The transcriptional corepressor SMRTER influences both Notch and ecdysone signaling during *Drosophila* development

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Summary

SMRTER (SMRT-related and ecdysone receptor interacting factor) is the *Drosophila* homologue of the vertebrate proteins SMRT and N-CoR, and forms with them a well-conserved family of transcriptional corepressors. Molecular characterization of SMRT-family proteins in cultured cells has implicated them in a wide range of transcriptional regulatory pathways. However, little is currently known about how this conserved class of transcriptional corepressors regulates the development of particular tissues via specific pathways. In this study, through our characterization of multiple *Smrter* (*Smr*) mutant lines, mosaic analysis of a loss-of-function *Smr* allele, and studies of two independent *Smr* RNAi fly lines, we report that SMRTER is required for the development of both ovarian follicle cells and the wing. In these two tissues, SMRTER inhibits not only the ecdysone pathway, but also the Notch pathway. We differentiate SMRTER's influence on these two signaling pathways by showing that SMRTER inhibits the Notch pathway, but not the ecdysone pathway, in a spatiotemporally restricted manner. We further confirm the likely involvement of SMRTER in the Notch pathway by demonstrating a direct interaction between SMRTER and Suppressor of Hairless [Su(H)], a DNA-binding transcription factor pivotal in the Notch pathway, and the colocalization of both proteins at many chromosomal regions in salivary glands. Based on our results, we propose that SMRTER regulates the Notch pathway through its association with Su(H), and that overcoming a SMRTER-mediated transcriptional repression barrier may represent a key mechanism used by the Notch pathway to control the precise timing of events and the formation of sharp boundaries between cells in multiple tissues during development.

Key words: SMRTER, Notch, Su(H), Ecdysone, EcR, Oogenesis

Introduction

The SMRT (Silencing Mediator of Retinoic and Thyroid hormone receptors) family of proteins is a well-conserved group of transcriptional corepressors that includes vertebrate SMRT (Chen and Evans, 1995) and N-CoR (Nuclear Hormone Receptor Co-Repressor) (Horlein et al., 1995), as well as *Drosophila* SMRTER (SMRT-related and Ecdysone Receptor-interacting factor) (Tsai et al., 1999). Vertebrate SMRT and N-CoR were first discovered through their associations with members of the nuclear hormone receptor superfamily (Jepsen and Rosenfeld, 2002; Lazar, 2003; Privalsky, 2004; Tsai and Fondell, 2004), which control a wide spectrum of biological processes, including reproductive organ development, metabolism, and neurogenesis (McKenna and O'Malley, 2002). At the molecular level, SMRT and N-CoR bind nuclear receptors in the absence of ligand. When ligand is present, ligand-bound nuclear receptors change their protein configuration, which leads to the release of SMRT and N-CoR and the recruitment of coactivators. These coupled events enable ligand-regulated nuclear receptors to convert from repressors to activators (Perissi et al., 2004; Perissi et al., 2008). Because SMRT and N-CoR interact with additional transcriptional cofactors and chromatin modifying factors, including Sin3A (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997), transducin beta-like 1X-linked proteins (TBL1/TLBR1) (Guenther et al., 2000; Li et al., 2000; Zhang et al., 2002; Yoon et al., 2003), and various HDACs (histone deacetylases) (Guenther et al., 2000; Huang et al., 2000; Kao et al., 2000; Li et al., 2000), these lines of evidence indicate that SMRT and N-CoR constitute a crucial part of the large multi-subunit transcriptional corepressor complexes that allow nuclear receptors to repress gene transcription.

In many respects, SMRTER behaves like its vertebrate counterparts (Tsai et al., 1999). It binds the ecdysone receptor (EcR), a member of the nuclear receptor superfamily (Koelle et al., 1991), in the absence of the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone). Moreover, SMRTER has been found to directly bind the fly homolog of Sin3A (Tsai et al., 1999) and the fly homolog of TBL1 (called Ebi) (Tsuda et al., 2002), and to form protein complexes with the fly HDAC (Pile and Wassarman, 2000; Pile et al., 2002). These results make it apparent that *Drosophila* SMRTER represents not only a structural, but also a functional homolog of SMRT and

N-CoR. Therefore, insights gained from studies of the *in vivo* properties of SMRTER may apply to SMRT and N-CoR in vertebrates as well.

Mounting evidence indicates that the functions of the SMRT-family proteins are not limited to nuclear receptor regulatory pathways. Since their discovery, SMRT and N-CoR have also been found to interact with myriad other DNA-binding transcription factors in mammalian cells, including CBF1 (C Promoter-binding Factor 1, also referred to as RBP-Jκ), PLZF (promyelocytic leukemia zinc finger protein), BCL6 (B-cell lymphoma 6), and MeCP2 (methyl-CpG binding protein 2), and with cofactors such as ETO/MTG8 (myeloid translocation gene 8), SKIP (Ski-interacting protein), SPEN (Split-ends)/SHARP, and ATXN1 (ataxin-1) and the closely related BOAT1 (Brother of ataxin-1) (Hong et al., 1997; Dhordain et al., 1998; Wang et al., 1998; Zhou et al., 2000; Ariyoshi and Schwabe, 2003; Stancheva et al., 2003; Tsai et al., 2004; Mizutani et al., 2005). Many of these factors are dedicated transcriptional repressors or corepressors implicated in various human disorders, including leukemia, lymphoma and neurodegeneration. Intriguingly, several of these SMRT/N-CoR associating factors are also functionally connected with the Notch pathway (Kao et al., 1998; Doroquez et al., 2007; Salat et al., 2008; Tong et al., 2011), raising the possibility that SMRT, N-CoR and some of their associating factors may converge in regulating the Notch pathway.

The Notch signaling pathway is well-conserved in metazoans, and is fundamental for pattern formation, cell fate specification and cell growth in multiple vertebrate and invertebrate tissues (Artavanis-Tsakonas et al., 1999; Lai, 2004; Schweisguth, 2004; Bray, 2006; Fortini, 2009; Kopan and Ilagan, 2009). Notch is a type I receptor that is expressed on the surface of the responding cells. Once it binds its ligands (Delta or Jagged/Serrated), which are expressed on the surface of neighboring signal-sending cells, Notch is activated in a juxtacrine manner. Activation of Notch triggers two protein cleavage events in Notch, leading to the generation of a truncated form of Notch that corresponds to its intracellular domain (NICD) (Struhl and Adachi, 1998; De Strooper et al., 1999; Struhl and Greenwald, 1999). Once NICD is generated, it is translocated from the membrane to the nucleus where it binds the DNA-binding transcription factor CSL (CBF1 in vertebrates; Suppressor of Hairless (Su(H)) in fly; and Lin12 and Glp-1 (LAG1) in worm). The association of CSL with NICD induces the former to release its transcriptional repressors and, concomitantly, to recruit transcriptional activators, such as Mastermind. Thus, NICD's operation in the Notch regulatory pathway is in many ways similar to the way steroid or thyroid hormones act in the nuclear receptor regulatory pathways: both cause their corresponding transcription factors (CSL and nuclear receptors) to be dissociated from their transcriptional corepressors.

Vertebrate CSL, known as CBF1, has been reported to interact directly or indirectly with various transcriptional corepressors, including SMRT/N-CoR, SPEN/SHARP, SKIP, CtBP (C-terminal interacting protein), CtIP (CtBP interacting protein), CIR (CBF1 interacting co-repressor), HDACs, and KyoT2 (Kao et al., 1998; Taniguchi et al., 1998; Zhou et al., 2000; Zhou and Hayward, 2001; Oswald et al., 2002; Oswald et al., 2005; Ehebauer et al., 2006). In addition, our lab has recently identified ATXN1 and the related BOAT1 as a new class of CBF1 corepressors (Tong et al., 2011). Based on these findings, CBF1 likely requires inputs from multiple transcriptional corepressors in tandem to repress gene transcription. So far, the developmental circumstances under which various transcriptional cofactors operate to influence the properties of CBF1 are largely unknown. Efforts to address these issues by means of *in vivo* studies using knockout mouse for the *Smr* gene or the *N-CoR* gene have been hampered by the fact that such mice are embryonic lethal (Jepsen et al., 2000; Jepsen et al., 2007), and that no conditional knockout line for either gene has yet been generated. Because SMRTER is the fly homolog of SMRT and N-CoR, the *Drosophila* system offers an opportunity to study how this important class of transcriptional corepressors controls the development of specific tissues by influencing the Notch and other signaling pathways.

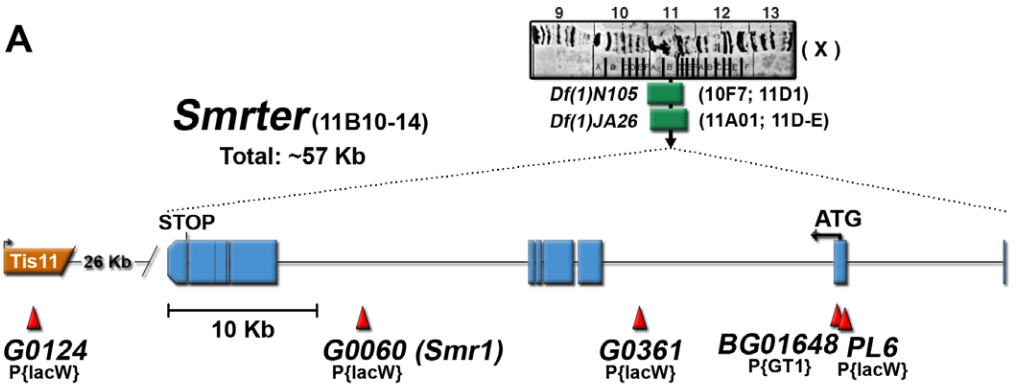
In our study, we have made use of multiple *Smrter (Smr)* mutant and RNA interference (RNAi) fly lines to investigate the functional properties of the *Smr* gene. Our studies led us to the discovery that SMRTER exerts an inhibitory effect not only on the ecdysone pathway, but also on the Notch pathway in both the ovarian follicle cells and the wing. In these two tissues, SMRTER appears to influence the transcriptional outputs of the ecdysone and Notch pathways in different manners. Our characterization of the involvement of SMRTER in both the Notch and ecdysone pathways in *Drosophila* suggests that SMRT-family proteins are employed repeatedly, but in diverse ways, by different transcription factors to regulate multiple biological processes throughout development in both vertebrates and invertebrates.

Results

The characterization of five *Smr* alleles

Smr, which codes for an unusually large protein of 3604 amino acids, is mapped to the 11B10-14 region on the X-chromosome. It is deleted from two deficiency lines, *Df(1)N105* and *Df(1)JA26* (Fig. 1A). A large number of transposable element insertion lines mapped to the *Smr* locus have been generated and are available from various stock centers. So far, however, a systematic characterization of these *Smr* fly lines has not been carried out. In this report, we used four fly lines (*BG01648*, *G0361, G0060* and *G0124*) obtained from the Bloomington stock center and one line (*PL6*) from Alain Vincent's group at CNRS/UPS (Bourbon et al., 2002) to characterize the functional properties of the *Smr* gene. Among these five putative *Smr* mutant lines, *BG01648* is a *p{GT1}-Gal4* trap line, and the rest are *p{lacW}-lacZ* trap lines. We performed inverse PCR to validate the accuracy of the mapped insertion sites. While our mapping results for *BG01648*, *G0361,* and *G0124* lines agreed with the reported information from the Flybase, we found that *G0060* was incorrectly annotated to another gene located on 5B4-B6. To confirm our results for *G0060*, we requested that the line be sequenced independently by Duke University Model System Genomics, whose result concurred with ours. The corrected insertion site for *G0060*, along with the confirmed insertion sites for the other lines, is shown in Fig. 1A. Interestingly, whereas the P-elements of four of the lines are inserted within the predicted *Smr* gene locus, the P-element of *G0124* is inserted into another gene locus, namely *Tis11*, which is immediately downstream of the *Smr* gene (Fig. 1A). Therefore, the *Smr* gene locus may include a further extended region in its 3′ end. Alternatively, *G0124* may affect the functions of both the *Smr* and the *Tis11* genes.

Our characterization of *PL6, G0361*, *G0060*, and *G0124* indicates that they cause lethality to both homozygous females



B

♂ \ ♀	BG01648/FM7	PL6/FM7	G0060/FM7	G0124/FM7	G0361/FM7
BG01648/Y	15.6%, n=128	0%	0%	0%	0%
PL6/Y-BSC5	5%, n=101	0%	0%	0%	0%
G0060/Y-BSC5	0%	0%	0%	0%	0%
G0124/Y-BSC5	0%	0%	0%	0%	0%

Note: *G0361/Y-BSC5* is not viable

Fig. 1. Characterization of five *Smr* mutant lines. (A) The gene structure of the *Smr* locus, showing insertion sites for five tested fly lines. Blue rectangles stand for exons; straight lines represent introns; red triangles mark the sites where the P-elements are inserted. *PL6* and *BG01648* are inserted within the 2nd exon and are located immediately upstream and downstream of the predicted translational start site of *Smr*, respectively; *G0361* is inserted within the 2nd intron; *G0060* (referred to as *Smr1* in this study) is inserted within the 6th intron; *G0124* is inserted within the *Tis11* gene locus downstream of *Smr*. **(B)** Complementation experiments for the tested five *Smr* fly lines. *BG01648* is a semi-lethal allele, while the rest are lethal alleles. Except *G0361*, the lethality in tested male flies can be rescued by *Y-BSC5*, a compound Y chromosome containing a fragment from the X-chromosome that harbors the *Smr* gene. In these complementation experiments, only a small number of homozygous *BG01648* females and heterozygous *PL6/BG01648* females derived from *BG01648/FM7* females are viable. All other combinations result in lethality. *n*=the number of females scored for their survival; *n*>100 for all combinations.

and hemizygous males. In contrast, *BG01648* is a semi-lethal allele. Lethality in *PL6, G0060*, and *G0124* males can be fully rescued by the presence of a compound Y chromosome, *Dp(1;Y)BSC5* (hereafter called *Y-BSC5*), which contains a fragment from the X-chromosome harboring the *Smr* gene locus. These male offspring also display no obvious defects. *Y-BSC5* cannot rescue the lethality in *G0361*, however, suggesting that this line may contain other lethal mutations in addition to *Smr*. We used *Y-BSC5*-rescued males and *BG01648* males, along with *FM7*-balanced females for all five lines, to carry out the complementation experiments shown in Fig. 1B. By scoring the female progenies ($n>100$) from these different genetic crosses, we found that, except for a few surviving homozygous *BG01648* (15.6% of total females scored; $n=128$) and trans-heterozygous *BG01648/PL6* females (5%, $n=101$; from the cross using *BG01648/FM7* females), all other combinations resulted in lethality. Based on this outcome, we conclude that the five tested fly lines all belong to the same complementation group. Judging by the survival rate of each tested *Smr* line and the phenotypes of the surviving females, we determined the following order of severity for these *Smr* alleles, from weak to strong: *BG01648*<*PL6*<*G0060*, *G0124*.

As noted above, *BG01648* is a semi-lethal allele, based on the observation that only ~15.6% *BG01648* homozygous females could be found in the *BG01648/FM7* stock. Examining the surviving homozygous *BG01648* females revealed that they show the following defects: (1) distorted wings and legs (~19%, $n=108$) (Fig. 2B); (2) ectopic bristle formation in the scutellum (~92%, $n=137$); and (3) reduced fecundity (Fig. 2D). These different defects indicate that SMRTER is required for the development of multiple tissues. Intriguingly, *BG01648/Y* males are viable, fertile, and show no pronounced defects like their homozygous female counterparts, indicating that the sexes respond to *BG01648* mutation differently.

SMRTER is required for the development of multiple tissues

Like homozygous *BG01648* females, trans-heterozygous *BG01648/PL6* females also display egg-laying defects (Fig. 2D) and wing and leg abnormality (not shown). Because these two lines are weak alleles and their resulting wing and leg phenotypes were found in less than 20% of the females, we decided to use stronger *Smr* alleles for additional investigation. Our characterization of *G0060* and *G0124* revealed that these two mutations represent strong alleles. While both alleles are lethal, homozygous clones from each decrease the expression of SMRTER substantially (shown later) and cause severe defects in various tissues (Fig. 2C and supplementary material Fig. S1). Concerned that *G0124* may affect the properties of both the *Smr* and *Tis11* genes, we used *G0060* (hereafter referred to as *Smr1)* for our *in vivo* investigation. Our characterization of *Smr1* indicates that this line does not contain additional mutations, since revertants generated from precise P-element excision are viable and show no visible defects.

We employed the FRT/FLP recombination system (Xu and Rubin, 1993) to generate mosaic tissues containing homozygous *Smr1* cells. Making use of two *FLP* lines, *T155-Gal4, UAS-FLP* (hereafter referred to as *T155-FLP)* and *Hsp70-FLP*, we are able to generate *Smr* mutant clones in various tissues including the wings, legs, and ovaries (the activity of *T155* in the imaginal discs and ovary are shown in supplementary material

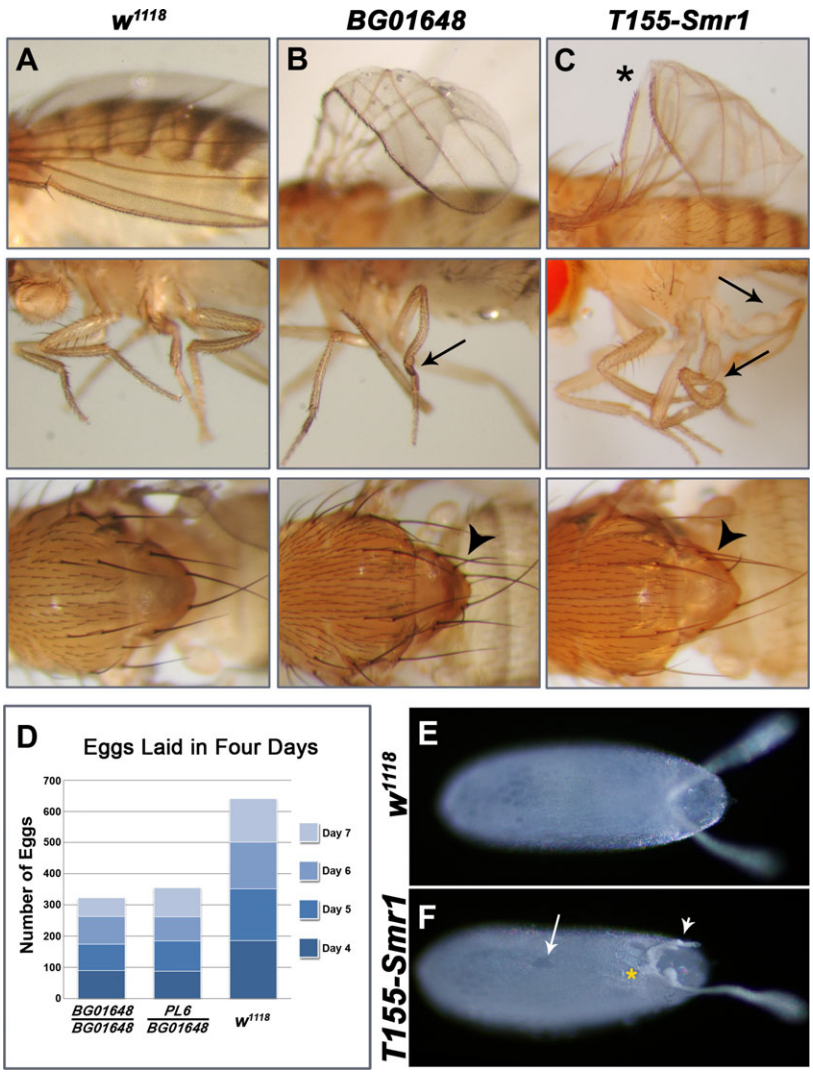


Fig. 2. SMRTER is required for the development of multiple tissues. The phenotypes of wing, leg, and scutellar bristles observed for the control w^{1118} females **(A)**, homozygous *BG01648* females **(B)**, and *T155-Smr1* females **(C)**. An asterisk marks the defective wing margin; arrows mark the deformed third pair legs; arrowheads mark extra scutellar bristles. **(D)** Number of eggs produced by the control, homozygous *BG01648,* and heterozygous *BG01648/PL6* females. Eggs were collected from the indicated female groups (6 females for each group) for four consecutive days, starting on day 4 after they were eclosed from the pupal cases. The number of eggs collected from each group in each single day is graphed into a box, whose height represents the number of eggs. **(E, F)** Egg shells and dorsal appendages found in embryos deposited by the control w^{1118} females (E) and by *T155-Smr1* females (F). Defective egg shell is denoted with an asterisk and an arrow; the shortened and branched dorsal appendage is marked with an arrowhead.

Fig. S1A–C). The *T155-FLP* line was particularly useful for our research on adult female flies, because *Smr1, FRT[9.2]/Ubi-GFP, FRT[9.2]; T155-FLP/+* (hereafter referred to as *T155-Smr1*) females are viable and yield highly reproducible phenotypes. As shown in Fig. 2C, *T155-Smr1* females (100%, $n=47$) show downwardly folded wings, severely distorted third pair legs (marked with arrows), and ectopic and defective bristle formation in the scutellum. Closer examination of the wings of *T155-Smr1* females revealed that their wing margin is defective as well (asterisk). These phenotypes are similar to, but more severe than those seen for homozygous *BG1648* females (Fig. 2B), which reaffirms our assessment that *Smr1* is a stronger allele.

Like *BG01648* homozygous mutants, *T155-Smr1* females also show egg-laying defects: not only do they produce fewer eggs, their laid embryos (~46%, $n=52$) also exhibit severe morphological defects, including scarred egg shells and shortened dorsal appendages (Fig. 2F). The formation of egg shell and dorsal appendages in the embryos depends on chorion proteins deposited by the ovarian follicle cells of the mother (Dobens and Raftery, 2000), and our immunostaining experiments reveal that wild-type SMRTER expression is enriched in these cells (supplementary material Figs S2, S3). We therefore postulated that the egg-laying defects seen for *Smr* mutant females result in part from disrupted genetic pathways that regulate the development of somatic follicle cells. Because oogenesis is one of the most studied developmental pathways in *Drosophila* (Spradling, 1993; McCall, 2004; Bastock and St Johnston, 2008; Wu et al., 2008), we decided to study the role of SMRTER in the development of ovarian follicle cells.

SMRTER inhibits ecdysone activity in follicle cells

The female fly has two ovaries, each containing approximately 16–20 ovarioles. Each ovariole is a string of egg chambers containing two cell types: internal germ cells, including 15 nurse cells and 1 oocyte, and the enveloping somatic follicle cells. Through the 14 successive stages of egg chamber development (S1–S14), the follicle cells, under the control of various signaling pathways, proceed through three distinct cell-cycle phases (mitosis, endocycle, and gene amplification) and ultimately are responsible for depositing the eggshell for the mature egg (see illustration in supplementary material Figs S2A, S3A).

The development and functioning of follicle cells is known to be regulated by ecdysone (Buszczak et al., 1999; Carney and Bender, 2000; Terashima et al., 2005; Hackney et al., 2007; Sun et al., 2008; Bernardi et al., 2009; Boyle and Berg, 2009; Romani et al., 2009). Having characterized SMRTER as a transcriptional

corepressor of EcR in cultured cells (Tsai et al., 1999), we first asked whether the expression of any known target genes of EcR is affected by *Smr* mutation in ovarian follicle cells. We chose Broad (Br) and Tramtrack 69 (Ttk69) for our investigation, because they have been characterized as ecdysone-responsive targets in follicle cells (Buszczak et al., 1999; Sun et al., 2008). In wild-type follicle cells, Br protein is first expressed at a low level during stages S6–S9. In response to a surge of ecdysone, its expression increases sharply in main body follicle cells at S9 (Buszczak et al., 1999). At later stages, however, the expression of Br is regulated by additional factors: starting with S10B, Br expression gradually declines in all follicle cells except those confined to the lateral-dorsal-anterior (LDA) region of the columnar follicle cells (Deng and Bownes, 1997; Tzolovsky et al., 1999; Yakoby et al., 2008). Ttk69 is expressed at a low level in follicle cells during the S1–S9 period, after which, in response to a surge of ecdysone, its expression level also increases substantially in all follicle cells at S9 (Sun et al., 2008). In more mature follicle cells, however, Ttk69 does not adopt the spatially restricted pattern of Br, but is evenly distributed (French et al., 2003; Jordan et al., 2006; Sun et al., 2008; Boyle and Berg, 2009). Therefore, although both Br and Ttk69 are ecdysone-responsive targets at S9–S10A, the genetic programs that control their expression in follicle cells differ at the later stages.

Our examination of mosaic *Hsp70-Smr1* and *T155-Smr1* female egg chambers showed that both Ttk69 and Br are increased in *Smr1* follicle cells at S9–S10A, compared to their expression in the SMRTER-positive follicle cells (Fig. 3A,B). These results are in line with our proposed role for SMRTER as a negative regulator of the ecdysone pathway (Tsai et al., 1999). In S10B egg chambers, higher Ttk69 levels persist in all *Smr1* follicle cells (marked by absent GFP signal), regardless of their location (Fig. 3C). For Br in age-matched egg chambers, however, elevated expression was observed only in *Smr1* cells confined to the LDA region (Fig. 3D). Since ectopic Br expression was not seen in *Smr1* follicle cells beyond the LDA region, it is possible that the repression of Br in follicle cells outside the LDA realm relies on inputs from transcription factors or cofactors other than EcR and SMRTER. Nevertheless, our demonstration that expression of Ttk69 and Br in *Smr1* follicle cells is increased supports our view that SMRTER acts to antagonize the ecdysone pathway in follicle cells. Because both Br and Ttk69 are required for follicle cells to form egg shell and dorsal appendages (Tzolovsky et al., 1999; French et al., 2003; Boyle and Berg, 2009), their altered expression in *Smr1* follicle cells may contribute to the defects seen for the embryos deposited by *T155-Smr1* females (Fig. 2F).

SMRTER inhibits Notch activity in follicle cells

While examining the expression of Ttk69 and Br in *Smr1* follicle cells of *T155-Smr1* egg chambers, we frequently observed that the nuclei of *Smr1* cells were larger than those of their neighboring heterozygous cells (Fig. 4A). We measured the area and the DNA content for a large number of SMRTER positive and negative nuclei found in DAPI-stained S9 *T155-Smr1* egg chambers, using the methods described in the materials and methods section. These two measurements revealed that homozygous *Smr1* nuclei, on average, are both larger (Fig. 4B) and contain approximately 1.6-fold more DNA than the control heterozygous nuclei (Fig. 4C,D). Because follicle cells undergo three rounds of genomic replication without cell division (endocycle) from S7 to S9/S10A (Royzman and Orr-Weaver,

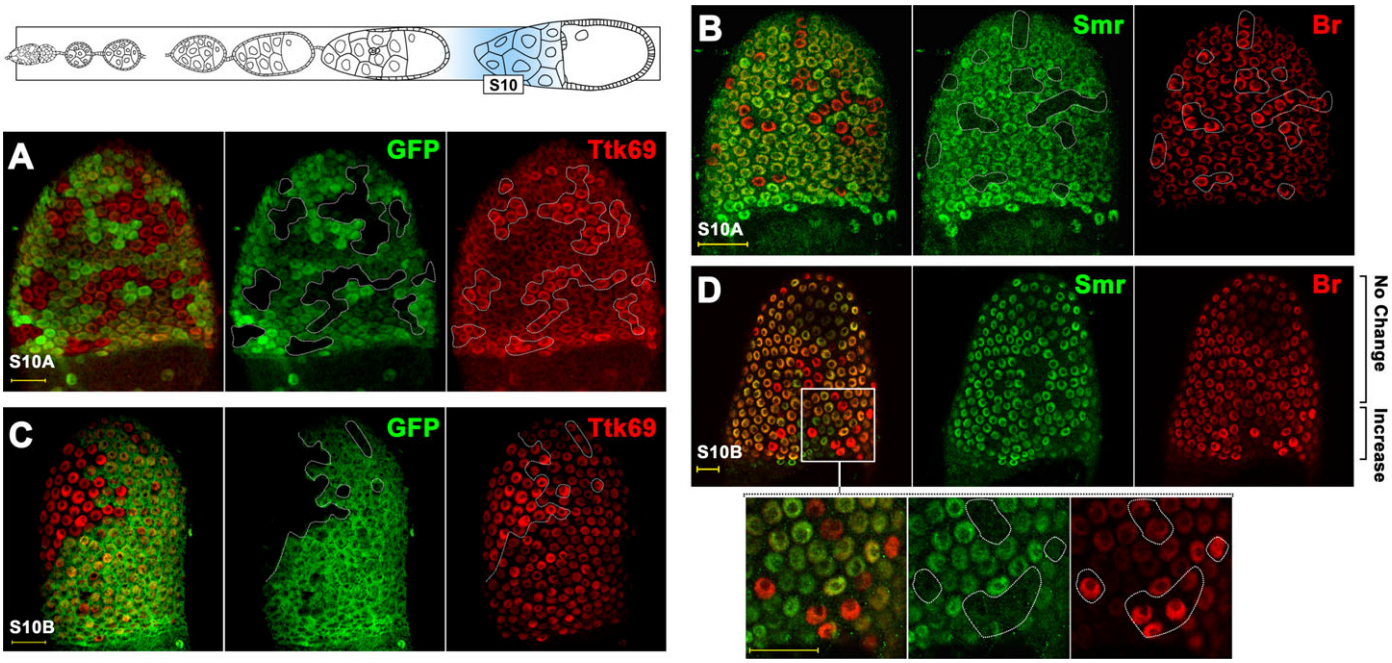


Fig. 3. *Smr* mutation affects the expression of EcR targets. *Hsp70-Smr1* **(A, C)** or *T155-Smr1* **(B, D)** egg chambers at S10A (A, B) and S10B (C, D), stained with antibodies against SMRTER or GFP and Tramtrack 69 (Ttk69) or Broad (Br). GFP marks the cells that express SMRTER. The enlarged images shown in the lower panel of (D) correspond to the boxed area. In the S10B egg chamber, increased Br expression can be seen only in *Smr1* columnar cells located within the lateral-dorsal-anterior (LDA) region (facing down). Scale=20 μm.

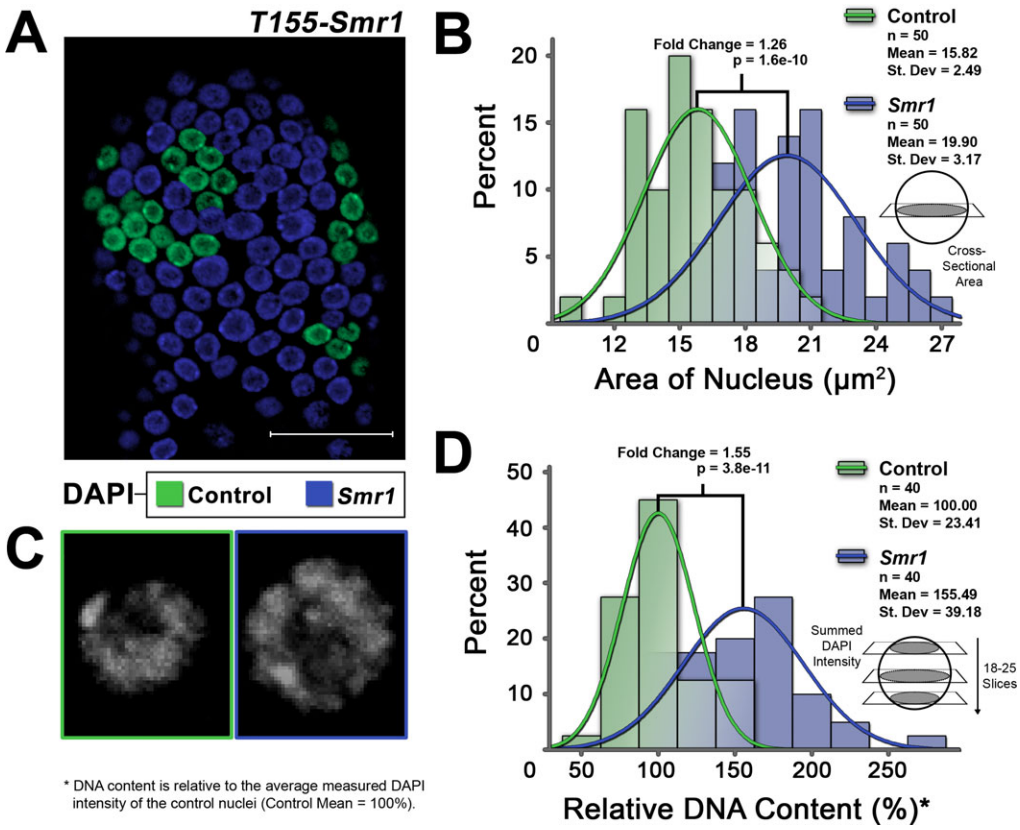


Fig. 4. *Smr* mutation affects endocycle in follicle cells. (A) A DAPI (4′,6-diamidino-2-phenylindole) stained S9 *T155-Smr1* egg chamber shows the difference in the size between control (green pseudocolor) and *Smr1* (blue pseudocolor) nuclei. Scale=20 µm. **(B)** A quantitative analysis of the cross-sectional areas measured for the control and *Smr1*nuclei found in A. *n*=the number of nuclei used for the analysis. **(C)** Compiled images derived from a series of z-sections collected for control and *Smr1* nuclei. **(D)** A quantitative analysis of the relative DNA content measured for control and *Smr1* nuclei derived from four different S9 *T155-Smr1* egg chambers. Detailed information for the methods used for these two analyses can be found in the Materials and Methods section.

1998; Klusza and Deng, 2011), we postulated that the increased size and DNA content of *Smr1* nuclei results from disruption of the genetic programs that control the endocycle process.

The endocycle is known to be positively regulated by the Notch pathway in follicle cells during S7–S10A (Deng et al., 2001; Schaeffer et al., 2004; Sun and Deng, 2005; Jordan et al., 2006). Notch is activated during this period as a result of stimulation from its ligand Delta, which is upregulated on the surface of the adjacent germ cells starting from S6 (Lopez-Schier and St Johnston, 2001). The activation of Notch triggers the S6 follicle cells to stop dividing, and maintains them in a state of genome replication from S7 to S9/10A. Consequently, loss-of-function *Notch* mutation has been shown to prevent follicle cells from exiting mitosis (Deng et al., 2001). Consistently, ectopic expression of a Notch target, Hindsight (Hnt) (Yip et al., 1997; Krejci et al., 2009), is sufficient to halt cell division and induce the endocycle (Sun and Deng, 2007), thereby generating egg chambers containing reduced number of follicle cells with larger nuclei. Because the phenotypes observed in *Smr1* follicle cells were strikingly similar to those produced by the misexpression of Hnt, we sought to investigate whether SMRTER plays a role in regulating Hnt expression, as well as Notch activity, during the endocycle. Making use of an antibody against Hnt, we observed that at S6, *Smr1* follicle cells within *T155-Smr1* egg chambers show increased Hnt expression (Fig. 5A). These results suggest that the Notch pathway may be negatively regulated by SMRTER in follicle cells during mid-oogenesis.

Although Hnt has been shown to be a direct target of Notch signaling (Krejci et al., 2009), the possibility remains that its upregulation by *Smr1* is independent of the Notch pathway. To address this concern further, we examined the expression of two additional Notch targets, Enhancer of Split-mβ [E(spl)mβ] and Cut. E(spl)mβ belongs to a family of helix-loop-helix proteins that have been characterized as the primary effectors of the Notch/Su(H) pathway (Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992; Bailey and Posakony, 1995; Furukawa et al., 1995). The expression of E(spl)mβ peaks during S7–S10A, concurrent with the expression of Hnt; the homeobox protein Cut, in contrast, is repressed by Notch (Sun and Deng, 2005; Sun and Deng, 2007). The repression of Cut by Notch is indirect and is mediated through Hnt, which acts directly to repress Cut (Sun and Deng, 2007). Consequently, in normal egg chambers, the expression patterns of Cut and Hnt are complementary to each other at different stages, with Cut expression limited to follicle cells at S1–S6 and after S10A (supplementary material Fig. S3). We analyzed the expression of *E(spl)mβ*, using a CD2 (a membrane-bound marker) reporter line, and of Cut in *T155-Smr1* egg chambers. In keeping with our observation for Hnt, E(spl)mβ-CD2 is also precociously expressed in some *Smr1* follicle cells at S6, coupled with premature loss of Cut (Fig. 5B,C). These results reinforce our view that SMRTER imposes a negative effect on the Notch pathway. The increase in Notch activity in *Smr1* follicle cells thus provides an explanation for why endocycle is affected by *Smr* mutation (Fig. 4).

SMRTER inhibits Notch activity in a temporally restricted manner

When we examined the expression of the three Notch targets discussed above in *T155-Smr1* egg chambers at different stages, we were surprised to find that their expression in *Smr1* follicle cells is altered in a temporally restricted manner. For instance, at S4, when Notch is inactive owing to the absence of its ligand

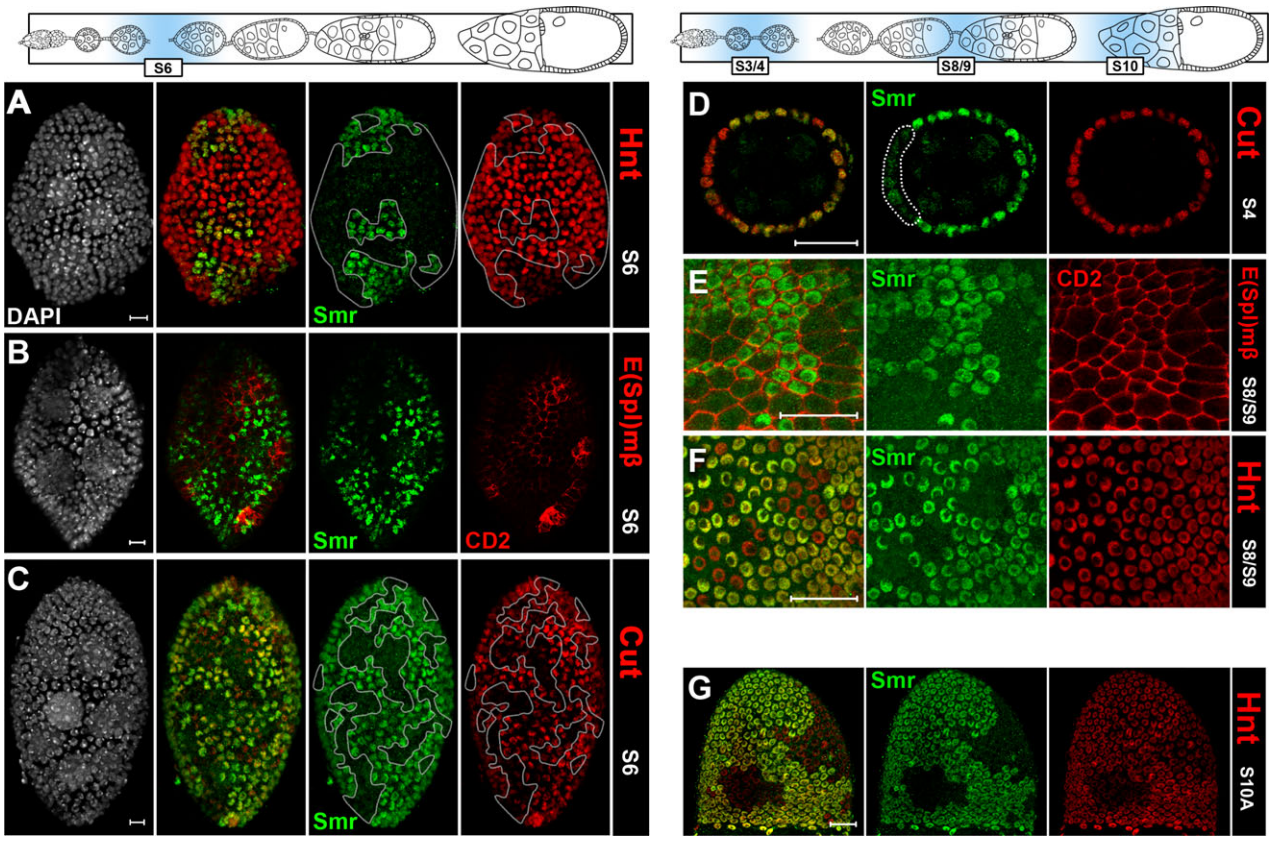


Fig. 5. *Smr* mutation affects the expression of Notch targets in a temporally-restricted manner. (A, B, C) Egg chambers at S6 stained with antibodies against SMRTER and Hindsight (Hnt) (A), CD2 (B), or Cut (C). Diagram shows the egg chambers at S6 during oogenesis. Membrane-bound CD2 is a reporter for E(spl)mβ. **(D)** An egg chamber at S4 stained with antibodies against SMRTER and Cut. **(E, F)** Egg chambers at S8/S9 stained with antibodies against SMRTER and CD2 (E) or Hnt (F). **(G)** An egg chamber at S10A stained with antibodies against SMRTER and Hnt. Diagram shows the egg chambers at S4, S8/9, and S10A during oogenesis. Genotypes: *T155-Smr1* (A, C, D, F, and G); *T155-Smr1, E(Spl)mβ-CD2* (B and E); Scale=5 µm.

Delta, no decrease in Cut (Fig. 5D) nor increase in Hnt and E(spl)mβ-CD2 (not shown) could be detected in *Smr1* cells. During S7–S10A, when Notch signaling has reached its full potency, the expression of E(spl)mβ and Hnt is not elevated in *Smr1* cells (Fig. 5E,F). At S9–10A, which marks the end of the Notch pathway and the beginning of the ecdysone pathway, the expression of Hnt is actually reduced in *Smr1* cells (Fig. 5G), in contrast to its response to *Smr* mutation at S6. These observations indicate that the inhibitory effect of SMRTER on the Notch pathway takes place in a temporally restricted fashion. Therefore, the extent to which SMRTER influences the Notch pathway depends not only on the strength of the Notch activity and the availability of its ligand, but may also be subject to influence from other competing factors or signaling pathways.

SMRTER inhibits Notch activity in a spatially restricted manner

Having observed a transient role for SMRTER in repressing the Notch pathway in follicle cells, we were interested in knowing whether SMRTER also affects the Notch pathway similarly in other tissues. One of the best-characterized developmental processes regulated by the Notch pathway is the formation of the dorsal-ventral boundary in the wing disc (Irvine and Rauskolb, 2001). Notch is specifically activated along the dorsal-ventral margin as it responds to inputs from its ligands Delta and Serrate (Fig. 6A). Upon its activation at the third instar larval stage, several Notch targets, including Cut, are specifically expressed at the wing margin (de Celis et al., 1996; Neumann and Cohen, 1996; de Celis and Bray, 1997; Micchelli et al., 1997). We therefore examined whether Cut expression is affected in *Smr1* cells in wing discs. We performed coimmunostaining on *Hsp70-Smr1* wing discs, using antibodies against GFP (a marker for SMRTER-positive cells) and Cut. Indeed, Cut is elevated in *Smr1* cells, but importantly, only in *Smr1* cells that are in juxtaposition to the wing margin (Fig. 6A; arrows). Contrastingly, in parallel experiments, we found that the level of Br, an ecdysone-responsive target, displayed sustained elevation in virtually all *Smr1* cells, with no regard to their locations (Fig. 6B).

Knockdown of SMRTER reproduces the effects caused by *Smr1*

To validate the results based on our mosaic analysis with the *Smr1* line, we conducted additional experiments in which we knocked down the expression of SMRTER, using the RNA interference (RNAi) approach, and measured the consequences. For this purpose, we generated a UAS line (*UAS-Smr·IR3*) that expresses *Smr* double stranded (ds) RNA using the Gal4-UAS system (Brand and Perrimon, 1993). Directed expression of *Smr* dsRNA in the region along the border between the anterior and posterior compartments in the wing disc, prepared from *patched-Gal4/UAS-Smr·IR3* larvae, reduces the expression of SMRTER significantly (Fig. 6C). Examining the expression of Cut and Br

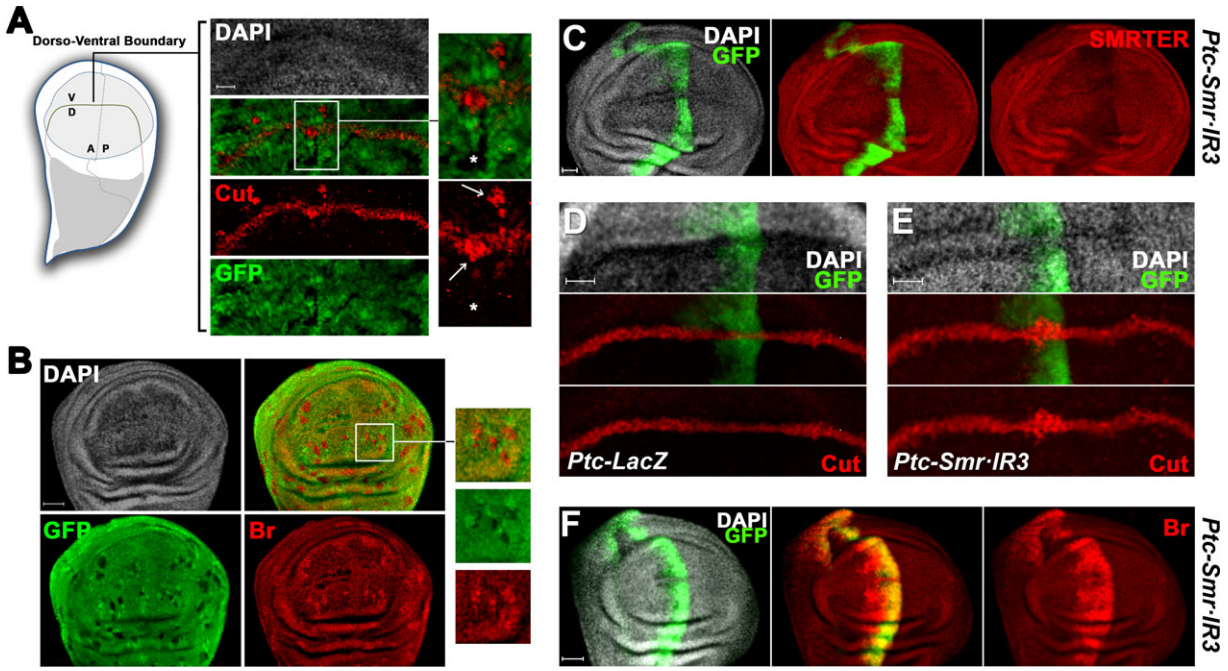


Fig. 6. *Smr* mutation and knockdown of SMRTER increase the expression of Cut and Br in wing discs. (A) *Hsp70-Smr1* wing disc stained with antibodies against GFP (marks for SMRTER-positive cells) and Cut. Diagram on the left shows the dorsal-ventral boundary in wing disc, whose formation is positively regulated by the activated Notch signaling pathway. The wing disc is divided into four compartments, including D: dorsal; V: ventral; A: anterior; and P: posterior. The enlarged images on the right correspond to the boxed areas on the left. Arrows mark the cells that express Cut ectopically; asterisks mark the cells that do not express Cut. **(B)** *Hsp70-Smr1* wing disc stained with antibodies against GFP and Br. A boxed area is enlarged to show an increased expression of Br. **(C, D, E, F)** Knockdown of SMRTER causes effects similar to *Smr* mutation. *Ptc-Smr.IR3* wing discs stained with antibodies against SMRTER (C), Cut (E), or Br (F). Wild-type Cut expression is shown in (D) for comparison. Wing discs were counterstained with DAPI (4′,6-diamidino-2-phenylindole). Scale=20 μm. Genotypes: *Hsp70-Smr1* (A, B), *patched (ptc)-Gal4, UAS-GFP/UAS-Smr.IR3* (C, E, F); *ptc-Gal4, UAS-GFP/UAS-lacZ* (D). *ptc-Gal4* is active along the border between the anterior-posterior compartments; *UAS-Smr.IR3* is a fly line for expressing double-stranded *Smr* RNA; *UAS-GFP* is a reporter line; *UAS-lacZ* is a control line. Wing discs are oriented anterior to the left. Scale=20 μm.

in these *ptc-Gal4/UAS-Smr·IR3* wing discs showed, consistently, that Cut is elevated only in SMRTER knocked-down cells that are in close proximity to the wing margin (Fig. 6E). In contrast, Br is increased in all regions as long as SMRTER expression is reduced (Fig. 6F). Since consistent results were obtained from our additional experiments using another *Smr* RNAi line from VDRC (106701/KK) (not shown), we conclude that the observed effects that *Smr1* exercises on the Notch and ecdysone pathways in the wing are specifically due to a loss of SMRTER activity.

SMRTER interacts with Su(H) in yeast

Having demonstrated that SMRTER is involved in the Notch pathway at the functional level, we next investigated how SMRTER participates in the Notch pathway at the molecular level. We focused on a possible direct interaction between SMRTER and Su(H), a transcription factor central to the Notch pathway (Furukawa et al., 1992; Schweisguth and Posakony, 1992), because a previous study showed a direct interaction between the vertebrate homologs of these two proteins (Kao et al., 1998). For this investigation, we employed yeast two-hybrid assays (Fields and Song, 1989), in which a positive protein-protein interaction enables transformed yeasts to grow on the nutrient-deficient selection plate. In our assays, we transformed AH109 cells with plasmids expressing a Gal4 DNA-binding-domain fused full-length Su(H) protein and each of the five shown Gal4 activating-domain fused SMRTER fragments. Based on the growth patterns of the transformed yeast shown in Fig. 7A, our data demonstrate that SMRTER indeed binds Su(H), and that their interaction is mediated through the 3068–3506 amino acid region of SMRTER. Since the interaction observed between SMRTER and Su(H) resembles that found between SMRT and CBF1 (Kao et al., 1998), we conclude that the formation of a CSL-SMRT repressor complex is a conserved feature for the Notch pathway in both vertebrates and invertebrates.

SMRTER and Su(H) bind overlapping chromosomal regions

Having demonstrated a physical interaction between SMRTER and Su(H), we asked whether their interaction can be visualized *in vivo*. Since both SMRTER and Su(H) are expressed in salivary gland cells, which contain polytene chromosomes, we examined whether SMRTER, by means of its interaction with Su(H), is recruited to the specific chromosomal regions that Su(H) binds. We performed coimmunostaining experiments on squashed salivary glands prepared from late third instar larvae stage, using antibodies against both SMRTER and Su(H). As shown in Fig. 7B, endogenous SMRTER binds many chromosomal regions that are positive for Su(H), which agrees with the notion that these two proteins interact physically with one another. Interestingly, not all chromosomal regions occupied by Su(H) are positive for SMRTER, nor do all the regions stained strongly by SMRTER show strong signal for Su(H). Therefore, although Su(H) can bind SMRTER physically in vitro, their interaction at the chromosomal level is likely influenced by other factors or by local chromatin structures.

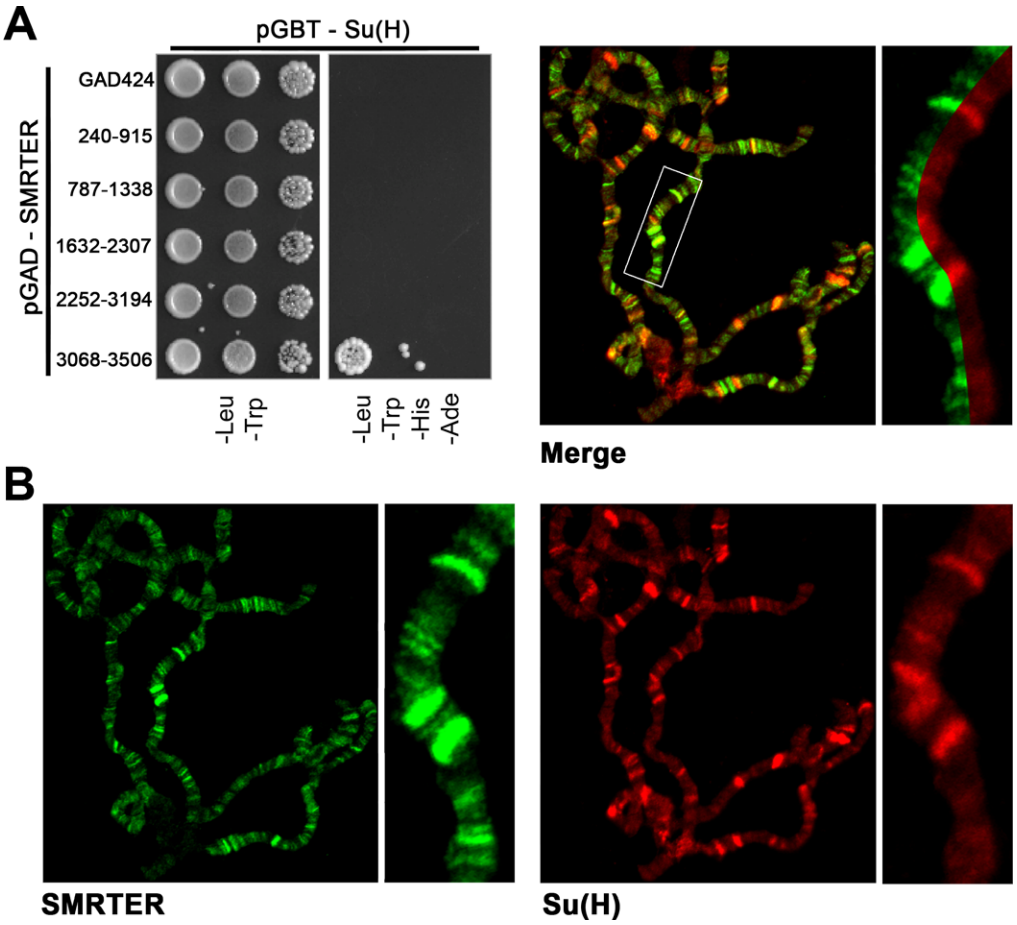


Fig. 7. SMRTER associates with Su(H). (A) Yeast two-hybrid assays for testing an interaction between SMRTER and Su(H). AH109 yeast cells were transformed with GBT-Su(H) (full-length) and each of the shown GAD-SMRTER constructs, which express the indicated regions of SMRTER; GBT is a Gal4-DNA binding domain-based construct; GAD is a Gal4-activating domain-based construct; GAD424 is an empty vector included as a negative control. Ten-fold serial dilutions of saturated transformed yeast were spotted on a double dropout (growth) plate and a quadruple dropout (selection) plate. Yeast that can grow on the selection plate indicates a positive protein-protein interaction. **(B)** Polytene chromosomes, prepared from salivary glands of w^{1118} late third instar larvae, stained with antibodies against SMRTER and Su(H). The enlarged images correspond to the boxed area. The merged image shows the colocalization of both proteins on some, but not all, chromosomal loci.

Discussion

SMRTER is a transcriptional corepressor that we previously identified as an interacting factor of EcR (Tsai et al., 1999). Since its discovery more than a decade ago, studies from our own and other labs have connected the properties of SMRTER to transcription factors or cofactors, including EcR (Tsai et al., 1999; Sedkov et al., 2003), the SIN3-RPD3 protein complex (Tsai et al., 1999; Pile and Wassarman, 2000; Pile et al., 2002), and Schnurri, a Smad cofactor involved in the TGF-β pathway (Cai and Laughon, 2009). Although these results have implicated SMRTER in various transcriptional regulatory pathways, no *in vivo* studies have been carried out to determine the involvement of SMRTER in specific developmental and genetic pathways in *Drosophila*. In this study, resting on our characterization of multiple *Smr* mutant lines, mosaic analysis with a loss-of-function *Smr* allele, and knockdown experiments with two *Smr.IR* fly lines, we report the involvement of SMRTER in the development of ovarian follicle cells and wing. In these two tissues, we provide evidence showing that SMRTER exerts a negative influence not only on ecdysone signaling, but also on the Notch pathway. We further show a direct interaction between SMRTER and Su(H) and the colocalization of both proteins at many chromosomal regions in the salivary glands. Based on our results, we conclude that SMRTER is an integral component of both the ecdysone and Notch signaling pathways in various *Drosophila* tissues.

SMRTER appears to differentially regulate the properties of EcR and Su(H)

Perhaps one of the most unexpected and potentially important results obtained from this study is that certain Notch target genes respond to *Smr* mutation in a temporally and spatially restricted manner. This phenomenon appears to be specific to the Notch pathway, since the ecdysone pathway is not similarly affected by the very same *Smr* mutation. For example, in the wing disc, ectopic expression of Cut, a Notch-responsive target, takes place only in *Smr1* clones that are in juxtaposition to the dorsal-ventral boundary where Notch is activated, whereas Br, a target of ecdysone, is elevated in *Smr1* cells throughout the wing disc (Fig. 6). While this difference might be explained by the relative restriction of Notch ligand in the wing as compared to the widely distributed ecdysone, such an explanation fails to account for the responses of E(spl)mβ and Hnt in the *T155-Smr1* ovary. The expression of these two direct targets of Notch is increased only in *Smr1* follicle cells within S6 egg chambers (Fig. 5A,B) – a stage coinciding with the onset of Notch's activation. Their expression is however not increased in *Smr1* follicle cells during S7–S9, even though Notch ligand, Delta, is expressed during this period. In contrast, once the expression of Ttk69, a target of ecdysone (Sun et al., 2008; Boyle and Berg, 2009), is elevated in *Smr1* follicle cells, its upregulated expression is sustained throughout the stages that follow (Fig. 3A,C).

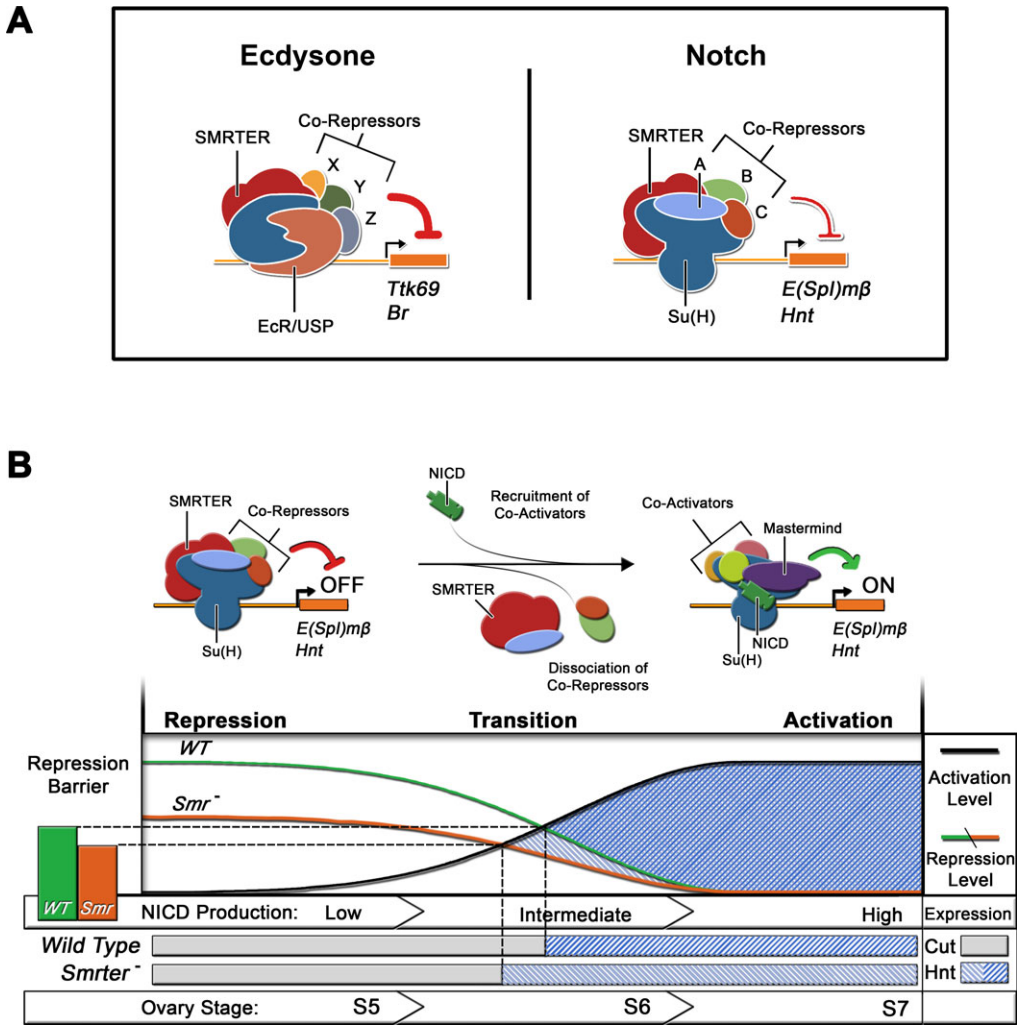


Fig. 8. Model depicting the properties of SMRTER found in this study. (A) A proposed model to explain how SMRTER exerts differential influence on the ecdysone- and the Notch- pathways. SMRTER is present in the multi-subunit transcriptional corepressor complexes of EcR and Su(H). In addition to SMRTER, these two transcription factors also recruit other transcriptional corepressors that are unique to each transcription factor. **(B)** A proposed model to explain how SMRTER influences the transcriptional output of the Notch pathway in follicle cells at different stages. Prior to S5, Su(H) acts primarily as a transcriptional repressor because the Notch pathway is inactive; at the transitional S6, Su(H) has intermediate transcriptional activating activity because Notch is partially activated; during S7–S10A, Notch's activity reaches its full potency. Notch activation produces a truncated and active form of Notch (NICD), which is translocated from the cell membrane to the nucleus, where it binds the DNA-binding transcription factor Su(H). Upon the formation of the Su(H)-NICD complex, the corepressor complex is dissociated from Su(H) and, concomitantly, the coactivator complex is recruited by Su(H). These NICD-induced events allow Su(H) to be converted from a repressor to an activator. The multi-subunit coactivator complex of Su(H) includes Mastermind and other coactivators. The multi-subunit transcriptional corepressor complex of Su(H) includes SMRTER and other corepressors. The multiple corepressors of Su(H) collaborate to form a "multi-corepressor-mediated repression barrier," which prevents Notch targets from being activated. Only when the positive activity from Notch exceeds the negative effect imposed by this repression barrier can the expression of Notch targets, such as Hnt and E(spl)mβ, be activated. When the repression barrier is lowered as a result of mutation of *Smr* or knockdown of SMRTER, a partially activated Notch pathway is sufficient for the precocious expression of its targets. Once Notch activity reaches its peak, the influence of its repressors, including SMRTER, on its transcriptional properties becomes negligible, because Su(H) no longer binds corepressors with high affinity. The relative expression level of Hnt is represented by the blue shaded area. Our results indicate that the timing (shaded bars), but not the maximum level of Hnt expression, is altered by *Smr* mutation.

How does SMRTER exert this differential effect on the Notch and ecdysone pathways? The most likely explanation is that the transcription factors EcR and Su(H) each employ different sets of corepressor complexes to repress gene transcription (see a model in Fig. 8A). While many components could be shared, the unique composition of each complex may influence the degree to which SMRTER contributes to the total repression. Based on our observations, SMRTER appears to play a more prominent role in enabling EcR to repress gene transcription, whereas its role in aiding Su(H) to silence gene expression is more subtle. To explain this phenomenon in the context of the Notch pathway, various factors essential for SMRTER to influence Su(H) may be spatiotemporally restricted, thereby limiting the scope of its influence. Alternatively, SMRTER may be only one of many components that have overlapping roles in modulating Su(H)'s repressive effects. In the latter case, multiple corepressors of Su(H) may join forces to form a "transcriptional repression barrier" that determines "ON/OFF" expression for Notch targets (see the model shown in Fig. 8B). In this model, only when the positive activity of ligand-bound Notch exceeds the negative

effect imposed by such a transcriptional barrier can the expression of Notch targets be activated. Mutation of *Smr* or knockdown of SMRTER expression inevitably lowers the threshold barrier that prevents NICD-bound Su(H) from activating its target genes. Consequently, even the partially activated Notch in *Smr1* follicle cells at S6 is sufficient to induce precocious expression of Hnt and E(spl)mβ (Fig. 5A,B). We reason that SMRTER has a negligible influence on the Notch pathway in follicle cells during S7–S10 because the fully activated NICD-Su(H) complex during this period no longer binds SMRTER with high affinity.

Which other transcriptional cofactors, then, may collaborate with SMRTER to assist Su(H) in repressing the expression of Notch targets? In vertebrates, several direct corepressors of CBF1 (the vertebrate Su(H)) have been identified, including the SPEN/SHARP-family proteins (Oswald et al., 2002) and the SKIP-family proteins (Zhou et al., 2000; Zhou and Hayward, 2001). Our group has also recently identified ATXN1 and its closely related factor, BOAT1, as a new class of corepressors of CBF1 (Tong et al., 2011). Interestingly, these CBF1-interacting factors have been found to bind SMRT/N-CoR directly (Zhou et al., 2000; Shi et al., 2001; Zhou and Hayward, 2001; Oswald et al., 2002; Tsai et al., 2004; Mizutani et al., 2005), suggesting that they may work with SMRT/N-CoR to silence Notch activity in vertebrates. Since homologs or related factors of these corepressors of CBF1 are present in *Drosophila* (Wieland et al., 1992; Wiellette et al., 1999; Kuang et al., 2000; Rebay et al., 2000; Mizutani et al., 2005; Tsuda et al., 2005), these fly homologs may interact and collaborate with SMRTER to silence the expression of Notch targets in *Drosophila*.

SMRTER regulates cell-cycle transitions in follicle cells

During oogenesis, under influence of various factors and signaling pathways, epithelial follicle cells proceed through three distinct phases of cell cycle (Klusza and Deng, 2011) (supplementary material Fig. S3A). During S1–6, Cut promotes mitosis; during S7–9, Notch stimulates endocycle; and during S10–14, ecdysone fuels a period of targeted gene amplification (Calvi et al., 1998; Sun et al., 2008). Our data suggest that SMRTER participates in regulating the timing for each of the two cell-cycle transitions: from mitosis to endocycle (M/E), and from endocycle to gene amplification (E/A). For instance, during the M/E switch, mutation of *Smr* causes the premature expression of Hnt and complementary repression of Cut, a mitosis-promoting factor (Fig. 5A,C). This premature activation is sufficient to force *Smr1* follicle cells to enter the endocycle earlier than their heterozygous counterparts, and likely contributes to fewer rounds of mitosis, as evidenced by the sparse distribution of nuclei within *Smr1* clones (Fig. 4A). Correspondingly, our results show that *Smr1* nuclei at S9 do indeed contain additional DNA. Because these mutant nuclei on the average possess only 1.6 fold more DNA than wild-type, we deduce that they have undergone, at most, one additional round of endocycle (each round of replication doubles the DNA content). This limited effect concurs with our model, in which the effect of *Smr* mutation on Notch signaling is only realized within a short window of time just prior to the S6 M/E transition. Therefore, although SMRTER is not required for the M/E switch, it appears to be involved in regulating the precise timing of this event.

Our observations of *Smr1* follicle cells at the later stages of oogenesis indicate that SMRTER appears to be involved in the E/A switch as well. Given that extended Notch activity has been shown to postpone the E/A switch (Sun et al., 2008), we at first predicted a delay in the E/A switch when *Smr* is mutated. Our results showed, however, that Hnt, a marker for the endocycle, is reduced at S10A in *Smr1* follicle cells. Because the expression of Ttk69 is elevated in *Smr1* clones at the same stage (Fig. 3A), the increased expression of this ecdysone target, which has been shown to antagonize Notch activity at the E/A switch (Sun et al., 2008), therefore provides a reasonable explanation for the untimely down-regulation of Hnt. Evidently, Ttk69 is able to override the ability of Notch/Su(H) to activate Hnt expression in *Smr1* cells. This observation not only agrees with the previous reports that ecdysone, through its activation of Ttk69, exerts a negative influence on Notch signaling (Sun et al., 2008; Boyle and Berg, 2009), but also substantiates our hypothesis that SMRTER plays a prominent role in enabling EcR to repress gene transcription. This phenomenon, combined with the role for SMRTER in regulating the M/E switch, indicates that SMRTER is required repeatedly at different stages during follicle cell maturation.

The regulation of Br by SMRTER may involve the Notch and EGFR pathways

Before this study, our lab showed that SMRTER is a transcriptional corepressor of EcR (Tsai et al., 1999). Our *in vivo* data here confirm that the ecdysone pathway indeed gains in activity when *Smr* is mutated. For instance, the expression of Ttk69 and Br, two known targets of EcR, is elevated in *Smr1* cells in both the follicle cell layer and wing (Fig. 3, Fig. 6B,F). However, the regulation of Br by SMRTER in follicle cells may involve factors or pathways in addition to EcR. One such potential pathway is Notch. We raise this possibility based on our observation that Br is elevated in *Smr1* follicle cells in egg chambers as early as at S6 (supplementary material Fig. S4A), which is prior to the ecdysone pathway's more prominent role after S8 (Buszczak et al., 1999). Moreover, the expression of Br is not significantly affected in *Smr1* follicle cells during S7–S10A (supplementary material Fig. S4B). This response of Br to *Smr* mutation during S7–S10A is similar to those found for Hnt and E(spl)mβ in *T155-Smr1* egg chambers (Fig. 5). Whether or not Br is indeed a target of Notch during S6–S9 remains an open question waiting to be investigated.

The repression of Br by SMRTER in the mature follicle cell may involve the EGFR pathway as well. This speculation is based on our observation that once *T155-Smr1* egg chambers have passed S10B, elevated Br expression takes place only in *Smr1* cells that are confined to the LDA domain (Fig. 3D). This response of Br differs significantly from that of Ttk69 in *Smr1* cells, as the latter is increased in virtually all *Smr1* follicle cells found in *T155-Smr1* egg chambers (Fig. 3C). The divergence in the responses of these two ecdysone targets to *Smr* mutation at the later stage is not entirely surprising, because the regulation of Br in follicle cells after S10B is known to be quite complex (Yakoby et al., 2008). Within the LDA domain, the expression of Br in follicle cells is known to be positively regulated by EGFR (Deng and Bownes, 1997), which is activated by its ligand Gurken, localized to the dorsal-anterior region of the oocyte (Nilson and Schupbach, 1999; Dobens and Raftery, 2000). Since a negative relationship has been reported between EGFR and

SMRTER in retinal cells (Tsuda et al., 2002; Tsuda et al., 2006), a similar relationship may exist between these two factors in follicle cells as well. In retinal cells, activation of EGFR was reported to cause SMRTER to be translocated from the nucleus to the cytoplasm (Tsuda et al., 2002). Such regulation of SMRTER by EGFR does not appear to apply to follicle cells because we found no evidence that the cellular pattern or the expression level of SMRTER is altered in follicle cells within the LDA domain (Fig. 3, supplementary material Fig. S3, not shown for egg chambers at the later stage). Therefore, a different mechanism may be employed by the EGFR pathway to influence the properties of SMRTER in follicle cells. We have identified two putative sites for phosphorylation by EGFR/MAPK in SMRTER, which may allow EGFR to down-regulate the properties of SMRTER. We speculate that phosphorylation of these two sites in SMRTER may compromise its transcriptional repressive properties or, alternatively, interfere with its interactions with specific transcription factors that repress Br. These possibilities are being investigated.

Additional developmental pathways that may be regulated by SMRTER

So far, we have focused on investigating the role of SMRTER in both the Notch and ecdysone pathways in the context of ovary and wing development. Since additional defects were seen in *Smr* mutant flies and larvae (Fig. 2) and since not all morphological defects displayed by *Smr* mutant adult flies entirely reflect the outcomes of increased Notch and ecdysone activities, we surmise that SMRTER may participate in further developmental and genetic pathways as well. These observations were not entirely unexpected because its vertebrate homologs, SMRT and N-CoR, are known to interact with other types of transcription factors.

One intriguing biological process that deserves attention in the future is SMRTER's differential influence on the development and survival of the sexes. For instance, the *BG01648* mutation disproportionally affects the survival rate and the development of females, but not males. So far, we have limited knowledge about how *BG01648* causes this sex-biased effect. Two recent studies, however, have shown that down-regulation of Notch activity by Sex-lethal (Sxl) is essential for the development of female flies (Penn and Schedl, 2007; Suissa et al., 2010). Since we have demonstrated a relationship between SMRTER and the Notch pathway, the demise or halted development of homozygous *BG01648* females may result from disruption of the Sxl-regulatory pathway when *Smr* is mutated.

Results from our complementation experiments also imply a possible maternal contribution of SMRTER to early embryonic development. As shown in Fig. 1B, surviving *BG01648/PL6* daughters were found only in the genetic experiments that used *BG01648/FM7* females, but not *PL6/FM7* females. Since our data indicate that *PL6* is a stronger allele than *BG01648*, the arrested development of *BG01648/PL6* embryos or larvae derived from the *PL6/FM7* females could result from an insufficient amount of maternally deposited SMRTER in the embryos. Further examination of the defects displayed by maternally and zygotically deprived *Smr1* mutant embryos or larvae may reveal which other genetic and developmental pathways – for example, segmentation, neurogenesis, or myogenesis – are also subject to regulation by SMRTER during early embryonic development.

Concluding remarks

In this study, we report that SMRTER is involved in the development of the ovary and the wing in *Drosophila*. In the context of these two developmentally distinct systems, we provide evidence showing that SMRTER participates not only in the ecdysone pathway, but also the Notch signaling pathway. Notch and nuclear receptors are important for the development of a wide variety of tissues both in vertebrates and invertebrates. Our results thus raise the possibility that SMRTER and its related factors may utilize mechanisms similar to those revealed in this study to influence the transcriptional properties of Notch and nuclear receptors in multiple developmental systems in both vertebrates and invertebrates. Our identification of *Smr1* as a loss-of-function allele and our characterization of two *Smr* RNAi lines that behave like *Smr1* also open up new possibilities for using these fly lines to study how this important class of transcriptional corepressors, under the influence of different signaling pathways, integrates the activities of different transcription factors, transcriptional cofactors, or chromatin modifying factors, in regulating the development and homeostasis of different tissues in *Drosophila*.

Materials and Methods

Fly Stocks

BG01648 (BL13116), *G0060* (referred to as *Smr1*in this study) (BL11653), *G0361*(BL11984), *G0124* (BL11915), *Df(1)N105* (BL962), *Df(1)JA26* (BL964), FRT9-2; *T155-Gal4*, *UAS-FLP* (BL5080), *Ubi-GFP*, *FRT[9-2]* (BL5154), and *UAS-Redstinger* lines (BL 8546, 8547) (Barolo et al., 2004) were obtained from the Bloomington Stock Center; *Smr* RNAi fly line (106701/KK) was obtained from the Vienna *Drosophila* RNAi Center (VDRC); *PL6* was obtained from Alain Vincent at the CNRS/UPS, France (Bourbon et al., 2002); *E(spl)mβ-CD2* was generated by Sarah Bray's lab (de Celis et al., 1998) and made available to us by Ken Irvine's lab. Inverse PCR experiments were carried out for *Smr* lines according to the protocol available from the Berkeley *Drosophila* Genome Project. The flanking sequences of the P-element insertion site of *G0060* are: GGCAGTTTAC ATTTAAGTTG AAGTTGGTAA TTAGTTTGTC AGCGC (Sp1 primer).

UAS-Smr.IR3 construct was generated by cloning *XbaI* digested fragments into the pWIZ vector (Lee and Carthew, 2003). The fragment was generated using two primers, 3-5′: ACG TCT AGA CAC ATG GGC ATG GTG GG and 3-3′: ACG TCT AGA TAG CTT GGG TGG CAC CT, and *Smr* cDNA as template. This construct was used to inject the w^{1118} embryos to generate the transformed flies using the Rainbow transgenic fly services. Multiple insertion lines were obtained and tested individually against different *Gal4* lines. Similar results were obtained from experiments using different *UAS-Smr.IR3* lines and a *Smr RNAi* line (106701/KK) from VDRC.

Egg quantification

Newly eclosed female w^{1118}, *BG01648*, and *BG01648/PL6* flies were isolated and kept on fresh food with males for 3 days at 25℃. On the third day, the flies were transferred to a fresh apple juice plate containing yeast paste. Plates were changed each day for the next four days, and the number of eggs laid on each plate per day was counted.

Generation of mosaic tissues

Recombined *G0060, FRT[9-2]* and *G0124, FRT[9-2]*were generated by recombination and selected based on darker eye color. To generate *G0060* (*Smr1*) or *G0124* somatic follicle cell clones, male *FRT[9.2]/Y; T155-Gal4,UAS-FLP* flies were crossed with female *Smr1, FRT[9-2]/FM7* or *G0124, FRT[9-2]/FM7* respectively. Offspring were raised at room temperature and 1–5 day old adult females carrying mutant clones were selected for ovary dissection on the basis of their apparent wing and leg phenotypes and the absence of *FM7* balancer. To generate larger clone size, females were kept at 25℃ for 2–3 days before ovary dissection.

To generate random mosaic clones in the imaginal discs or ovaries, male *Ubi-GFP, FRT[9.2]; Hsp70-FLP* was crossed with female *Smr1, FRT[9-2]/FM7*. First and second instar larvae were heat-shocked at 37℃ for 1.5 hours and were recovered in room temperature. Imaginal discs were dissected from third instar larvae, and mutant clones were identified by the absence of GFP or SMRTER. For ovaries, adult females lacking the *FM7* balancer were selected and heat shocked for 1 hour twice a day for three days. Ovaries were dissected 24 hours after the

final heat shock, and mutant clones were identified by the lack of GFP or SMRTER signal.

Immunostaining

Ovaries and larval tissues were dissected in PBS and fixed for 20 minutes with 4% paraformaldehyde in 1 × PBS. Samples were washed at least three times following fixation and subsequently blocked in a solution of 0.4% Triton X-100 in PBS (PBT) containing 1–2% Normal Donkey Serum (NDS). Primary antibodies were added to 200 µL of 1 × PBT/NDS solution, and samples were incubated with the primary antibodies at 4℃ overnight. The following day, samples were washed with 1 × PBS three times for at least five minutes per wash. Samples were incubated with the secondary antibodies in 200 µL of 1 × PBT/NDS solution at room temperature for 3 hours, and again washed 3 times for 15 minutes with 1 × PBS. Discs and ovaries were then dissected and conterstained with DAPI prior to being mounted in either Vectashield or Fluoro-Gel mounting mediums.

Monoclonal Hnt (IG9; mouse), Cut (2B10; mouse), and Br (25E9-D7; mouse) antibodies were obtained from the Developmental Studies Hybridoma Bank and were used at dilutions of 1:20, 1:50, and 1:100 respectively; Rabbit anti-Ttk69 was obtained from Andrew Travers' lab as used at a dilution of 1:1000 (Murawsky et al., 2001); Chicken anti-GFP (ab13970) was obtained from Abcam and used at a dilution of 1:1000; Mouse anti-CD2 (MCA154R) was obtained from AbD Serotec and used at a dilution of 1:100; Goat anti-Su(H) (dC-20) was obtained from Santa Cruz and used at a dilution of 1:50; Rat anti-Su(H) was a gift of François Schweisguth (Gho et al., 1996). Polyclonal rabbit anti-SMRTER antibody was used at a dilution of 1:400–1:800 (Tsai et al., 1999).

Methods for preparing and immunostaining squashed polytene chromosomes prepared from late third instar larvae are described in (Wang et al., 2008).

Nuclear size quantification

Confocal microscopy (Zeiss LSM 510 META) was used to capture images corresponding to the central regions of follicle cells found in S9 egg chambers from *T155-Smr1* flies. *Smr1* follicle cells and the neighboring control cells were determined by the absence or presence of SMRTER as detected by the antibody. DAPI (4′,6-diamidino-2-phenylindole)- stained nuclei were traced and filled in with Photoshop, and area measurements were taken with ImageJ 1.43 according to the scale of the original image. Statistical analysis including means, standard deviations, and *t*-tests were conducted in Excel, and histograms with normal distribution were generated in Minitab 15 and assembled in Photoshop.

Relative DNA quantification

DNA content measurements were based on the concept that the fluorescent signal from DAPI is proportional to the DNA content of nuclei (Manzini et al., 1983). Confocal microscopy (Zeiss LSM 510 META) was used to generate Z-sections for four independent early S9 egg chambers, as identified by morphology. The images were acquired under a 63 × water immersion objective, N.A. 0.8 with excitation wavelength at 364 nm and emission at 385 nm–470 nm. Each section was taken at 0.3 micron intervals with a pinhole size of 76 microns. Sufficient sections were scanned (18–25) to incorporate the entire follicle cell nucleus from top to bottom. To avoid signal contamination from neighboring follicle cells and the high-DNA nurse cells, only those follicle cells surrounding the oocyte, without overlapping the germinal vesicle or adjacent follicle cells, were considered. As per the area measurements, control and *Smr1* cells were identified by the absence and presence of SMRTER, respectively. Nuclei (40 for each control and *Smr1*) were grouped accordingly and their circumferences were traced at their widest points in ImageJ. ImageJ was then used to calculate the mean gray value (average signal intensity over the selected area) and integrated density (mean gray value multiplied by the selected area) for the DAPI signal in each of the 18–25 slices in each nucleus selected for our study. Integrated densities were summed in Excel to yield a total DAPI signal over the entire volume of each nucleus. Data from each of the four egg chambers were normalized to the mean of the control nuclei (control mean=100), and statistical analysis including means, standard deviations, and *t*-tests were conducted in Excel. Histograms with normal distribution were generated in Minitab and assembled in Photoshop.

Yeast Two-Hybrid

AH109 yeast cells were transformed with both pGBT9- and pGAD424-based constructs according to manufacturer's instructions (Clontech). SMRTER constructs were described previously (Tsai et al., 1999; Tong et al., 2011). Full length Su(H) was generated via polymerase chain reaction (PCR) using a cDNA library as a template; the resulting PCR fragment was subcloned into the pGBT9 (Clontech) vector. Transformed yeast cells were selected on SD –Leu/-Trp plates at 30℃ for 2 days. Interactions between the tested proteins were determined via spotting assay: five microliters of ten-fold serial dilutions of liquid saturated yeast culture were spotted onto the selective SD –Leu/-Trp/-His/-Ade plates or onto the control SD –Leu/-Trp growth plates. Growth differences were recorded following incubation of the plates at 30℃ for two days.

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