Regulation of the endocycle/gene amplification switch by Notch and ecdysone signaling

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The developmental signals that regulate the switch from genome-wide DNA replication to site-specific amplification remain largely unknown. *Drosophila melanogaster* epithelial follicle cells, which begin synchronized chorion gene amplification after three rounds of endocycle, provide an excellent model for study of the endocycle/gene amplification (E/A) switch. Here, we report that down-regulation of Notch signaling and activation of ecdysone receptor (EcR) are required for the E/A switch in these cells. Extended Notch activity suppresses EcR activation and prevents exit from the endocycle. Tramtrack (Ttk), a zinc-finger protein essential for the switch, is regulated negatively by Notch and positively by EcR. Ttk overexpression stops endoreplication prematurely and alleviates the endocycle exit defect caused by extended Notch activity or removal of EcR function. Our results reveal a developmental pathway that includes down-regulation of Notch, activation of the EcR, up-regulation of Ttk to execute the E/A switch, and, for the first time, the genetic interaction between Notch and ecdysone signaling in regulation of cell cycle programs and differentiation.

Introduction

Chromosomal DNA replication is restricted to once per cell cycle in eukaryotes. Incomplete or continuous DNA replication without cell division can cause aneuploidy and disturb genomic stability. During metazoan development, however, some cells do not follow this once per cycle rule. For example, cells such as trophoblasts in mammalian placenta and salivary gland cells in dipterans undergo endoreplication, producing multiple copies of their nuclear DNA without dividing (Edgar and Orr-Weaver, 2001). In some organisms, certain genes are amplified in some cells to meet massive demand for their products at particular developmental stages, such as the single locus in the *Sciara coprophila* puffII/9A region and ribosomal DNA genes in *Tetrahymena thermophila* and *Xenopus laevis* (Tower, 2004). These variants of DNA replication are essential for cellular function in metazoan development.

Drosophila melanogaster epithelial follicle cells provide an excellent model for study of developmental regulation of cell cycle programs, DNA replication, and cell differentiation. The single monolayer of follicle cells that surrounds 16 interconnected germline cells to form the egg chamber undergo three distinctive cell cycle programs during oogenesis. In early stages (1–6), they carry out the normal mitotic cycle, including complete G1, S, G2, and M phases. After stage 6, they undergo three rounds of endocycle, duplicating their genomic DNA without division. At stage 10B, genomic DNA replication stops, and the main body follicle cells (columnar cells that surround the oocyte rather than those that cover the nurse cells) switch from endoreplication to synchronized amplification of some genomic loci (Calvi et al., 1998). During amplification, continuous origin firing occurs without obvious gap phases. The amplified genomic regions encode eggshell proteins, which are in high demand during late oogenesis.

At the switch of cell cycle programs, follicle cells also change the expression pattern of molecular markers such as Cut, Hindsight (Hnt), and Fasciclin III (Sun and Deng, 2005, 2007). The mitotic cycle/endocycle (M/E) switch is induced by Delta-Notch signaling originating from the germline cells (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Notch activates expression of Hnt, a zinc-finger protein, in follicle cells during endocycle stages. Hnt mediates the role of Notch in suppressing

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Abbreviations used in this paper: CycE, cyclin E; Dl, Delta; DN, dominant negative; E/A, endocycle/gene amplification; EcR, ecdysone receptor; FLP, flipase; FRT, FLP recombinase target; Hnt, Hindsight; M/E, mitotic cycle/endocycle; NICD, Notch intracellular domain; ORC, origin recognition complex; PonA, ponasterone A; Ttk, Tramtrack.

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the expression of a homeobox gene, *cut*, and the Cdc25 phosphatase, *string*, to regulate the M/E transition. Hnt also suppresses hedgehog signaling, which promotes follicle cell proliferation in early oogenesis (Sun and Deng, 2007).

However, the developmental signal triggering the endocycle gene/amplification (E/A) switch remains unknown, although amplification has been demonstrated to use the same DNA replication machinery as is used in the mitotic cycle (Claycomb and Orr-Weaver, 2005). Gene amplification requires the replication origins at specific genomic regions to be fired repeatedly without cell division, whereas all other origins in the genome are inhibited. Several proteins and protein complexes required for forming the prereplication complex are involved, including the origin recognition complex (ORC), Cdt1 (encoded by *double parked* in *Drosophila*), and the minichromosome maintenance complex (Landis et al., 1997; Whittaker et al., 2000; Schwed et al., 2002). ORC2 moves from a diffused nuclear distribution into a localized distribution at the amplification foci in stage 10 egg chambers but is undetectable at stage 12 (Royzman et al., 1999). Restriction of ORC2 to amplification foci depends on dE2F1, which forms a heterodimer with dDp and acts as a transcriptional activator (Royzman et al., 1999; Bosco et al., 2001). The $dE2F1^{i1}$ mutant lacks punctate ORC2 staining and shows decreased intensity of chorion gene amplification (Royzman et al., 1999). dE2F1 and dE2F2 forming complexes with Rbf1, the *Drosophila* homologue for retinoblastoma, are also involved in suppression of genomic replication during amplification (Cayirlioglu et al., 2003). $Rbf1^{120a}/Rbf1^{14}$ follicle cells have an additional round of genomic DNA replication (Cayirlioglu et al., 2003). dE2F2 and Rbf also belong to the Myb-Muv B–dREAM complex that constrains genomic DNA replication during amplification (Beall et al., 2002, 2004, 2007; Georlette et al., 2007). Some evidence also indicates that chromatin modification regulates origin activity during amplification (Aggarwal and Calvi, 2004; Hartl et al., 2007).

The steroid hormone ecdysone and its receptor may be involved in chorion gene expression. Ecdysone functions during postembryonic development, including larval molts and metamorphosis. 20-hydroxyecdysone, the active hormone produced in the peripheral tissues by metabolism of ecdysone, binds to the ecdysone receptor (EcR), which forms heterodimers with the *Drosophila* RXR homologue USP (encoded by the gene *ultraspiracles*), and activates the ecdysone signaling pathway, which turns on the early response genes, including Broad, E75, and E74, during metamorphosis (Thummel, 1996). In adult flies, ecdysone is mainly synthesized in ovaries, and the ecdysteroid hierarchy is known to regulate *Drosophila* oogenesis and reproduction (for review see Kozlova and Thummel, 2000). Egg chambers with germline clones of *ecdysoneless*, which disrupts production of ecdysone, arrest before vitellogenesis (Gaziova et al., 2004). Both EcR-A and EcR-B1, two EcR isoforms, are expressed in nurse and follicle cells during oogenesis. Removal of EcR function in germline cells causes abnormal egg chamber formation, loss of vitellogenic egg chambers, and nurse cell degeneration (Carney and Bender, 2000). Recently, EcR activity was shown to be required for follicle cell migration, dorsal appendage tube formation, and chorion gene expression and amplification. The EcR activity in the dorsal anterior region is also sensitive to Ras signaling (Hackney et al., 2007).

Here, we report that Notch signaling is down-regulated in main body follicle cells at stage 10B. Extended Notch signaling causes an extra round of genomic DNA replication and suppresses ecdysone signaling and the up-regulation of the zinc-finger protein Tramtrack (Ttk), both key regulators of the E/A switch. Overexpression of Ttk69 before stage 10 is sufficient to induce exit from the endocycle. Our findings elucidate a developmental pathway that includes down-regulation of Notch activity, activation of the EcR pathway, and up-regulation of Ttk to regulate the E/A switch.

Results

Extended Notch activity prolongs endocycle and prevents synchronous gene amplification

The important role of Notch signaling in follicle cell proliferation and differentiation led us to examine the temporal and spatial pattern of Notch activity during oogenesis. Three independent reporter lines, *Gbe-Su(H)m8-lacZ* (*Gbe-lacZ*), *E(Spl)mβ-CD2* (*mβ-CD2*), and *E(spl)m7-lacZ* (*m7-lacZ*; de Celis et al., 1998; Furriols and Bray, 2001; Assa-Kunik et al., 2007), showed similar patterns of Notch activity in follicle cells, as revealed by costaining of anti–β-galactosidase or anti-CD2 antibodies with Cut antibody for egg chamber staging. During stages 1–6, Notch activity was limited to anterior and posterior ends of the egg chamber (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200802084/DC1; and not depicted), which is related to the role of Notch in polar and stalk cell differentiation. At around stage 7, Notch activity was detected in the entire follicle cell epithelium and reached its highest level at stage 8 (Fig. 1 A and Fig. S1 A). It then slowly decreased until stage 10A in main body cells. No Notch activity was observed in these follicle cells at stage 10B (Fig. 1 A), when follicle cells have already left the endocycle and begun synchronous site-specific DNA replication. The residual levels of reporter gene detected may result from the longevity of the gene product. The coincidence of Notch down-regulation and initiation of gene amplification in follicle cells was verified by a BrdU incorporation assay in the *Gbe-lacZ* reporter line. Normally, cells undergoing gene amplification have a unique punctate BrdU incorporation pattern. Each nucleus contains four BrdU incorporation foci representing the *Drosophila* amplicons in follicle cells (Fig. S1, C and C′; Calvi et al., 1998; Claycomb et al., 2004). Sometimes the fifth focus was also detected with our sensitized BrdU labeling protocol (Fig. S1 C). Notch activity was absent from the main body cells that began to show gene amplification (Fig. S1, B and B′), suggesting a potential connection between Notch down-regulation and the E/A switch.

To determine whether down-regulation of Notch signaling is necessary for the E/A switch, we induced misexpression of a constitutively active form of Notch, Notch intracellular domain (NICD), using the flip-out act-Gal4/UAS system in follicle cells. NICD-misexpressing cells showed high levels of Notch activity after stage 10B (not depicted), when such activity had already diminished in the wild type (Fig. 1 A and Fig. S1 A). Through BrdU incorporation analysis, we found that NICD-misexpressing cells did not have the punctate BrdU incorporation pattern but instead

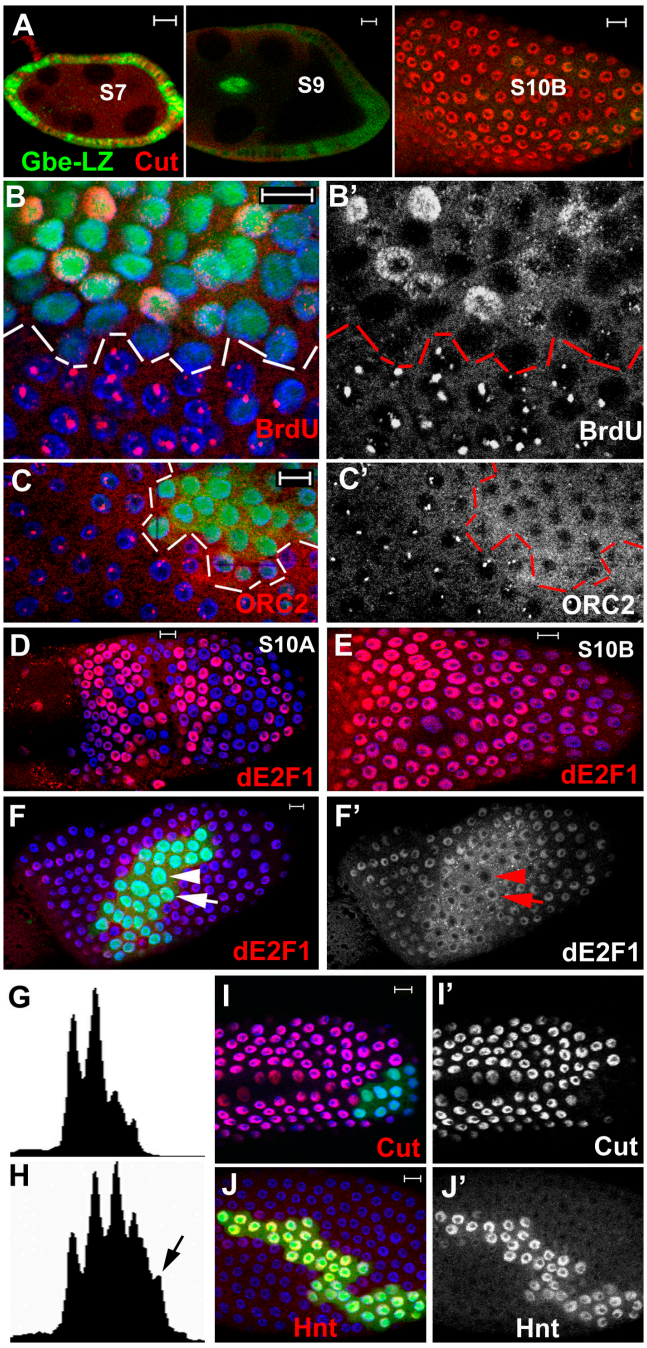

Figure 1. **Down-regulation of Notch activity is required for the proper E/A switch and cell differentiation.** DAPI (B–F, I, and J; blue) was used to mark cell nuclei. Follicle cells with NICD misexpression are marked by GFP (B, C, F, I, and J; green). Bars, 10 μm. (A) The pattern of Notch activity illustrated by *Gbe-lacZ* expression (green). Cut staining (red) was used to mark the egg chambers before stage 7 and after stage 10A. (B and B′) Genomic BrdU incorporation (B, red; B′, white) was found in follicle cells with NICD misexpression in a stage 10B egg chamber (top). Dashed outlines separate the follicle cells with NICD overexpression and wild-type cells. Cells above the line are NICD-overexpressing cells. (C and C′) Restricted subnuclear ORC2 localization (C, red; C′, white) was not detected in NICD-misexpressing follicle cells (outlined) in a stage 10B egg chamber. (D and E) dE2F1 showed an oscillating expression pattern in follicle cell nuclei at stage 10A (D) but was accumulated in all follicle cell nuclei at stage 10B (E). (F and F′) dE2F1 (F, red; F′, white) showed an oscillating pattern in NICD-misexpressing follicle cells in a stage 10B egg chamber. Arrows point to follicle cells with dE2F1 staining, and arrowheads point to those without dE2F1 staining. (G and H) Fluorescence-activated cell-sorting analyses of DNA contents in GFP-negative (G, wild type) and GFP-positive (H, NICD misexpressing) follicle cells. The fifth peak, which indicates a 32C DNA content, was found in NICD-misexpressing cells (H, arrow). (I and I′) Cut (I, red; I′, white) was not expressed in NICD-misexpressing follicle cells in a stage 10B egg chamber. (J and J′) Hnt (J, red; J′, white) was continuously expressed in follicle cells with NICD misexpression at stage 10B.

showed an oscillating genomic BrdU incorporation pattern at stage 10B and later (Fig. 1 B; >300 egg chambers counted with 100% penetrance), so they are in either the mitotic cycle or the endocycle. Staining with mitotic markers cyclin B and phosphohistone 3 (Deng et al., 2001) showed that the NICD-misexpressing cells stopped expressing these markers after stage 6, like the wild-type cells (unpublished data), so they are probably not maintained in the mitotic cycle. Together, these data suggest that extended Notch signaling in main body follicle cells keeps them in the endocycle when they would otherwise have proceeded to synchronous gene amplification.

To determine whether NICD-misexpressing cells complete an extra round of the endocycle, we isolated follicle cells from NICD mosaic egg chambers (marking the NICD-misexpressing cells with GFP) and analyzed the genomic DNA content by fluorescence-activated cell sorting. The wild-type follicle cells (GFP negative) showed four different cell populations, with 2C, 4C, 8C, and 16C DNA contents, consistent with three endocycle rounds (Fig. 1 G). In contrast, NICD-misexpressing (GFP positive) cells additionally showed a fifth peak indicating 32C DNA content (Fig. 1 H), which suggests that they undergo an extra round of the endocycle. Normally, cells with more copies of genomic DNA have larger nuclei, and indeed, nuclei of the NICD-misexpressing cells were larger than those of their wild-type neighbors (Fig. 1 B).

Gene amplification requires that replication origins be fired repeatedly without cell division. To fulfill this task, ORC2 becomes restricted at the amplification foci in stage 10B egg chambers (Royzman et al., 1999). Staining ORC2 in NICD mosaic egg chambers, we found that the punctate ORC2 pattern normally seen in wild-type cells was not detected in follicle cells with continued Notch activity at stage 10B (Fig. 1 C; $n = 50$). These follicle cells showed unrestricted localization of ORC2 (Fig. 1, C and C′), suggesting that continued Notch signaling blocks the transition to competency for gene amplification.

Extended Notch activity disrupts dE2F1, Hnt, and Cut expression during late oogenesis

Restriction of ORC2 to amplification foci depends on dE2F1 (Royzman et al., 1999). Staining wild-type egg chambers with a polyclonal antibody against dE2F1, we found that dE2F1 expression oscillated in follicle cell nuclei until about stage 10A (Fig. 1 D), a pattern probably related to the involvement of dE2F1 during the onset of S phases in both the mitotic cycle and the endocycle (Follette et al., 1998; Reis and Edgar, 2004). Starting at stage 10B, dE2F1 was expressed in all main body follicle cell nuclei (Fig. 1 E), coincident with the E/A switch, but this uniform expression of dE2F1 was disrupted in cells with NICD misexpression.

dE2F1 expression continued to oscillate in stage 10B and older follicle cells (Fig. 1 F; >20 egg chambers counted). The other member of the *Drosophila* E2F transcription factor, dE2F2, which is also involved in promoting the E/A switch (Cayirlioglu et al., 2001), is expressed in all follicle cells throughout oogenesis (Fig. S2, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200802084/DC1), but continued Notch activity did not change dE2F2 expression in follicle cells (Fig. S2 B).

dE2F1 has been shown to regulate cyclin E (CycE) expression, and CycE/Cdk2 activity is required for gene amplification (Duronio and O'Farrell, 1995; Calvi et al., 1998). To determine whether that activity is altered by extended Notch activity, we used the MPM2 antibody, which detects the CycE/Cdk2-dependent phosphoepitope at the histone locus body (White et al., 2007) to stain NICD-misexpressing mosaic egg chambers. MPM2 appeared in an oscillating dotted pattern in follicle cells in the endocycle, but, as Calvi et al. (1998) reported, it accumulated in all main body follicle cells at stage 10B in the wild type. In contrast, NICD-misexpressing follicle cells showed an oscillating pattern of the MPM2 signal at stage 10B (Fig. S2, C–C′′′), consistent with the conclusion that these cells are still in the endocycle (Fig. 1 B). In addition, some of the NICD-misexpressing follicle cells had higher MPM2 signal intensity than did their wild-type neighbors (Fig. S2 C′′′), suggesting that continued Notch activity affects CycE/Cdk2 activity during the E/A switch.

Cut expression is down-regulated by Notch signaling during the endocycle but resumes at around stage 10B (Sun and Deng, 2005). This resumption is not essential for the E/A switch, as *cut* mutant clones at stage 10B showed normal gene amplification (Sun and Deng, 2005). Nonetheless, expression of Cut in late follicle cells serves as a marker for late follicle cell differentiation. Staining Cut in egg chambers with extended Notch activity, we found that NICD misexpression sufficed to block Cut expression during late oogenesis (Fig. 1 I; >100 egg chambers counted), suggesting that down-regulation of Notch signaling in follicle cells is required for resumption of Cut expression in those cells.

Hnt, a positive target of Notch signaling, is normally expressed in follicle cells during stages 7–10A and down-regulated in main body cells at stage 10B (Sun and Deng, 2007). In main body cells with NICD misexpression during late oogenesis, Hnt expression continued (Fig. 1 J; $n = 100$), indicating that extended Notch signaling maintains Hnt expression. The maintenance of the middle stage expression patterns of dE2F1, Hnt, and Cut in NICD-misexpressing cells during late oogenesis suggests that extended Notch activity suffices to keep the middle stage (endocycle) identity of follicle cells.

Ttk function is required for the E/A switch

To elucidate regulation of the E/A switch further, we performed a mosaic screen to isolate *P* element insertion mutations that disturb late Cut expression in follicle cells. The target gene of such a mutation, *l(3)S144802*, turned out to be *ttk*, which encodes a zinc-finger domain protein and is required in early follicle cell differentiation and dorsal appendage morphogenesis (French et al., 2003; Jordan et al., 2006; Sun and Deng, 2007). To determine

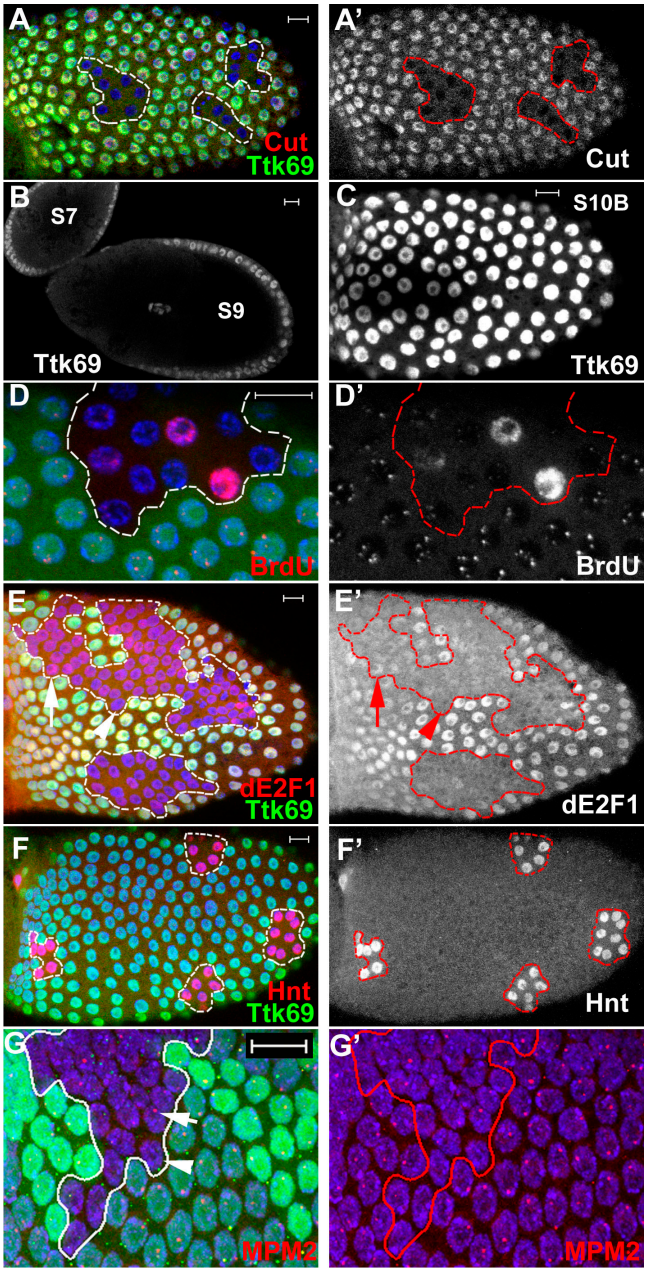

Figure 2. **Up-regulation of Ttk69 is required for the proper E/A switch and cell differentiation.** Images in A and E–G are collapsed images of the z stacks. ttk^{1e11} mutant clones are marked by the absence of Ttk69 (A, E, and F; green) or GFP (D and G; green). (A and A′) Cut (A, red; A′, white) was not detected in *ttk* mutant clones (outlined) in a stage 10B egg chamber. (B and C) The expression pattern of Ttk69 in follicle cells. Ttk69 was steadily expressed at a low level in follicle cells before stage 10 (B) but significantly up-regulated at stage 10B (C). (D and D′) *ttk* mutant follicle cells (outlined) at stage 10B had oscillating genomic BrdU incorporation (D, red; D′, white). (E and E′) dE2F1 (E, red; E′, white) showed an oscillating expression pattern with an overall low level in *ttk* mutant clones (outlined) in a stage 10B egg chamber. The arrow points to a follicle cell with dE2F1 expression, and the arrowhead points to one without dE2F1 expression. (F and F′) Hnt (F, red; F′, white) was still detected in stage 10B *ttk* mutant clones. Outlining indicates the ttk mutant follicle cells. (G and G′) MPM2 staining (red) retained an oscillating pattern in *ttk* mutant clones in a stage 10B egg chamber. The arrow points to a cell with MPM2 signal, and the arrowhead points to a cell without MPM2 signal. Outlining indicates the ttk mutant follicle cells. Bars, 10 µm.

whether *ttk* is indeed required for late Cut expression in follicle cells during the E/A switch, we generated *ttk* mosaic clones with a known *ttk*-null allele, ttk^{1e11} (French et al., 2003). Consistently, late Cut expression was disrupted in *ttk* mutant follicle cells (Fig. 2 A; >45 stage 10B or later egg chambers bearing ≥10 *ttk* mutant follicle cells). In contrast, early Cut expression during mitotic and endocycle stages was unaffected in *ttk* mutant cells (Jordan et al., 2006; unpublished data).

To determine whether the function of *ttk* in late follicle cells is reflected in its expression pattern, we reexamined its protein distribution in wild-type egg chambers using antibodies against different Ttk isoforms. Consistent with a previous study, only the 69-kD isoform (Ttk69) was steadily expressed, albeit at a relatively low level, in follicle cells before stage 10 (Fig. 2 B; Sun and Deng, 2007). At around stage 10B, Ttk69 expression was significantly up-regulated in all main body cells (Fig. 2 C), concurrent with the E/A switch and the resumption of Cut expression.

To determine whether Ttk69 up-regulation is functionally related to the E/A switch, we monitored BrdU incorporation in ttk^{1e11} mosaic egg chambers. Interestingly, *ttk* mutant follicle cells showed oscillating genomic BrdU incorporation during or after stage 10B, when adjacent wild-type cells showed synchronized gene amplification (Fig. 2 D). The *ttk* mutant cells also lacked the punctate ORC2 localization pattern at stage 10 (unpublished data). These defects resemble those of the NICD-misexpressing follicle cells (Fig. 1). Further analyses of the *ttk* mutant clones with MPM2 and antibodies against dE2F1 and Hnt supported the conclusion that these mutant cells are kept in the endocycle. dE2F1 expression and MPM2 still showed an oscillating pattern in *ttk* mutant cells after stage 10B, when the wild type showed uniform expression (Fig. 2, E and G). Hnt was also detected in *ttk* mutant cells after stage 10B, when its expression in the wild type had already ceased (Fig. 2 F), but the *ttk* mutation did not disturb Hnt expression during stages 7–10A of oogenesis, when Hnt is normally expressed, consistent with our previous study (Sun and Deng, 2007). Together, these data suggest that loss of *ttk* and extended Notch signaling produce similar defects in the E/A switch and cell differentiation in late follicle cells.

Ttk function in the E/A switch in late oogenesis is separable from its earlier function

Clonal analysis of *ttk* function in the E/A switch is based on flipase (FLP)/FLP recombinase target (FRT) mitotic recombination, which requires generation of clones when follicle cells are still in the mitotic cycle. As previously reported, Ttk is required during early oogenesis for the suppression of *cubitus interruptus* and *string* and for up-regulation of *fizzy related* during the M/E switch (Jordan et al., 2006; Sun and Deng, 2007). To determine whether the phenotypes of *ttk* mutation during the E/A switch are unrelated to its earlier role, we used the RNAi technique to knock down *ttk* expression in follicle cells only after the M/E switch. We used the temperature-sensitive Gal80/Gal4 (McGuire et al., 2004) and RNAi techniques to remove *ttk* function specifically after stage 7 in posterior follicle cells with the C204 Gal4 driver (Manseau et al., 1997). When adult flies were shifted to the restrictive temperature, Ttk69 was significantly down-regulated in the posterior half of the main body cells relative to that in the anterior half at stage 10B (Fig. 3 A′). Consistent with the *ttk* mutant phenotypes, C204/*ttk* RNAi caused extended Hnt expression and suppression of Cut expression after stage 10B in the posterior half of the main body cells (Fig. 3 A′′; and not depicted). In addition, these cells showed oscillating genomic DNA replication (Fig. 3 B), suggesting that specific knockdown of *ttk* after the M/E switch suffices to disrupt the E/A switch and supporting our conclusion that up-regulation of Ttk69 at stage 10B is required for the proper E/A switch and follicle cell differentiation.

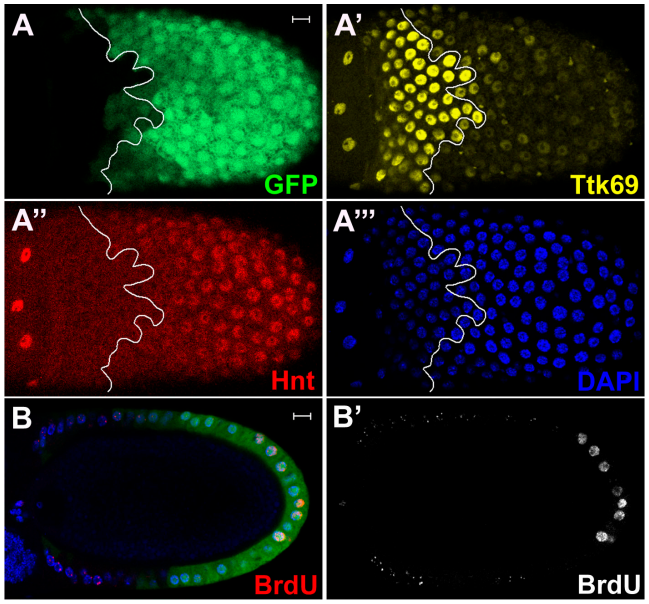

Figure 3. **Ttk function in late oogenesis is separable from its earlier function.** (A–A′′) A stage 10B egg chamber with overexpression of double-stranded RNA targeting *ttk* mRNA driven by C204 Gal4 driver. UAS-eGFP expression (A, green) indicated a C204 expression pattern, and Ttk69 antibody staining (A′, yellow) confirmed that Ttk69 was knocked down in the posterior half of the egg chamber with strong GFP expression. Hnt staining (A′′, red) revealed continuous expression in these follicle cells with low levels of Ttk69. White lines separate follicle cells with ttk^{RNAi} and wild-type cells. (B and B′) Oscillating genomic BrdU incorporation (B, red; B′, white) was found in follicle cells in the posterior half of a stage 10B egg chamber. Bars, 10 μm.

Premature up-regulation of Ttk69 results in precocious exit from the endocycle

To determine whether Ttk69 overexpression during midoogenesis could cause premature exit from the endocycle, we first compared the sizes of nuclei in follicle cells overexpressing Ttk69 and in adjacent wild-type cells and found no obvious difference until stage 8. At around stage 9, the nuclei of Ttk69-overexpressing follicle cells were slightly smaller (unpublished data); the difference was much more obvious at around stage 10B or later, when the wild-type cells had finished three rounds of the endocycle and had 16 copies of the genomic content (Fig. 4 A). We then performed the BrdU incorporation assay in Ttk69-overexpressing mosaic egg and control egg chambers containing GFP-overexpressing clones. Consistent with the nuclear size defect, Ttk69 overexpression significantly reduced genomic DNA replication; the replication ratio was 12-fold lower in clone areas of stage 9 egg

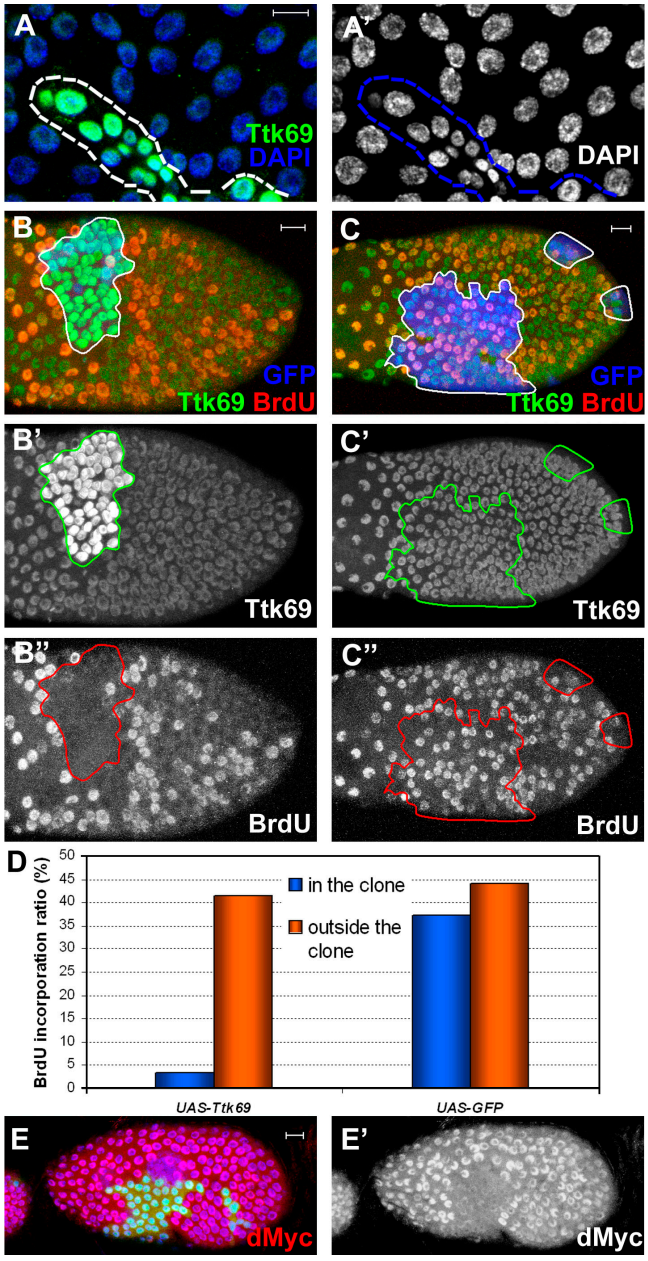

Figure 4. **Overexpression of Ttk69 causes premature exit from the endocycle.** B, C, and E are collapsed images of the z stacks. (A and A′) Cell nuclei (stained with DAPI) in Ttk69-overexpressing follicle cells (A, outlined and green) were much smaller than those in the wild-type cells in a stage 10B egg chamber. (B–B′′) A stage 9 egg chamber with Ttk69-overexpressing follicle cells marked by the presence of GFP (B, blue) and high levels of Ttk69 (B, green; B′, white). No or very little BrdU incorporation (B, red; B′′, white) was found in these follicle cells (outlined). (C–C′′) A control egg chamber with misexpression of GFP (C, blue) but not Ttk69 (C, green; C′, white). The GFP-positive cells (outlined) had normal BrdU incorporation (C, red; C′′, white) similar to that of the GFP-negative cells. (D) Quantitative analysis of the BrdU incorporation ratio in the clone area (blue) and outside of the clone (orange) in *UAS-Ttk69* and *UAS-GFP* egg chambers. In total, 235 Tt69-overexpressing and 703 wild-type follicle cells from three *UAS-Ttk69* egg chambers were counted. 113 GFP-positive and 236 GFP-negative follicle cells were counted from one *UAS-GFP* egg chamber (C). (E and E′) dMyc (E, red; E′, white) was down-regulated in Ttk69-misexpressing follicle cells in a stage 8 egg chamber. Bars, 10 µm.

chambers than elsewhere (Fig. 4, B′′ and D). In the control egg chamber, the GFP clone and areas outside did not differ significantly (Fig. 4, C′′ and D). Together, these data suggest that Ttk69 overexpression causes premature exit from the endocycle at around stage 9, but no precocious gene amplification was observed (Fig. 4 B′′). In addition, Ttk69-overexpressing cells showed premature down-regulation of dMyc (Fig. 4 E), a transcription factor required for the endocycle (Maines et al., 2004).

Extended Notch activity suppresses Ttk69 up-regulation

Because extended Notch activity and loss of *ttk* function produced similar defects in the E/A switch, we explored the relationship between Notch signaling and *ttk* in late oogenesis. Using polyclonal antibody against Ttk69, we found during late oogenesis that NICD-misexpressing follicle cells maintained low levels of Ttk69 protein, whereas their wild-type neighbors had elevated levels (Fig. 5 A). Extended Notch activity may therefore suppress Ttk69 up-regulation at stage 10B. In contrast, Notch activity, as revealed by the *mβ-CD2* reporter, was unaffected in *ttk* mutant follicle cells (Fig. 5 B; and not depicted).

Next, to determine whether extended Notch activity caused the E/A switch and cell differentiation defects by suppressing Ttk69 up-regulation, we co-overexpressed NICD and Ttk69 in follicle cells and found that one copy of *UAS-ttk69*, ttk^{1e11} could restore Ttk69 to wild-type or higher levels in the presence of NICD (Fig. 5, compare C′ with A). These egg chambers showed normal expression of Cut (compare Fig. 5 C′′′ with Fig. 1 I) and lack of Hnt expression after stage 10B (compare Fig. 5 D with Fig. 1 J). The BrdU incorporation assay revealed that gene amplification was present in some of the follicle cells with NICD/Ttk69 co-overexpression at stage 10B, although at a lower intensity than in the wild type (Fig. 5 E, red circles). The majority of the NICD/Ttk69 cells had neither genomic nor site-specific BrdU incorporation, similar to the follicle cells with Ttk69 overexpression alone (Fig. 5 E, white circles; and not depicted). The nuclei of the NICD/Ttk69 follicle cells were either smaller than or similar in size to those of the adjacent wild-type cells, unlike those produced by NICD overexpression alone (compare Fig. 5, C′′ and D with Fig. 1 B). Together, these results suggest that Ttk69 overexpression can partially reverse the E/A switch defects caused by extended Notch activity.

EcR activity is required for Ttk69 up-regulation and proper E/A switch

Although extended Notch activity sufficed to induce an extra round of endoreplication in follicle cells, premature down-regulation of Notch activity during the endocycle did not obviously shift the E/A transition to an earlier stage (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200802084/DC1), so we investigated other signaling pathways that might affect the E/A switch. Disruption of EcR activity by misexpression of a dominant-negative (DN) form of the EcR causes defects in chorion gene expression and reduces gene amplification (Hackney et al., 2007). Consistently, using an ecdysone sensor line *hs-Gal4-EcR, UAS-nlacZ* (Kozlova and Thummel, 2002), we found that the EcR pathway was activated in main body follicle cells at around

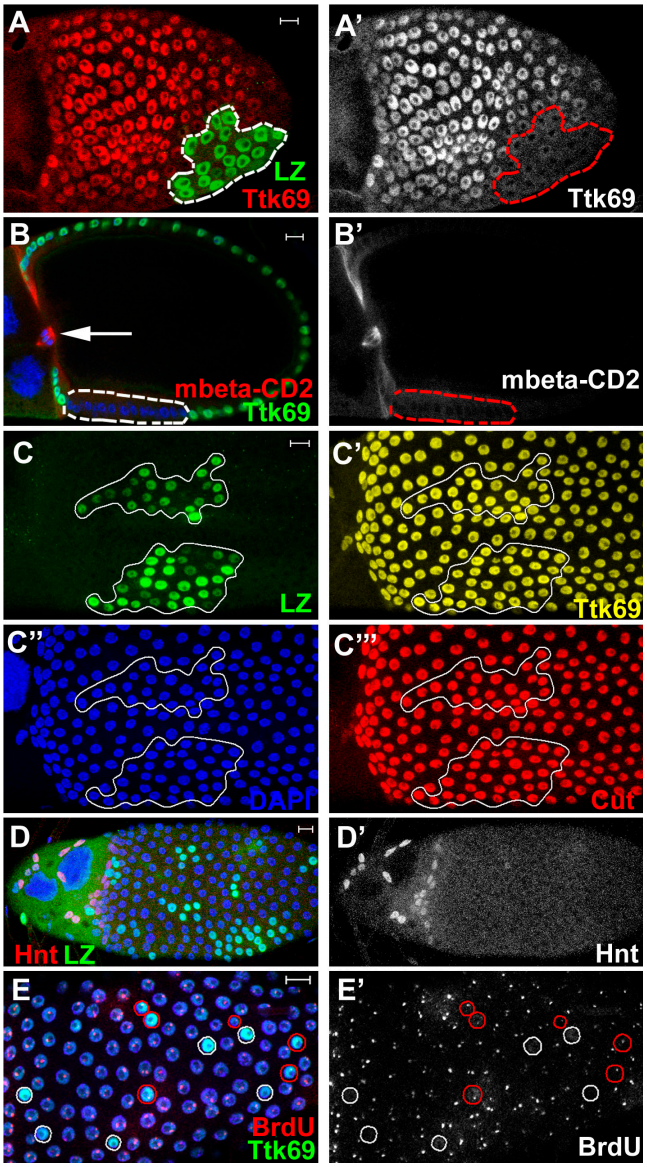

Figure 5. **The relationship between Notch signaling and Ttk.** Images in C–D′ are collapsed images of the z stacks. (A and A′) Ttk69 (A, red; A′, white) was down-regulated in follicle cells with NICD misexpression (outlined and marked by UAS-lacZ expression; A, green) in a stage 10B egg chamber. (B and B′) Notch activity (illustrated by *mβ-CD2* expression; B, red; B′, white) was not detected in *ttk* mutant clones (outlined) in a stage 10B egg chamber. The arrow points to the polar cell/border cell cluster with normal *mβ-CD2* expression. (C–C‴) A stage 11 egg chamber with co-overexpression of NICD (outlined and marked by UAS-lacZ expression; C, green) and Ttk69 (C′, yellow). Cut expression (C‴, red) was detected in these follicle cells. (D and D′) Hnt (D, red; D′, white) was not detected in main body follicle cells with co-overexpression of NICD and Ttk69 (marked by UAS-lacZ; D, green) in a stage 11 egg chamber. (E and E′) Site-specific BrdU incorporation (E, red; E′, white) was detected at a low level in some follicle cells with co-overexpression of NICD and Ttk69 (E, green; red circles). The majority of the follicle cells with NICD/Ttk69 co-overexpression (white circles) had no BrdU incorporation. Bars, 10 μm.

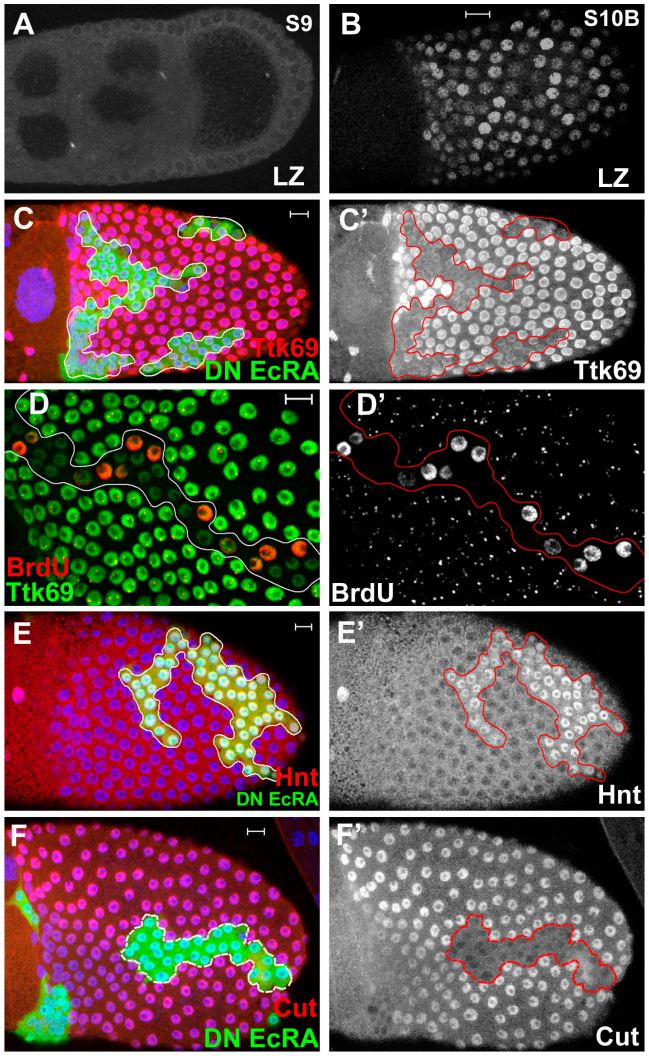

Figure 6. **EcR activity is required for the proper E/A switch and cell differentiation.** Images in C, E, and F are collapsed images of the z stacks. Follicle cells with DN EcR-A misexpression were marked by EcR-A staining (C, E, and F; green) or Ttk69 down-regulation (D, green). (A and B) The pattern of EcR activity illustrated by the *EcR-LBD-Gal4, UAS-nlacZ* system. EcR activity, marked by the LacZ (LZ) expression, was not observed in follicle cells before stage 10 (A), but it was strongly detected in follicle cells at stage 10B and later (B). (C and C′) Ttk69 (C, red; C′, white) was down-regulated in follicle cells with DN EcR-A (outlined) at a stage 10B egg chamber. (D and D′) Oscillating genomic BrdU incorporation (D, red; D′, white) was observed in DN EcR-A–misexpressing follicle cells (outlined) in a stage 10B egg chamber. (E and E′) Hnt (E, red; E′, white) was still detected in follicle cells with DN EcR-A (outlined) at stage 10B. (F and F′) Cut (F, red; F′, white) was not detected in follicle cells with DN EcR-A (outlined) in a stage 10B egg chamber. Bars, 10 μm.

stage 10B but not earlier (Fig. 6, A and B). β-Galactosidase antibody labeling of these egg chambers together with Cut or Ttk69 antibody revealed that EcR activation was concurrent with the up-regulation of Ttk69 and the E/A switch in follicle cells (unpublished data). To determine whether the two are related, we stained Ttk69 in follicle cells with misexpression of a DN form of the EcR-A isoform EcR-A (F645A) to suppress the EcR activity using flip-out Gal4. Ttk69 was kept at a low level in follicle cells with DN EcR-A misexpression when the adjacent wild-type cells already had high levels of Ttk69 expression at stage 10B (Fig. 6 C), suggesting that suppression of EcR activity is sufficient to block Ttk69 up-regulation at this stage.

Because Ttk69 up-regulation at stage 10B is crucial for the E/A switch, we asked whether disruption of EcR signaling keeps follicle cells in the endocycle during late oogenesis.

EcR-A (F645)–misexpressing follicle cells showed oscillating patterns of genomic DNA replication after stage 10B, whereas the adjacent wild-type cells had punctate BrdU incorporation (Fig. 6 D). The genomic BrdU incorporation pattern was also detected in follicle cells misexpressing other forms of DN EcR, such as EcR-A (W650A), EcR-B2 (F645A), and EcR-B2 (W650A). In addition, follicle cells with DN EcR had diffused localization of ORC2 and oscillating expression of dE2F1 at stage 10B (not depicted) like the NICD-overexpressing follicle cells (Fig. 1, C and F). Suppression of EcR activity by DN EcR was also sufficient to extend Hnt expression and suppress late Cut expression after stage 10B (Fig. 6, E and F), similar to the defects caused by removal of *ttk* function or extension of the Notch activity. To determine whether EcR signaling acts through Ttk69 to regulate the E/A switch and follicle cell differentiation, we examined BrdU incorporation and Hnt and Cut expression in late follicle cells with coexpression of DN EcR-A (F645A) and Ttk69. These cells had almost normal levels of Cut expression and no Hnt expression at stage 10B or later (Fig. S3, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200802084/DC1), like those with NICD and Ttk69 coexpression. Most DN ECR/Ttk69 cells had no detectable BrdU incorporation, but a few had low levels of chorion gene amplification (Fig. S3 C), suggesting that Ttk69 alleviates the cell differentiation and gene amplification defects caused by DN EcR expression.

Extended Notch activity antagonizes EcR activity during the E/A switch

Because both Notch and ecdysone signaling are involved in regulating Ttk69 expression, we investigated the relationship between these two pathways during the E/A switch. First, we found that in DN EcR–misexpressing follicle cells, Notch signaling was properly down-regulated at stage 10B, as revealed by the Notch reporter *m7-lacZ* in these cells (Fig. 7 A). Second, by overexpressing NICD with the *hsp70* promoter, we found that ectopic NICD suppressed activation of EcR signaling, whereas the control egg chambers without NICD misexpression had normal reporter expression at stage 10B (Fig. 7, B and C).

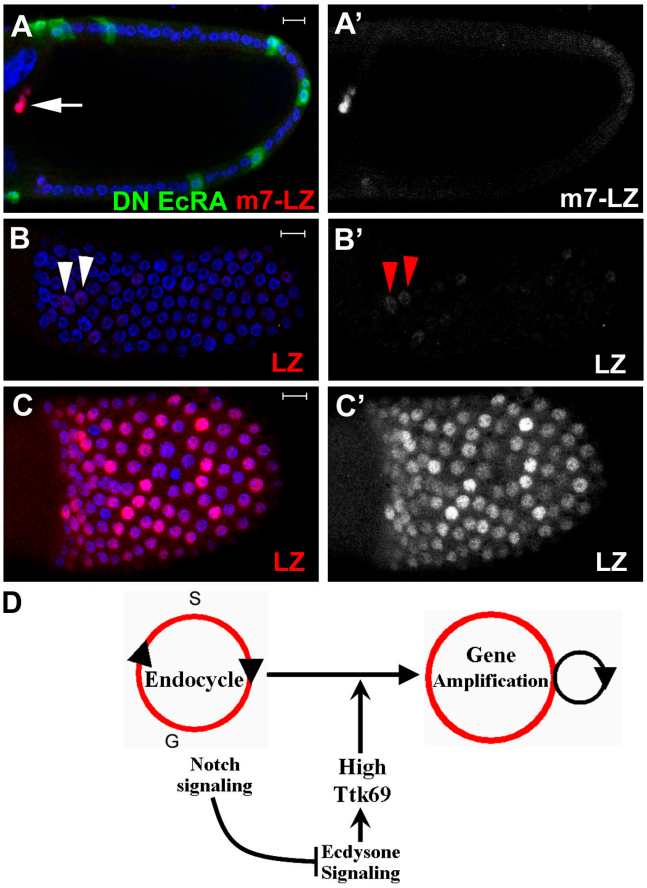

Figure 7. **Notch signaling antagonizes EcR activity during the E/A switch.** (A and A′) Notch activity (marked by *m7-lacZ* expression; A, red; A′, white) was not detected in follicle cells with DN EcR-A (A, green) in a stage 10B egg chamber or in the adjacent wild type. The arrow points to the polar cells with *m7-lacZ* expression. (B and B′) EcR activity (illustrated by the *LBD-Gal4, UAS-nlacZ* expression; B, red; B′, white) was dramatically suppressed in follicle cells with NICD misexpression driven by the *hsp70* promoter in a stage 10B egg chamber. The arrowheads point to follicle cells with weak EcR activity. (C and C′) A control egg chamber without NICD misexpression during the same stage had high levels of EcR activity (C, red; C′, white) in follicle cells. Red channels in B and C had the same settings when the images were acquired. (D) A schematic drawing of the involvement of Notch and ecdysone signaling and high levels of Ttk69 in the E/A switch. Bars, 10 μm.

Discussion

In *Drosophila* follicle cells, cell cycle program switches and DNA replication patterns are tightly associated with different developmental stages of oogenesis. Here, we report that inactivation of the Notch signaling pathway in late oogenesis is important for cell differentiation and cell cycle regulation. Down-regulation of Notch is required for main body follicle cells to switch from genomic DNA replication in the endocycle stage to site-specific DNA replication in the gene amplification stage. The developmental pathway involved in this E/A switch also includes the activation of ecdysone signaling and up-regulation of a zinc-finger transcriptional repressor, Ttk. On the basis of our current findings, we propose the following model to explain the developmental regulation of the E/A switch in follicle cells (Fig. 7 D). At around stage 10, main body cells down-regulate Notch signaling as the prerequisite for activation of ecdysone signaling, which promotes the up-regulation of Ttk69. High levels of Ttk69 stop endoreplication and allow the cells to enter the synchronous gene amplification stage. Our data suggest that the Notch and EcR pathways have opposite function in the regulation of Ttk69 expression and the E/A switch. Their interaction is probably indirect because Notch signaling is usually involved in activation of gene expression.

Our results did not reveal a developmental signal sufficient to trigger the E/A switch. Although extended Notch activity sufficed to drive an extra round of endocycle, premature down-regulation of Notch activity by an N^{ts} allele caused neither premature exit from the endocycle (Fig. S4 B), probably because it was insufficient for premature up-regulation of Ttk69 (Fig. S4 D), nor premature entry into the gene amplification stage (Fig. S4 C). Down-regulation of Notch therefore provides only a permissive signal for the E/A switch. Our data also indicate that EcR signaling is required for the E/A switch. Is ectopic activation of

EcR in follicle cells sufficient to drive premature E/A switch? Treating egg chambers with ponasterone A (PonA) can activate the EcR sensor in follicle cells before stage 10 (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200802084/DC1; Hackney et al., 2007), but it did not cause any up-regulation of Ttk69 or defects in BrdU incorporation (Fig. S5 A′; and not depicted). Notch activity in PonA-treated follicle cells was normal during midoogenesis (Fig. S5 A′′′), so ectopic EcR activity could not interfere with Notch signaling. The combination of Notch down-regulation and EcR activation in midoogenesis did not result in premature E/A transition either (Fig. S5 B). Although ectopic EcR activity may therefore not suffice to induce premature E/A switch, we could not rule out that 5-h incubation is not long enough for EcR to up-regulate Ttk69 indirectly. In addition, the sensor line might be more sensitive to PonA treatment, whereas the target gene expression requires higher levels of EcR activity. Unfortunately, no constitutively active EcR transgenes are currently available. In summary, these analyses have not ruled out that EcR is the inductive signal. Alternatively, the E/A switch may require involvement of other developmental signals.

Although we proposed a model that involves Notch, EcR, and Ttk in regulating the E/A switch, the relationships among Notch, ECR, Ttk69, and a Notch target, Hnt, are complicated. Extended Notch signaling suppresses Ttk69 up-regulation. Loss of *ttk* function does not affect Notch activity but causes continuous expression of Hnt, whereas misexpression of Ttk69 suffices to down-regulate Hnt expression early (unpublished data). Similarly, DN EcR misexpression affects Hnt expression but not Notch activity reporters, suggesting that EcR regulates Hnt through Ttk69. Indeed, coexpression of DN EcR and Ttk69 restored almost normal expression patterns of Hnt and Cut in late follicle cells (Fig. S3, A and B). These data also suggest that Hnt is probably not a direct target of the NICD–Su(H) activator complex and that Ttk69 intersects with Notch signaling downstream of Su(H) but upstream of Hnt. Our previous study showed that *hnt* mutant cells had only one extra round of mitotic cycle (Sun and Deng, 2007) and that Hnt misexpression had no obvious defects in the E/A switch. Notch may therefore have additional targets to maintain the endocycle in follicle cells.

Notch signaling has been implicated in a wide range of biological and pathological processes (Bolos et al., 2007) and has prompted much research into the biological consequences of its activation. Notch activation at stages 6 and 7 is necessary for follicle cells to enter the endocycle (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). Our current findings indicate that its down-regulation is also a critical step for them to continue to differentiate and exit the endocycle. Both up- and down-regulation of Notch may therefore play important roles in temporal regulation of follicle cell differentiation and cell cycle programs. In accordance with the temporal pattern of Notch activity in follicle cells, Delta (Dl), the ligand for Notch signaling in follicle cells, was gradually down-regulated from its peak level at stages 7 and 8, especially in the oocyte (Fig. S1, D and E; Bender et al., 1993). By stage 10, no Dl expression was detected in oocyte membranes adjacent to main body follicle cells (Fig. S1 F), so Dl down-regulation is probably the cause of the down-regulation of Notch activity during late oogenesis. An interesting exception is the centripetal cells, which are located anterior to the main body cells and migrate centripetally to separate the nurse cells and the oocyte at stage 10B. In these cells, Notch activity is still detected after stage 10B (Dobens et al., 2005), but how it is maintained remains unclear. The centripetal cells do enter gene amplification, but not at the same time as main body cells; we often observe genomic DNA replication in centripetal cells at stage 10B when main body cells have obvious gene amplification. Other developmental signaling pathways are also known to be active in these cells, and they could regulate the expression of cell cycle machinery genes that are responsible for gene amplification in the presence of Notch activity.

Use of a tissue-specific Gal4 (*slbo<Gal4*) has been reported to reduce amplification in follicle cells expressing DN EcR (Hackney et al., 2007), in contrast to our finding that the DN EcR cells stay in the endocycle. These discrepancies could be caused by the different Gal4 lines used. The expression of *slbo<Gal4* is probably too low to significantly block EcR activity in main body cells. Several lines of evidence suggest that EcR regulates the E/A switch at the transcriptional level. First, expression levels of Ttk69 are reduced by the DN form of EcR, which does not block EcR binding to DNA but does block transcriptional activation (Cherbas et al., 2003). Second, a transcriptional target of EcR during metamorphosis, the broad complex, also confers a fragile eggshell phenotype when mutated (Tzolovsky et al., 1999).

Ttk was first isolated as a transcriptional repressor of the *fushi tarazu* segmentation gene (Harrison and Travers, 1990) and is also an inhibitor of the neuronal fate of cells such as photoreceptors (Xiong and Montell, 1993). In the peripheral nervous system, Ttk is positively regulated by Notch signaling to fulfill the suppression of the neuronal fate (Guo et al., 1996). It is generally expressed in follicle cells throughout oogenesis, but its expression is not regulated by Notch during the M/E switch (Sun and Deng, 2007). The involvement of Ttk in late follicle cells appears to be negatively regulated by Notch because up-regulation of Ttk69 at stage 10B was completely blocked by extended Notch activity (Fig. 5 A). Notch most likely blocks Ttk69 up-regulation by suppressing EcR activity. How exactly does the Notch–EcR–*ttk* pathway regulate endocycle exit as well as gene amplification? It probably acts by regulating dE2F1 and CycE/Cdk2 activity, as follicle cells with extended Notch activity or disruption of EcR or *ttk* function retained oscillating patterns of dE2F1 and MPM2 staining at stage 10B (Fig. 1 F; Fig. 2, E and G; Fig. S2 C; and not depicted). Some NICD cells had levels of MPM2 signal higher than the uniform level in the wild-type cells undergoing gene amplification (Fig. 2 G and Fig. S2 C), indicating higher Cdk2 activity in those cells. To initiate genomic DNA replication, levels higher than the uniform levels of CycE/Cdk2 activity in late follicle cells are probably required. Ttk up-regulation during the E/A switch is probably important for lowering the CycE/CDK2 activity to a level too low to initiate an additional round of genomic replication yet sufficient to allow the amplification of specific loci. Consistently, *ttk* can suppress CycE expression in *Drosophila* glial cells and bristle cell lineage and thus exit from the cell cycle (Badenhorst, 2001; Audibert et al., 2005). Similarly, other cell cycle–related genes such as *dacapo* (CDK2 inhibitor; Lane et al., 1996), *dmyc* (Maines et al., 2004), and *dmyb*

(Beall et al., 2002) could be targets of *ttk*. Indeed, extended Notch activity, which suppresses Ttk69 up-regulation, suffices to prolong the expression of dMyc after stage 10B (Fig. S2 D), whereas misexpression of Ttk69 suffices to down-regulate dMyc expression precociously (Fig. 4, E and E′). Determining whether any of these genes is a direct target of Ttk69 will be interesting.

We also show that an increase in Ttk expression suffices to induce precocious exit from the endocycle, but the cells did not then go into gene amplification, perhaps because the Gal4-UAS–induced expression of Ttk69 is much higher than the endogenous level of Ttk69 during late oogenesis. Because different levels of Ttk69 have profound effects on DNA replication patterns in follicle cells, these much higher levels could have eliminated all DNA replication in follicle cells. The ability of different expression levels of transcription factors to induce a different cellular differentiation status is well documented in early *Drosophila* development. Gradients of morphogens, mostly transcription factors, are the major source of segmentation and pattern formation in embryos. Follicle cells also adopt this rule to allow different cellular functions of Ttk at different oogenesis stages. Low levels of Ttk69 contribute to stage 6 entry into the endocycle, whereas high levels of Ttk69 permit exit from the endocycle and further differentiation at stage 10. Notch signaling, probably in concert with other signals, ensures the proper timing of up-regulation of Ttk69 and thus proper egg chamber development.

Materials and methods

Genetic strains

N^{ts} allele was obtained from the Bloomington Stock Center (BSC) and recombined to the *FRT18D* chromosome. *FRT82B* ttk^{1e11} and *UAS-Ttk69,* ttk^{1e11} were obtained from C. Berg (University of Washington, Seattle, WA; French et al., 2003). *UAS-*N^{intra} (BSC) was used to express the constitutively active form of Notch, and *hs-*N^{intra}*,* ry^{506} was provided by G. Struhl (Columbia University, New York, NY). *UAS–EcR-A (F645A), UAS–EcR-A (W650A), UAS–EcR-B2 (F645A), UAS–EcR-B2 (W650A), tubP-Gal80*^{ts}, and C204 (Manseau et al., 1997) were obtained from the BSC. ttk^{dsRNA} was obtained from the Vienna Drosophila RNAi Center. Ecdysone sensor *hs-Gal4-*EcR^{LBD}*, UAS-*$nlacZ^{7.4}$ was provided by C.S. Thummel (University of Utah, Salt Lake City, UT; Kozlova and Thummel, 2002). We used three Notch activity reporters: *Gbe-Su(H)m8-lacZ* (Furriols and Bray, 2001), *E(Spl)mβ-CD2* (de Celis et al., 1998), and *E(spl)m7-lacZ* (Assa-Kunik et al., 2007). The following marker lines were used: *yw, hsFLP; act<CD2<Gal4, UAS-GFP/TM3; yw, hsFLP; act<y+<Gal4, UAS-nlacZ/Cyo; yw, hsFLP; FRT82B ubiGFP/TM3; and hGFP FRT18D/FM7; MKRS, hsFLP/TM6B.*

Generation of mosaics

Flies were raised under standard conditions at 25°C except for N^{ts} flies, which were raised at 18°C. Mitotic clones were generated by FLP/FRT-mediated recombination. Adult female flies were heat shocked for 1 h at 37°C and incubated at 25°C for 3–4 d for generation of loss-of-function follicle cell clones. For generation of flip-out clones, adult females were heat shocked for 45 min at 37°C and incubated at 25°C for 2–3 d before dissection. For ectopic expression of ttk^{dsRNA} driven by C204, we raised adult females at 29°C after hatching to remove Gal80^{ts} function. For examination of EcR activity, flies were heat shocked for 45 min at 37°C and allowed to recover at 29°C for 16 h before dissection.

To bypass the early requirement for Notch in follicle cells, we used the N^{ts} mutant to induce follicle cell clones but grew the flies at the permissive temperature (18°C) for 4–7 d and shifted to the restrictive temperature (30°C) for 18 h to remove the Notch function sufficiently from the mutant clone cells. Notch activity reporter E(spl)mβ-CD2 and Cut staining were used to monitor the Notch activity in these experiments (Fig. S5, B and D).

Mosaic screen

A small-scale mosaic screen was performed on the 3R collection of the FRT-SXXXXX stocks from the Szeged Stock Center (Bellotto et al., 2002). Male flies from individual stocks were crossed with female virgins of the *hsFLP, 82BGFP/TM3* stock. Generation of mosaics followed the protocol described in the previous section. Anti-Cut antibodies were used to stain the ovaries for late Cut expression analysis. Target genes were identified according to the inverse PCR protocol.

Immunohistochemistry, BrdU labeling, and imaging

Immunohistochemistry and BrdU labeling were performed as previously described (Sun and Deng, 2005) with the following antibodies: rabbit anti-Ttk69 and rat anti-Ttk69 (1:200; provided by P. Badenhorst, University of Birmingham, Edgbaston, Birmingham, UK), guinea pig anti-dE2F1 (1:500; provided by T. Orr-Weaver, Whitehead Institute, Cambridge, MA), rabbit anti-dE2F2 (provided by N. Dyson, Harvard Medical School, Boston, MA), rabbit anti-ORC2 (1:3,000; provided by S.P. Bell, Whitehead Institute, Cambridge, MA), rabbit anti-Cut (1:500; provided by Y.N. Jan, University of California, San Francisco, CA), mouse anti-Cut (2B10; 1:15), anti-Dl (C594.9B; 1:15), anti–EcR-A (15G1a; 1:50), anti-Hnt (1G9; 1:15), anti–cyclin B (F2F4; 1:50; Developmental Studies Hybridoma Bank), mouse anti-BrdU (1:50; BD Biosciences), mouse anti-CD2 (1:50; AbD Serotec), mouse anti-Ser/Thr-ProMPM-2 (1:1,000; Millipore), rabbit anti–β-galactosidase (1:5,000; Sigma-Aldrich), and rabbit anti–phosphohistone 3 (1:200; Millipore). Images were acquired with a confocal microscope (LSM510; Carl Zeiss, Inc.) with a Plan Neofluar 40× 1.3 NA lens, a camera (Hamamatsu Photonics), and a photo multiplier tube at 72°F assembled with Photoshop (7.0.1; Adobe).

Fluorescence-activated cell-sorting analysis

Follicle cell isolation was conducted as described previously (Bryant et al., 1999). Ovaries of 80–100 females per experiment were dissected in Grace's insect medium supplemented with 10% fetal calf serum and 1× antibiotic antimycotic. Ovaries were washed three times in calcium-free phosphate-buffered saline and incubated in 0.7 ml of 0.25% trypsin with intermittent vortexing at room temperature for 15 min. Supernatant was passed through a 40-µm nylon filter into 1 ml of Grace's medium and pelleted at 4,000 rpm for 7 min in an Eppendorf Minispin. Trypsinization and filtration steps were repeated two to three additional times or until the supernatant became clear. Follicle cells were then resuspended in 0.5 ml of Grace's medium containing 1 µl of 5 mM Vybrant DyeCycle DNA-specific stain, incubated at room temperature for 30 min, washed once in calcium-free phosphate-buffered saline, and stored on ice. A flow cytometer (FACSAria; Becton Dickinson) determined follicle cell ploidy by fluorescence-activated cell-sorting analysis of Vybrant DyeCycle–stained cell preparations with excitation at 407 nm for Vybrant DyeCycle stain and at 488 nm for GFP.

In vitro culture of egg chambers and PonA treatment

Egg chambers were dissected from the abdomen region and cultured in Grace's medium with 1× antibiotic antimycotic (Invitrogen) and physiological levels of an agonist of ecdysone signaling (10^{-6} M PonA) to activate ecdysone signaling at room temperature or in the 29°C incubator for the N^{ts} clones. After a 5-h incubation, egg chambers were fixed and stained with the correct antibodies according to the standard staining procedure (Sun and Deng, 2005).

Online supplemental material

Fig. S1 shows that down-regulation of Notch activity is concurrent with the E/A switch. Fig. S2 shows that MPM2 and dMyc, but not dE2F2, are affected by ectopic Notch activity. Fig. S3 that shows overexpression of Ttk69 alleviates the defects caused by DN EcR-A. Fig. S4 shows that premature down-regulation of Notch activity is not sufficient to induce a premature E/A switch. Fig. S5 shows that EcR activity alone or with down-regulation of Notch activity is not sufficient to up-regulate Ttk69 precociously. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200802084/DC1.

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