREST* mutant FCs, upregulation of negative regulator(s) would lead to greater activity of negative than positive regulators, resulting in disruption of Notch signaling. Both suggested models of the direct and indirect transcriptional role of CoREST are consistent with our results, given that the *CoREST* mutant phenotype could be suppressed by removal of one copy of H, CtBP or Gro, components of the Notch repressor complex.

More recently, epigenetic mechanisms have emerged as an important interface regulating context-dependent and stage-specific gene regulation. Mammalian CoREST acts as a scaffold for recruitment of transcriptional regulators such as REST, and epigenetic factors such as the enzymes HDAC1, HDAC2 and LSD1 (Lakowski et al., 2006; Qureshi et al., 2010). In *Drosophila*, using two-hybrid interaction, CoREST was also shown to interact with Su(VAR)3-3 (*Drosophila* homolog of LSD1) and Rpd3 (HDAC1) (Dallman et al., 2004). In this study, we showed that the levels of H3K27me3 and H4K16 acetylation are significantly and specifically increased in the *CoREST* mutant FCs. Recently, the H3K27me3 demethylase UTX was shown to act as a suppressor of Notch- and Rb-dependent tumors in *Drosophila* eyes (Herz et al., 2010), and in addition to increased level of H3K27me3 staining, an excessive activation of Notch was detected in *Utx* mutant eye discs. The observation of increased levels of H3K27me3 coupled to cell overproliferation and modified Notch signaling in both of these cases [Hertz et al. (Herz et al., 2010) and this study] suggests that the increased H3K27me3 results in epigenetic regulation of genes involved in Notch signaling and/or of Notch target genes. However, in the eye tumor system, this increase in H3K27me3 promotes Notch signaling, whereas in the follicle cells, it reduces Notch signaling. This indicates a strong context-dependent effect on Notch signaling by certain chromatin modifications. Thus, these chromatin modifications might be involved in cell-context-dependent Notch target gene silencing and/or activation (Schwanbeck et al., 2011). Interestingly, many Notch-regulated genes are highly enriched in a characteristic chromatin modification pattern, termed a bivalent domain, consisting of regions of H3K4me3, a marker for actively expressed genes, and H3K27me3, a marker for stably repressed genes; and Notch signaling could be involved in resolving these domains, leading to gene expression (Schwanbeck et al., 2011). Therefore, the

increased level of H3K27me3 in *CoREST* mutant FCs might lead to a repression of certain Notch target genes, for instance *hnt*.

CoREST regulates Notch signaling independently of the function of known CoREST transcription cofactors, Chn and Ttk

To further understand the function of the *Drosophila* CoREST in Notch pathway regulation, identification of other CoREST essential and specific binding partners would be useful. One previously identified partner for CoREST is Chn (Tsuda et al., 2006). Given that we observed wild-type expression of Hnt and Cut in *chn* mutant cells, this factor does not appear to partner CoREST in regulation of Notch signaling in FCs. Using yeast two-hybrid analyses and an embryonic cDNA fusion protein library, it was shown that all three splice variants of *Drosophila* CoREST interact with the unique C-terminus of Tramtrack88 (Ttk88), a known repressor without homology to REST (Dallman et al., 2004). In addition, a Ttk69 splice variant can form a complex with CoREST and Ttk88 (Dallman et al., 2004). However, Ttk88 was not detected in the ovary by immunofluorescence or western blot analysis, and disruption of Ttk88 does not have any impact on oogenesis (French et al., 2003). Conversely, Ttk69 is steadily expressed in FCs before stage 10 (French et al., 2003; Sun and Deng, 2007) and it is required for the M/E transition (Jordan et al., 2006). However, in contrast to CoREST, which acts upstream of Hnt, Hnt expression is not affected in ttk^{1e11} mutant FCs, indicating a role of Ttk69 downstream of Hnt in the control of the M/E switch (Sun and Deng, 2007). Additionally, Ttk69 is not required for cell differentiation, as expression of FasIII, a cell fate marker for immature follicle cells, is normal in ttk^{1e11} mutant FCs (Jordan et al., 2006). From these important phenotypic differences between Ttk69, Ttk88 and CoREST, it appears that CoREST plays a Ttk-independent role in Notch pathway regulation in the FCs. Future work to identify transcription regulators that act as binding partners of CoREST will help in determining the precise biochemical role of CoREST in modulating Notch signaling.

Higher sensitivity of the PFCs to loss of CoREST

Our results demonstrate an unexpected role for CoREST in positively regulating Notch signaling. The effect of the loss of CoREST is particularly strong in the PFCs and relatively mild in the lateral and anterior follicle cells. This implies that CoREST is crucially required in cells that are more sensitive to loss of Notch signaling. The difference between the PFCs and the other follicle cells is established at approximately stages 6–7 of oogenesis by EGF receptor activation in response to Gurken produced by the oocyte (Gonzalez-Reyes et al., 1995; Roth et al., 1995). EGF signaling, therefore, is active around the same time as the Notch pathway and hence it is probable that downstream effector(s) of EGFR signaling result in the increased sensitivity of PFCs to the loss of CoREST. In our model of CoREST negatively affecting a repressor of Notch signaling, we would expect EGFR signaling to act positively to enhance expression and/or activity of a Notch repressor. Thus, loss of CoREST from the PFCs would occur in a cell type where repressor activity is already augmented, which would explain our observation of differential loss of Notch signaling in the PFCs.

In summary we have shown that CoREST, a component of transcriptional repressor complexes, acts positively in Notch signaling in the ovarian follicle cells of *Drosophila*. The results also show that different cell types are differentially sensitive to loss of this repressor. Future identification of partners and targets of CoREST in the follicle cells should further elucidate how activity of EGFR and other signaling pathways are integrated in this process.

Materials and Methods

Drosophila stocks and genetics

The $CoREST^{GF60}$, hnt^{FG47} and N^{FU42} mutations were isolated in a mosaic screen for EMS-induced mutations in *y w FRT19* flies (Denef et al., 2008). The UAS-Notch line was a gift from Gary Struhl (Struhl et al., 1993). UAS-NEXT and UAS-NICD lines were gifts from Spyros Artavanis-Tsakonas (Rebay et al., 1993), to assay Notch activity *E(Spl)-mβ-CD2* line was used (de Celis et al., 1998). The $CtBP^{P1590}$ (Poortinga et al., 1998) and gro^{e47} (Orian et al., 2007) lines were gifts from Susan Parkhurst. We obtained the N^{XK11} line from Eric Wieschaus (Wieschaus et al., 1984; Hoppe and Greenspan, 1986). chn^{ECJ1} FRT42D was a gift from Juan Modolell and Sonsoles Campuzano (Escudero et al., 2005). Dl^{7}, hnt^{EP55}, H^{1} and various duplication and P-element lines used for mapping were obtained from the Bloomington *Drosophila* Stock Center. $CoREST^{GF60}$ was recombined with the hnt^{EP55} mutation to create the double mutant chromosome.

Follicle cell clones were generated using the FRT/UAS-Flp/GAL4 system (Duffy et al., 1998) using a Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp and hs-Flp; FRT42D Ubi-GFP lines obtained from the Bloomington *Drosophila* Stock Center. Follicle cell clones that also expressed Notch constructs were generated using the MARCM system (Lee and Luo, 1999), involving FRT19A, hsFLP122 tub-Gal80; UAS-GFP; Act-Gal4-containing flies.

Mapping of the $CoREST^{GF60}$ mutation

We used recombination with visible recessive markers to map the lethal mutation in the $CoREST^{GF60}$ mutant to the region proximal to *f* (proximal to 15F7). The lethal phenotype was rescued by duplications covering the genomic region 18B6-18C2; 19A2 (Dp(1;Y)BSC134 and Dp(1;Y)BSC135). Subsequent SNP mapping (Berger et al., 2001; Hoskins et al., 2001; Chen et al., 2008; Chen et al., 2009) placed the lethal mutation between 18C8 and 18D3. We sequenced PCR products of at least two independent genomic isolations covering the region, and compared the sequences with those of the FRT19A control.

Constructs for transgenesis

To generate the pTIGER-CoREST and pTIGER-CoREST-3′HA rescue constructs, the coding region of CoREST was obtained by amplification of LD26250 (Drosophila Genomic Resource Center) cloned into pTIGER in frame with or without a C-terminal HA tag. pTIGER was derived from pUASp with enhancements that confer compatibility with ΦC31 integration strategies while retaining P-element compatibility and enhanced restriction enzyme selection (constructed by Scott Ferguson in T. Schupbach's laboratory; unpublished; details available upon request).

Immunofluorescence staining and microscopy

Ovaries were dissected in PBS, fixed for 20 minutes in 4% paraformaldehyde at room temperature, and stained according to standard procedures (Ashburner, 1989). Primary antibodies used were rabbit polyclonal anti-CoREST (1:1000; a gift from Gail Mandel) (Dallman et al., 2004), mouse anti-FasIII (1:10; 7G10, DSHB), rabbit anti β-gal (1:2000; Millipore), mouse anti-Cut (1:10; 2B10, DSHB), mouse anti-Hnt (1:20; 1G9, DSHB), mouse anti-CycB (1:20, F2F4, DSHB), mouse anti-Eya (1:10, 10H6, DSHB), mouse anti-rat CD2 (1:50, Serotec), mouse anti-HA (1:500; Santa Cruz), rabbit anti-PH3 (1:500; Upstate), mouse anti-Arm (1:50; N2 7A1, DSHB), rabbit anti-H3K4me2 (1:200; Cell Signaling Technology), rabbit anti-H3K4me3 (1:200; Cell Signaling Technology), mouse anti-H3K9me1 (1:200; Millipore), rabbit anti-H3K9me2 (1:200; Millipore), rabbit anti-H3K9me3 (1:200; Upstate), rabbit anti-H3K27me2 (1:200; Cell Signaling Technology), rabbit anti-H3K27me3 (1:200; Cell Signaling Technology), rabbit anti-H3K9 acetylated (1:200; Abcam), rabbit anti-H4K20me1 (1:200; Cell Signaling Technology), rabbit anti-H4K20me2 (1:200; Cell Signaling Technology), rabbit anti-H4K5 acetylated (1:100; Millipore), rabbit anti-H4K8 acetylated (1:100; Millipore), rabbit anti-H4K12 acetylated (1:100; Millipore), rabbit anti-H4K16 acetylated (1:100; Millipore). Secondary antibodies were conjugated Alexa Fluor 488, 568 and 647 (1:1000; Molecular Probes). Phalloidin conjugates and Hoechst were from Molecular Probes. Images were taken on a Zeiss LSM510 confocal microscope.

Acknowledgements

We thank Spyros Artavanis-Tsakonas, Sonsoles Campuzano, Gail Mandel, Susan Parkhurst, Gary Struhl, Eric Wieschaus, Leonard Dobens, the Developmental Studies Hybridoma Bank and the Bloomington stock center for sending *Drosophila* stocks and for providing antibodies. We also thank Natalie Denef and Yan Yan for work on the mutagenesis screen, Scott Ferguson for providing pTiger vector, Stefano Di Talia for advice on statistical analysis, Gail

Barcelo for technical help, Joe Goodhouse for support with confocal microscopy, and members of the Schupbach and Wieschaus laboratories for feedback and suggestions. We also thank Olivier Mauti, Yi Sun and Shawn Little for critical reading and helpful comments on the manuscript.

Funding

This work was supported by the Howard Hughes Medical Institute and by the US Public Health Service [grant number RO1 GM077620 to T. S.]. Deposited in PMC for release after 6 months.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.089797/-/DC1

References

Abrajano, J. J., Qureshi, I. A., Gokhan, S., Zheng, D., Bergman, A. and Mehler, M. F. (2009a). Differential deployment of REST and CoREST promotes glial subtype specification and oligodendrocyte lineage maturation. *PLoS ONE* **4**, e7665.

Abrajano, J. J., Qureshi, I. A., Gokhan, S., Zheng, D., Bergman, A. and Mehler, M. F. (2009b). REST and CoREST modulate neuronal subtype specification, maturation and maintenance. *PLoS ONE* **4**, e7936.

Abrajano, J. J., Qureshi, I. A., Gokhan, S., Molero, A. E., Zheng, D., Bergman, A. and Mehler, M. F. (2010). Corepressor for element-1-silencing transcription factor preferentially mediates gene networks underlying neural stem cell fate decisions. *Proc. Natl. Acad. Sci. USA* **107**, 16685-16690.

Andres, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., Dallman, J., Ballas, N. and Mandel, G. (1999). CoREST: a functional corepressor required for regulation of neural-specific gene expression. *Proc. Natl. Acad. Sci. USA* **96**, 9873-9878.

Artavanis-Tsakonas, S. and Muskavitch, M. A. (2010). Notch: the past, the present, and the future. *Curr. Top. Dev. Biol.* **92**, 1-29.

Ashburner, M. (1989). *Drosophila: a Laboratory Manual*. Vol. 2, pp. xxiii+424. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Bai, J. and Montell, D. (2002). Eyes absent, a key repressor of polar cell fate during *Drosophila* oogenesis. *Development* **129**, 5377-5388.

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.

Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J. et al. (2001). Regulation of neuronal traits by a novel transcriptional complex. *Neuron* **31**, 353-365.

Berger, J., Suzuki, T., Senti, K. A., Stubbs, J., Schaffner, G. and Dickson, B. J. (2001). Genetic mapping with SNP markers in Drosophila. *Nat. Genet.* **29**, 475-481.

Borggrefe, T. and Oswald, F. (2009). The Notch signaling pathway: transcriptional regulation at Notch target genes. *Cell Mol. Life Sci.* **66**, 1631-1646.

Bruce, A. W., Donaldson, I. J., Wood, I. C., Yerbury, S. A., Sadowski, M. I., Chapman, M., Gottgens, B. and Buckley, N. J. (2004). Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc. Natl. Acad. Sci. USA* **101**, 10458-10463.

Cau, E. and Blader, P. (2009). Notch activity in the nervous system: to switch or not switch? *Neural Dev.* **4**, 36.

Chen, D., Ahlford, A., Schnorrer, F., Kalchhauser, I., Fellner, M., Viragh, E., Kiss, I., Syvanen, A. C. and Dickson, B. J. (2008). High-resolution, high-throughput SNP mapping in Drosophila melanogaster. *Nat. Methods* **5**, 323-329.

Chen, D., Berger, J., Fellner, M. and Suzuki, T. (2009). FLYSNPdb: a high-density SNP database of Drosophila melanogaster. *Nucleic Acids Res.* **37**, D567-D570.

Dallman, J. E., Allopenna, J., Bassett, A., Travers, A. and Mandel, G. (2004). A conserved role but different partners for the transcriptional corepressor CoREST in fly and mammalian nervous system formation. *J. Neurosci.* **24**, 7186-7193.

de Celis, J. F., Tyler, D. M., de Celis, J. and Bray, S. J. (1998). Notch signalling mediates segmentation of the *Drosophila* leg. *Development* **125**, 4617-4626.

de la Calle-Mustienes, E., Modolell, J. and Gomez-Skarmeta, J. L. (2002). The Xiro-repressed gene CoREST is expressed in Xenopus neural territories. *Mech. Dev.* **110**, 209-211.

Denef, N., Chen, Y., Weeks, S. D., Barcelo, G. and Schupbach, T. (2008). Crag regulates epithelial architecture and polarized deposition of basement membrane proteins in Drosophila. *Dev. Cell* **14**, 354-364.

Deng, W. M., Althauser, C. and Ruohola-Baker, H. (2001). Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in *Drosophila* follicle cells. *Development* **128**, 4737-4746.

Duffy, J. B., Harrison, D. A. and Perrimon, N. (1998). Identifying loci required for follicular patterning using directed mosaics. *Development* **125**, 2263-2271.

Eimer, S., Lakowski, B., Donhauser, R. and Baumeister, R. (2002). Loss of spr-5 bypasses the requirement for the C.elegans presenilin sel-12 by derepressing hop-1. *EMBO J.* **21**, 5787-5796.

Escudero, L. M., Caminero, E., Schulze, K. L., Bellen, H. J. and Modolell, J. (2005). Charlatan, a Zn-finger transcription factor, establishes a novel level of regulation of the proneural achaete/scute genes of *Drosophila*. *Development* **132**, 1211-1222.

French, R. L., Cosand, K. A. and Berg, C. A. (2003). The Drosophila female sterile mutation twin peaks is a novel allele of tramtrack and reveals a requirement for Ttk69 in epithelial morphogenesis. *Dev. Biol.* **253**, 18-35.

Fryer, C. J., Lamar, E., Turbachova, I., Kintner, C. and Jones, K. A. (2002). Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes Dev.* **16**, 1397-1411.

Furukawa, T., Kobayakawa, Y., Tamura, K., Kimura, K., Kawaichi, M., Tanimura, T. and Honjo, T. (1995). Suppressor of hairless, the Drosophila homologue of RBP-J kappa, transactivates the neurogenic gene E(spl)m8. *Jpn. J. Genet.* **70**, 505-524.

Gonzalez-Reyes, A., Elliott, H. and St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature* **375**, 654-658.

Grammont, M. (2007). Adherens junction remodeling by the Notch pathway in Drosophila melanogaster oogenesis. *J. Cell Biol.* **177**, 139-150.

Grammont, M. and Irvine, K. D. (2001). fringe and Notch specify polar cell fate during *Drosophila* oogenesis. *Development* **128**, 2243-2253.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Guo, Y., Livne-Bar, I., Zhou, L. and Boulianne, G. L. (1999). Drosophila presenilin is required for neuronal differentiation and affects notch subcellular localization and signaling. *J. Neurosci.* **19**, 8435-8442.

Herz, H. M., Madden, L. D., Chen, Z., Bolduc, C., Buff, E., Gupta, R., Davuluri, R., Shilatifard, A., Hariharan, I. K. and Bergmann, A. (2010). The H3K27me3 demethylase dUTX is a suppressor of Notch- and Rb-dependent tumors in Drosophila. *Mol. Cell Biol.* **30**, 2485-2497.

Hoppe, P. E. and Greenspan, R. J. (1986). Local function of the Notch gene for embryonic ectodermal pathway choice in Drosophila. *Cell* **46**, 773-783.

Horne-Badovinac, S. and Bilder, D. (2005). Mass transit: epithelial morphogenesis in the Drosophila egg chamber. *Dev. Dyn.* **232**, 559-574.

Hoskins, R. A., Phan, A. C., Naeemuddin, M., Mapa, F. A., Ruddy, D. A., Ryan, J. J., Young, L. M., Wells, T., Kopczynski, C. and Ellis, M. C. (2001). Single nucleotide polymorphism markers for genetic mapping in Drosophila melanogaster. *Genome Res.* **11**, 1100-1113.

Jarriault, S. and Greenwald, I. (2002). Suppressors of the egg-laying defective phenotype of sel-12 presenilin mutants implicate the CoREST corepressor complex in LIN-12/Notch signaling in C. elegans. *Genes Dev.* **16**, 2713-2728.

Jordan, K. C., Schaeffer, V., Fischer, K. A., Gray, E. E. and Ruohola-Baker, H. (2006). Notch signaling through tramtrack bypasses the mitosis promoting activity of the JNK pathway in the mitotic-to-endocycle transition of Drosophila follicle cells. *BMC Dev. Biol.* **6**, 16.

Klusza, S. and Deng, W. M. (2011). At the crossroads of differentiation and proliferation: Precise control of cell-cycle changes by multiple signaling pathways in Drosophila follicle cells. *BioEssays* **33**, 124-134.

Koch, U. and Radtke, F. (2010). Notch signaling in solid tumors. *Curr. Top. Dev. Biol.* **92**, 411-455.

Kovall, R. A. (2007). Structures of CSL, Notch and Mastermind proteins: piecing together an active transcription complex. *Curr. Opin. Struct. Biol.* **17**, 117-127.

Krejci, A., Bernard, F., Housden, B. E., Collins, S. and Bray, S. J. (2009). Direct response to Notch activation: signaling crosstalk and incoherent logic. *Sci. Signal.* **2**, ra1.

Kuppuswamy, M., Vijayalingam, S., Zhao, L. J., Zhou, Y., Subramanian, T., Ryerse, J. and Chinnadurai, G. (2008). Role of the PLDLS-binding cleft region of CtBP1 in recruitment of core and auxiliary components of the corepressor complex. *Mol. Cell Biol.* **28**, 269-281.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-973.

Lakowski, B., Roelens, I. and Jacob, S. (2006). CoREST-like complexes regulate chromatin modification and neuronal gene expression. *J. Mol. Neurosci.* **29**, 227-239.

Lecourtois, M. and Schweisguth, F. (1995). The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. *Genes Dev.* **9**, 2598-2608.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.

Lopez-Schier, H. and St Johnston, D. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. *Genes Dev.* **15**, 1393-1405.

Lopez-Schier, H. and St Johnston, D. (2002). Drosophila nicastrin is essential for the intramembranous cleavage of notch. *Dev. Cell* **2**, 79-89.

Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G. et al. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* **298**, 1747-1752.

Maier, D., Stumm, G., Kuhn, K. and Preiss, A. (1992). Hairless, a Drosophila gene involved in neural development, encodes a novel, serine rich protein. *Mech. Dev.* **38**, 143-156.

Micchelli, C. A. and Blair, S. S. (1999). Dorsoventral lineage restriction in wing imaginal discs requires Notch. *Nature* **401**, 473-476.

Mulligan, P., Yang, F., Di Stefano, L., Ji, J. Y., Ouyang, J., Nishikawa, J. L., Toiber, D., Kulkarni, M., Wang, Q., Najafi-Shoushtari, S. H. et al. (2011). A SIRT1-LSD1 corepressor complex regulates notch target gene expression and development. *Mol. Cell* **42**, 689-699.

Nagel, A. C., Krejci, A., Tenin, G., Bravo-Patino, A., Bray, S., Maier, D. and Preiss, A. (2005). Hairless-mediated repression of notch target genes requires the combined activity of Groucho and CtBP corepressors. *Mol. Cell. Biol.* **25**, 10433-10441.

Orian, A., Delrow, J. J., Rosales Nieves, A. E., Abed, M., Metzger, D., Paroush, Z., Eisenman, R. N. and Parkhurst, S. M. (2007). A Myc-Groucho complex integrates EGF and Notch signaling to regulate neural development. *Proc. Natl. Acad. Sci. USA* **104**, 15771-15776.

Panin, V. M. and Irvine, K. D. (1998). Modulators of Notch signaling. *Semin. Cell Dev. Biol.* **9**, 609-617.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912.

Poortinga, G., Watanabe, M. and Parkhurst, S. M. (1998). Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *EMBO J.* **17**, 2067-2078.

Portin, P. (2002). General outlines of the molecular genetics of the Notch signalling pathway in Drosophila melanogaster: a review. *Hereditas* **136**, 89-96.

Qureshi, I. A., Gokhan, S. and Mehler, M. F. (2010). REST and CoREST are transcriptional and epigenetic regulators of seminal neural fate decisions. *Cell Cycle* **9**, 4477-4486.

Rebay, I., Fehon, R. G. and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. *Cell* **74**, 319-329.

Roth, S., Neuman-Silberberg, F. S., Barcelo, G. and Schupbach, T. (1995). cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. *Cell* **81**, 967-978.

Schwanbeck, R., Martini, S., Bernoth, K. and Just, U. (2011). The Notch signaling pathway: molecular basis of cell context dependency. *Eur. J. Cell Biol.* **90**, 572-581.

Schweisguth, F. (2004). Regulation of notch signaling activity. *Curr. Biol.* **14**, R129-R138.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A. and Casero, R. A. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**, 941-953.

Shyu, L. F., Sun, J., Chung, H. M., Huang, Y. C. and Deng, W. M. (2009). Notch signaling and developmental cell-cycle arrest in Drosophila polar follicle cells. *Mol. Biol. Cell* **20**, 5064-5073.

Steiner, H., Fluhrer, R. and Haass, C. (2008). Intramembrane proteolysis by gamma-secretase. *J. Biol. Chem.* **283**, 29627-29631.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.

Struhl, G. and Greenwald, I. (1999). Presenilin is required for activity and nuclear access of Notch in Drosophila. *Nature* **398**, 522-525.

Struhl, G. and Greenwald, I. (2001). Presenilin-mediated transmembrane cleavage is required for Notch signal transduction in Drosophila. *Proc. Natl. Acad. Sci. USA* **98**, 229-234.

Sun, J. and Deng, W. M. (2005). Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in *Drosophila* follicle cells. *Development* **132**, 4299-4308.

Sun, J. and Deng, W. M. (2007). Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation. *Dev. Cell* **12**, 431-442.

Sun, J., Smith, L., Armento, A. and Deng, W. M. (2008). Regulation of the endocycle/gene amplification switch by Notch and ecdysone signaling. *J. Cell Biol.* **182**, 885-896.

Tontsch, S., Zach, O. and Bauer, H. C. (2001). Identification and localization of M-CoREST (1A13), a mouse homologue of the human transcriptional co-repressor CoREST, in the developing mouse CNS. *Mech. Dev.* **108**, 165-169.

Tsuda, L., Kaido, M., Lim, Y. M., Kato, K., Aigaki, T. and Hayashi, S. (2006). An NRSF/REST-like repressor downstream of Ebi/SMRTER/Su(H) regulates eye development in Drosophila. *EMBO J.* **25**, 3191-3202.

Vachias, C., Couderc, J. L. and Grammont, M. (2010). A two-step Notch-dependant mechanism controls the selection of the polar cell pair in Drosophila oogenesis. *Development* **137**, 2703-2711.

Wieschaus, E., Nusslein-Volhard, C. and Jürgens, G. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. III. Zygotic loci on the X-chromosome and fourth chromosome. *Roux's Arch. Dev. Biol.* **193,** 296-307.

Wu, L., Aster, J. C., Blacklow, S. C., Lake, R., Artavanis-Tsakonas, S. and Griffin, J. D. (2000). MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat. Genet.* **26**, 484-489.

Yan, Y., Denef, N. and Schupbach, T. (2009). The vacuolar proton pump, V-ATPase, is required for notch signaling and endosomal trafficking in Drosophila. *Dev. Cell* **17**, 387-402.

Zhang, L., Tran, D. T. and Ten Hagen, K. G. (2010). An O-glycosyltransferase promotes cell adhesion during development by influencing secretion of an extracellular matrix integrin ligand. *J. Biol. Chem.* **285**, 19491-19501.

Zusman, S., Patel-King, R. S., Ffrench-Constant, C. and Hynes, R. O. (1990). Requirements for integrins during *Drosophila* development. *Development* **108**, 391-402.

Zusman, S., Grinblat, Y., Yee, G., Kafatos, F. C. and Hynes, R. O. (1993). Analyses of PS integrin functions during *Drosophila* development. *Development* **118**, 737-750.