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The Osa-containing SWI/SNF chromatin-remodeling complex regulates stem cell commitment in the adult *Drosophila* intestine

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SUMMARY

The proportion of stem cells versus differentiated progeny is well balanced to maintain tissue homeostasis, which in turn depends on the balance of the different signaling pathways involved in stem cell self-renewal versus lineage-specific differentiation. In a screen for genes that regulate cell lineage determination in the posterior midgut, we identified that the Osa-containing SWI/SNF (Brahma) chromatin-remodeling complex regulates *Drosophila* midgut homeostasis. Mutations in subunits of the Osa-containing complex result in intestinal stem cell (ISC) expansion as well as enteroendocrine (EE) cell reduction. We further demonstrated that Osa regulates ISC self-renewal and differentiation into enterocytes by elaborating Notch signaling, and ISC commitment to differentiation into EE cells by regulating the expression of Asense, an EE cell fate determinant. Our data uncover a unique mechanism whereby the commitment of stem cells to discrete lineages is coordinately regulated by chromatin-remodeling factors.

KEY WORDS: Chromatin-remodeling factor, SWI/SNF, Osa, Intestinal stem cells, Self-renewal, Differentiation, *Drosophila*

INTRODUCTION

Adult tissue homeostasis is maintained by adult stem cells, which are multipotent cells that can self-renew and differentiate into functional cell types throughout the lifetime of the organism. The differentiation into multiple mature cell types and the self-renewal of adult stem cells are well balanced, and alterations in this equilibrium may cause diseases such as premature aging and carcinogenic transformation.

Like its mammalian counterpart, the adult midgut of *Drosophila* is maintained by multipotent intestinal stem cells (ISCs). After an asymmetrical division (de Navascués et al., 2012; Goulas et al., 2012; O'Brien et al., 2011), ISCs give rise to one new ISC (self-renewal) and one immature daughter cell, an enteroblast (EB). The EB can further differentiate into either an absorptive enterocyte (EC) or a secretory enteroendocrine (EE) cell without mitotic division (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Notch (N) signaling plays a major role in regulating ISC self-renewal and differentiation, and its loss leads to ISC expansion at the expense of ECs and to increased numbers of EE cells, probably because of the elevated expression of EE cell fate determinants *scute* (*sc*) and *asense* (*ase*), whereas N overactivation results in ISC differentiation into ECs (Bardin et al., 2010; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). The ligand of the N pathway, Delta (Dl), is specifically expressed in ISCs and unidirectionally switches the N signaling pathway on in neighboring EBs to promote differentiation toward ECs.

Stem cell fate is orchestrated by both intrinsic programs within the stem cells and extrinsic cues, namely the stem cell niche (Decotto and Spradling, 2005). Epigenetic programming, such as DNA methylation, histone modification and chromatin remodeling, which can generate variable patterns of gene expression from an invariant regulatory DNA sequence, has been identified as a major intrinsic mechanism for stem cell fate regulation (Hochedlinger and Plath, 2009; Juliandi et al., 2010). However, the epigenetic regulation of stem cell self-renewal and differentiation *in vivo* is not well understood.

SWI/SNF is an evolutionarily conserved and well characterized ATP-dependent chromatin-remodeling complex (Bouazoune and Brehm, 2006). A growing body of evidence indicates that many counterparts of SWI/SNF in mammals have a widespread role in tumor suppression; a high frequency of mutations in several SWI/SNF subunits have been identified in various cancers (Clapier and Cairns, 2009; Wilson and Roberts, 2011). There are at least two subtypes of the SWI/SNF (Brahma) complex in *Drosophila*: BAP and PBAP (Bouazoune and Brehm, 2006; Mohrmann et al., 2004). BAP and PBAP share common subunits including Brahma (Brm), Snr1 and Moira (Mor) but contain different signature proteins. Osa defines the BAP complex, which is required for normal embryonic segmentation and antagonizes Wingless signaling (Collins and Treisman, 2000; Treisman et al., 1997). The BAP complex also plays a role in the regulation of gene expression in response to Egfr signaling in the *Drosophila* wing (Terriente-Félix and de Celis, 2009). The mammalian homologs of Osa, BAF250a (ARID1A) and BAF250b (ARID1B), are required for maintaining the pluripotency of embryonic stem cells (Gao et al., 2008; Yan et al., 2008).

To further understand the molecular mechanisms of ISC self-renewal and differentiation, we carried out a transgenic RNAi screen and identified that the Osa-containing SWI/SNF complex regulates *Drosophila* ISC commitment to differentiation into discrete lineages. Loss-of-function mutations of subunits of the Osa-containing SWI/SNF complex resulted in ISC-like cell expansion at the expense of differentiated EC and EE cells. We demonstrated that Osa binds to the promoters of *Dl* and *ase* to regulate their expression, thus controlling ISC self-renewal and commitment to differentiation into EC and EE cells.

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MATERIALS AND METHODS

Fly strains

The following fly strains were used: *esg-Gal4* (Shigeo Hayashi, Riken); *esg-lacZ* (Stephen DiNardo, University of Pennsylvania); *mira-GFP* (Francois Schweisguth, CNRS); *Dl-lacZ* (Bruce Edgar, University of Heidelberg); *UAS-N$^{\Delta 34a}$* (Ken Irvine, Rutgers); *UAS-N^{DN}* (Mark Fortini, Thomas Jefferson University); *Su(H)GBE-lacZ* (Sarah Bray, University of Cambridge); *UAS-ase* (Yuh Nung Jan, UCSF); *ase-Gal4* (Tzumin Lee, Janelia Farm); *FRT^{82B}-osa^{2}* (James Kennison, NIH); and *FRT^{82B}-Snr1^{R3}* (Andrew Dingwall, Loyola University). *UAS-Dl, act-Gal4*, *UAS-sc*, *UAS-2XEYFP*, *tub-Gal80^{ts}* and fly strains used for MARCM clones (*FRT^{82B}-piM*; *act>y^{+}>Gal4, UAS-GFP*; *SM6, hs-flp*; *FRT^{82B} tub-Gal80*) were obtained from the Bloomington Drosophila Stock Center (BDSC) at Indiana University. The following transgenic RNAi lines were obtained from BDSC or the Vienna Drosophila RNAi Center (VDRC): *UAS-osa^{RNAi}* (V7810 and BL31266), *UAS-Snr1^{RNAi}* (V12645, V108599 and BL32372), *UAS-brm^{RNAi}* (V37721 and BL31712), *UAS-mor^{RNAi}* (V6969 and V110712) and *UAS-ase^{RNAi}* (V108511).

The *UAS-Snr1* transgene was generated by cloning full-length cDNA of *Snr1* into pUAST (Brand and Perrimon, 1993), and injecting purified DNA into the embryo using standard protocols.

MARCM clone assay

To induce MARCM clones of *FRT^{82B}-piM* (as a wild-type control), *FRT^{82B}-osa^{2}*, *FRT^{82B}-Snr1^{R3}* and *FRT^{82B}-Dl^{RevF10}*, we generated the following flies: *act>y^{+}>Gal4, UAS-GFP/SM6, hs-flp; FRT^{82B} tub-Gal80/FRT^{82B} mutant*. The genotype of flies used to generate *N* and *osa^{2}* double-mutant clones is *hs-flp, FRT^{19A} tub-Gal80/FRT^{19A} N^{55e11}; act>y^{+}>Gal4, UAS-GFP; FRT^{82B}-osa^{2}/FRT^{82B} tub-Gal80*. Three- or four-day-old adult female flies were heat shocked twice at an interval of 8-12 hours, at 37°C for 60 minutes. The flies were transferred to fresh food daily after the final heat shock, and their posterior midguts were processed for staining at the indicated times.

RNA interference (RNAi)-mediated gene depletion

Four male *UAS-RNAi* transgenic flies were crossed with eight virgin *act-Gal4, esg-lacZ/Cyo; tub-Gal80^{ts}/tub-Gal80^{ts}* or *esg-Gal4, UAS-2XEYFP/Cyo; tub-Gal80^{ts}/tub-Gal80^{ts}* virgin female flies at 18°C. One-week-old adult progenies of the correct genotype were transferred to new vials at 29°C for 7 days before dissection.

Quantitative PCR (qPCR) and chromatin immunoprecipitation (ChIP) assay

Total RNA from adult guts was isolated using the RNeasy Mini Kit (Qiagen) with on-column DNase digestion to remove genomic DNA. cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen). Real-time PCR analysis was performed on the Mastercycler Realplex real-time PCR system (Eppendorf) using SYBR Green PCR Master Mix (Clontech). qPCR results are represented as mean ± s.e.m. of three biological replicates. Primer pairs for qPCR are listed in supplementary material Table S1.

Two hundred fly intestines were dissected for ChIP assays using the Magna ChIP G Tissue Kit (Millipore) with some modification. The primer pairs used to detect immunoprecipitated DNA are listed in supplementary material Table S1.

Antibody production

Polyclonal rabbit antisera were raised against 6×His fusion proteins containing amino acids 2-180 of the Snr1 protein. To produce the 6×His fusion protein, a 537 bp fragment of *Snr1* was amplified by PCR using primers 5′-AGTAGAATTCGCACTGCAGACATACGGGGA-3′ and 5′-AGTAGCGGCCGCTCACTCTAGCTCCATGTCCAGTC-3′ (restriction sites underlined). The amplified fragments were cloned into the *Eco*RI and *Not*I sites of PET-28a (+) (Novagen). 6×His-Snr1 fusion protein was expressed in *E. coli* BL21(DE3) pLysS, purified on Ni-NTA agarose columns (Qiagen) and used to immunize rabbits as described previously (Zeng et al., 2007).

Histology and image capture

The fly intestines were dissected in PBS and fixed in PBS containing 4% formaldehyde for 20 minutes. After three 5-minute rinses with PBT (PBS + 0.1% Triton X-100), the samples were blocked with PBT containing 5% normal goat serum overnight at 4°C. Then, the samples were incubated with primary antibody at room temperature for 2 hours and then with fluorescent secondary antibody for 1 hour at room temperature. Samples were mounted in Vectashield mounting medium with DAPI (Vector Laboratories). We used the following antibodies: mouse anti-β-Gal (1:200; Clontech); mouse anti-Dl [1:50; Developmental Studies Hybridoma Bank (DSHB)]; mouse anti-Pros (1:50; DSHB); nc82 (1:20; DSHB); rabbit polyclonal anti-Pdm1 (1:1000; a gift from X. Yang, Zhejiang University); rabbit anti-Spdo (1:1000; a gift from J. Skeath, Washington University in St Louis); mouse anti-Osa (1:20; DSHB); rabbit anti-Snr1 (1:1000; this study); rabbit anti-Ase (1:2000; a gift from Yuh Nung Jan); guinea pig anti-Sc (1:1000; a gift from S. Crews, UNC-Chapel Hill) and chicken anti-GFP (1:3000; Abcam). Secondary antibodies were goat anti-mouse, anti-chicken and anti-rabbit IgG conjugated to Alexa 488, Alexa 568 or Alexa 649 (1:400; Molecular Probes). Images were captured with a Zeiss LSM 510 confocal system and processed with LSM5 Image Browser (Zeiss) and Adobe Photoshop.

Quantification and statistical analysis

To quantify the number of *escargot* (*esg*)$^{+}$ or Prospero (Pros)$^{+}$ cells in Fig. 1 and Fig. 5, the *esg*$^{+}$ or Pros^{+} cells were counted in a 5×10^{3} μm^{2} area of the field. In Fig. 4, all of the images were taken with the same confocal settings and the fluorescence intensity was measured using LSM5 Image Browser. All data were analyzed using Student's *t*-test and sample size (*n*) is shown in the text.

RESULTS

Knockdown of the Osa-containing SWI/SNF chromatin-remodeling complex results in the expansion of *esg*-expressing cells

To identify new regulators of ISCs, we carried out a screen in which a collection of transgenic RNAi lines from the Vienna *Drosophila* RNAi Center and the Bloomington Stock Center (Dietzl et al., 2007; Ni et al., 2009) were crossed with *act-Gal4, esg-lacZ; Tub-Gal80^{ts}* (referred to as *act^{ts}, esg-lacZ*) flies. One-week-old adult flies were shifted to the restrictive temperature (29°C) for 1 week, dissected and stained, and then examined for *esg-lacZ*-labeled progenitors.

One of the first genes identified in this screen was *osa*. Knockdown of *osa* by transgenic RNAi (*osa^{RNAi}*, V7810) resulted in a dramatic expansion of *esg*$^{+}$ cells (average of 49.5 *esg*$^{+}$ cells/5×10^{3} μm^{2}, *n*=31; Fig. 1B,B′,F) compared with the wild-type (7.8 *esg*$^{+}$ cells/5×10^{3} μm^{2}, *n*=24; Fig. 1A,A′,F) posterior midgut. To test whether Osa functions specifically in progenitors, we knocked down *osa* specifically in ISCs and EBs using *esg-Gal4, UAS-GFP; tub-Gal80^{ts}/+* (referred to as *esg^{ts}*). Compared with the wild-type control (8.6 *esg*$^{+}$ cells/5×10^{3} μm^{2}, *n*=30; Fig. 1C,C′,G), knockdown of *osa* using *esg^{ts}* also caused dramatic expansion of the *esg*$^{+}$ cells (V7810; 46.1 *esg*$^{+}$ cells/5×10^{3} μm^{2}, *n*=33; Fig. 1D,D′,G). Knockdown of genes by dsRNAs often produces false-positive phenotypes because of off-target effects (Kulkarni et al., 2006). We ruled out the possibility of false-positive effects and confirmed the *osa* phenotype with a second transgenic RNAi line (BL31266; 44.7 *esg*$^{+}$ cells/5×10^{3} μm^{2}, *n*=42; Fig. 1E,E′,G) generated from independent sequences (Ni et al., 2009). We further stained wild-type and *osa^{RNAi}* midguts for phospho-Histone H3 (pH3), a specific marker for mitotic cells. More pH3^{+} cells were found in *osa^{RNAi}* posterior midguts than in wild type (supplementary material Fig. S1), indicating that these *esg*$^{+}$ cells kept dividing to achieve the cell expansion.

Osa is a component of the BAP SWI/SNF complex (supplementary material Fig. S2E) (Bouazoune and Brehm, 2006; Clapier and Cairns, 2009; Collins et al., 1999; Vázquez et al., 1999). Knockdowns of three other components (*Snr1*, *brm* and *mor*) by RNAi also resulted in significant increases of *esg-lacZ*$^{+}$ cells in the

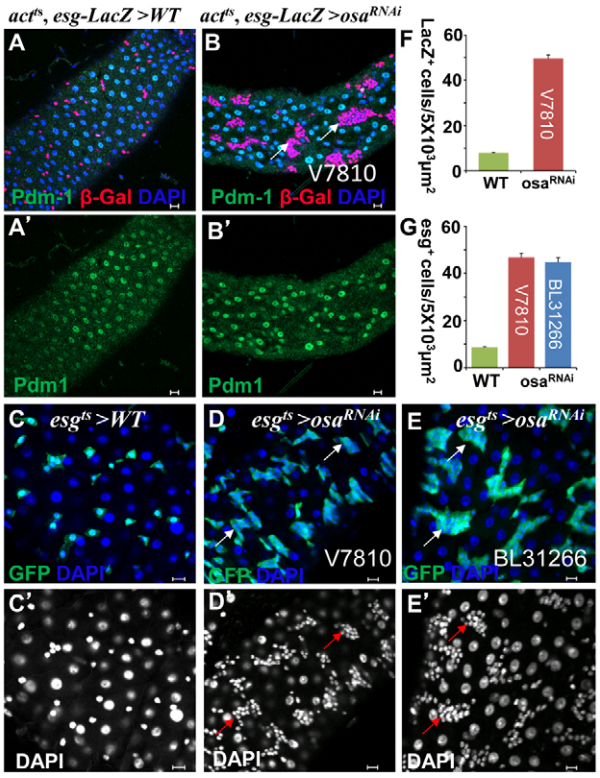

Fig. 1. Knockdown of *Drosophila osa* results in the expansion of *esg*$^+$ cells. (**A-B′**) Compared with the wild-type control (A,A′), the knockdown of *osa* (B,B′) by ubiquitous driver *act*^{ts} results in the dramatic expansion of *esg*$^+$ cells (marked by *lacZ* expression detected by β-Gal antibody, red, arrows). These *esg*$^+$ diploid cells do not express the EC-specific marker Pdm1 (Nubbin – FlyBase) (green). DAPI, blue. (**C-E′**) In comparison to the wild-type control (C,C′), the knockdown of *osa* by the progenitor-specific driver *esg*^{ts} also leads to the expansion of *esg*$^+$ cells (arrows). Two independent RNAi lines (V7810 in D,D′ and BL31266 in E,E′) show the same phonotype. GFP, green; DAPI, blue or white. (**F**) Quantification of *esg*$^+$ cells in wild type (WT) and *osa*^{RNAi} driven by *act*^{ts} in the posterior midgut. (**G**) Quantification of *esg*$^+$ cells in wild type and *osa*^{RNAi} (V7810 and BL31266) driven by *esg*^{ts} in the posterior midgut. Data are mean ± s.e.m. Scale bars: 20 μm in A-B′; 10 μm in C-E′.

posterior midguts (supplementary material Fig. S2A-D,F). However, the phenotypes of *brm* or *mor* knockdowns are generally much weaker than those of *osa* or *Snr1* knockdowns, suggesting that the other components in the complex might partially compensate the loss of function of *brm* or *mor* in ISCs, or that ISC fate regulation is a mechanistically novel function of a subset of SWI/SNF complexes. We focused on analyzing the loss-of-function phenotypes of *osa* and *Snr1*.

Based on the important function of Osa and Snr1 in ISCs, we examined their expression patterns in the midgut using antibodies. In the wild-type flies, Osa and Snr1 were expressed in all cells, including ISCs and EBs, in the posterior midgut and displayed nuclear localization (supplementary material Fig. S3A,A′,C,C′), which is consistent with their role in chromatin remodeling. As expected, in GFP-labeled *osa*^{RNAi} and *Snr1*^{RNAi} cells, the protein levels were reduced to an undetectable level, suggesting that the RNAi almost completely depletes Osa and Snr1 expression (supplementary material Fig. S3B,B′,E,E′). However, higher expression of Snr1 was detected in ISCs and EBs when *UAS-Snr1* was expressed in midguts by *esg*^{ts} (supplementary material Fig. S3D,D′), which confirms the specificity of our new anti-Snr1 serum.

Knockdown of Osa produces excess ISC-like cells

Esg is a marker of both ISCs and EBs (Micchelli and Perrimon, 2006). To characterize expanded *esg*$^+$ cells in *osa*^{RNAi} midguts, we analyzed the expression of *Su(H)GBE-lacZ*, a marker of EBs (Ohlstein and Spradling, 2007), in wild-type and *osa*^{RNAi} posterior midguts. In wild-type midguts, only some *esg*$^+$ cells expressed *Su(H)GBE-lacZ* (Fig. 2A,A′), which is consistent with its reported expression in EBs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). In *osa*^{RNAi} posterior midguts (Fig. 2B,B′), *Su(H)GBE-lacZ*-expressing cells were missing, suggesting that the expanded *esg*$^+$ cells in *osa*^{RNAi} posterior midguts are not EBs. *miranda* promoter-GFP (Mira-GFP) is a target of Daughterless-dependent bHLH transcriptional activity and is specifically expressed in ISCs (Bardin et al., 2010). Interestingly, Mira-GFP is indeed expressed in all expanded *esg*$^+$ cells in *osa*^{RNAi} posterior midguts (supplementary material Fig. S4A,A′), which suggests that these *esg*$^+$ cells might be ISC-like cells.

Dl and Sanpodo (Spdo) are markers of ISCs (Ohlstein and Spradling, 2007; Perdigoto et al., 2011). We first examined Dl expression in the wild-type (*esg*^{ts}*>WT*; Fig. 2C,C′) and *osa*^{RNAi} (*esg*^{ts}*>osa*^{RNAi}; Fig. 2D,D′) posterior midguts. In wild-type midguts, some of the *esg*$^+$ cells that are ISCs express cytoplasmic Dl (Fig. 2C,C′). Surprisingly, none of the *esg*$^+$ cells in the *osa*^{RNAi} midguts expressed Dl (Fig. 2D,D′). We also found that Dl expression was undetectable in *Snr1*^{RNAi} midguts (*esg*^{ts}*>Sn1*^{RNAi}; supplementary material Fig. S4B,B′). In wild-type posterior midguts, some of the *esg*$^+$ cells that are ISCs expressed Spdo (Fig. 2E,E′). In the *osa*^{RNAi} midguts (Fig. 2F,F′), all *esg*$^+$ cells expressed Spdo, further suggesting that the *esg*$^+$ cells are ISC-like cells.

Osa and Snr1 autonomously regulate ISC self-renewal and differentiation

To further determine the function of Osa in regulating ISC self-renewal or differentiation, we generated wild-type and *osa*^{2} (Vázquez et al., 1999) clones using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999). Clones marked homozygous for wild type (Fig. 3A,A′,C) and *osa*^{2} (Fig. 3B,B′,D) were generated in the posterior midgut and identified by GFP expression. Eight days after clone induction (ACI) we observed that, in GFP-labeled wild-type clones (Fig. 3A,A′,C), there were differentiated EC cells with large nuclei (asterisk in Fig. 3A,A′), Pros$^+$ EE cells (arrowheads in Fig. 3A,A′), Dl$^+$ ISCs (arrows in Fig. 3A,A′) and Spdo$^+$ ISCs (arrow in Fig. 3C). However, *osa*^{2} mutant clones were almost devoid of EC and EE cells, and all cells were Dl$^-$ and Spdo$^+$ ISC-like cells (Fig. 3B,B′,D).

To more precisely assess the phenotypes, we counted the number of Spdo$^+$ ISC-like cells and Pros$^+$ EE cells in GFP-labeled MARCM clones of wild type, *osa*^{2} and *Dl*^{RevF10} (a null allele of *Dl*; supplementary material Fig. S5) (Heitzler and Simpson, 1991) midguts at 4 and 8 days ACI (Fig. 3E,F). In the wild-type control clones, 23% and 18% of cells were Spdo$^+$ ISCs at 4 and 8 days ACI (*n*=20, 21), respectively. However, 91% and 98% of cells were Spdo$^+$ ISC-like cells in *osa*^{2} clones at 4 and 8 days ACI (*n*=20, 21), respectively; and 70% and 67% of cells were Spdo$^+$ ISC-like cells in *Dl*^{RevF10} clones at 4 and 8 days ACI (*n*=20, 21), respectively. The Pros$^+$ EE cells were dramatically reduced in *osa*^{2} mutant clones. At 4 and 8 days ACI, respectively, 9.7% and 11% of cells were EE cells in wild-type clones and only 1.8% and 0.9% of cells were EE cells

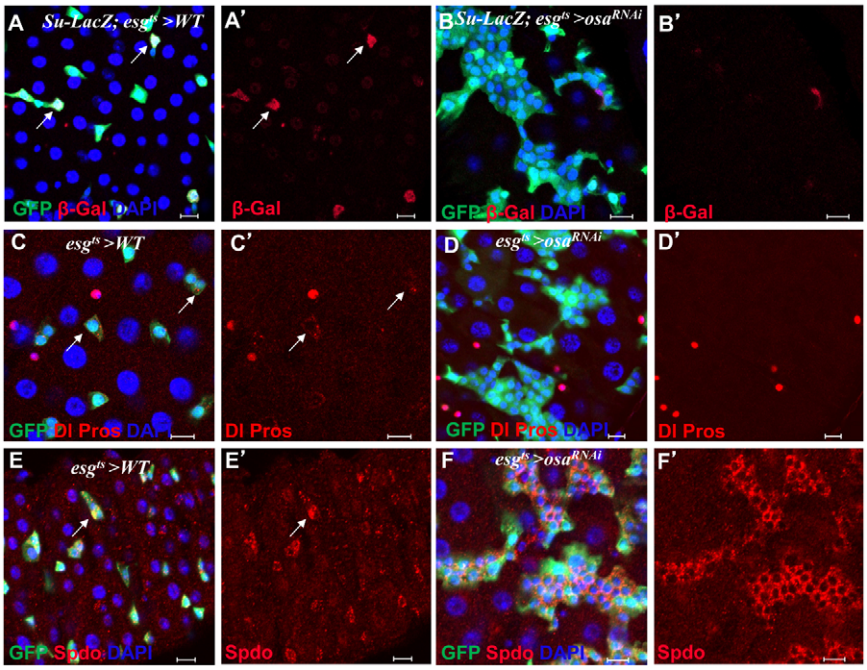

Fig. 2. Knockdown of *osa* produces excess ISC-like cells. The wild-type control and osa^{RNAi} were expressed in the posterior midgut using the esg^{ts} driver. (**A-B′**) In the wild-type midgut, some esg^+ cells that are EBs express *Su(H)GBE-lacZ* (arrows in A,A′). However, no *Su(H)GBE-lacZ*$^+$ EBs were found in the esg^+ cell cluster in osa^{RNAi}, suggesting that these cells are not EBs (B,B′). (**C-D′**) Dl is expressed in ISCs in the wild-type midgut (arrows in C,C′), whereas no Dl expression was detected in the expanded esg^+ cells caused by osa^{RNAi} (D,D′). (**E-F′**) In the wild-type midgut, some of the esg^+ cells are Spdo$^+$ ISCs (arrow in E,E′). All esg^+ cells in the osa^{RNAi} midgut are Spdo$^+$ (F,F′), suggesting that these cells are ISC-like cells. Scale bars: 10 μm.

in osa^2 clones, whereas 24.9% and 31.8% of cells were EE cells in Dl^{RevF10} clones.

We similarly generated $Snr1^{R3}$ (Zraly et al., 2003) MARCM clones and found that *Snr1* mutation also resulted in ISC expansion without differentiation (supplementary material Fig. S6). Together, these results suggest that Osa and Snr1 might regulate ISC self-renewal and differentiation into both EC and EE cells.

Osa regulates *Dl* expression in ISCs

The undetectable level of Dl expression, as judged by antibody staining, encouraged us to further examine the expression of *Dl* using *Dl-lacZ*, an enhancer trap at the *Dl* locus (Beebe et al., 2010; Jiang et al., 2009; Zeng et al., 2010). In wild-type posterior midguts, *Dl-lacZ* was highly enriched in ISCs (fluorescence intensity of 3991, n=56; Fig. 4A,A′,C). However, the expression of *Dl-lacZ* was dramatically reduced in osa^{RNAi} posterior midguts (esg^{ts}>osa^{RNAi}; fluorescence intensity of 200, n=58; Fig. 4B,B′,C). We also detected *Dl* mRNA expression by qPCR and found that the level of *Dl* mRNA was significantly reduced in the osa^{RNAi} midguts (Fig. 4D). These data suggest that Osa regulates *Dl* expression at the transcriptional level in the posterior midgut.

Expression of the *Dl* receptor N was unaffected, as N expression can be detected in the osa^{RNAi} midguts as well as in the wild type (supplementary material Fig. S4C-D′). These data suggest that Osa might specifically regulate *Dl* expression at the transcriptional level to control ISC self-renewal and differentiation of ISCs into ECs.

Dl expression is sufficient to rescue the ISC tumor phenotype but not the EE cell phenotype of *osa* mutants

When an activated form of N is expressed in the posterior midgut (esg^{ts}>$N^{\Delta 34a}$), all ISCs differentiate into ECs (compare supplementary material Fig. S7B with S7A). In the osa^{RNAi} midgut (esg^{ts}>osa^{RNAi}; supplementary material Fig. S7C), excess ISC-like cells were found at the expense of differentiated EC and EE cells. To determine the epistatic relationship between Osa and the N signaling pathway, we expressed the constitutively activate form of N ($N^{\Delta 34a}$) in the osa^{RNAi} midgut (esg^{ts}>osa^{RNAi}+$N^{\Delta 34a}$; supplementary material Fig. S7D). After shifting the adult flies to the restrictive temperature (29°C) for 7 days, all ISCs had differentiated into ECs. These results support the idea that Osa functions upstream of N in regulating EC fate.

Since Osa functions upstream of N signaling, and *Dl* expression is blocked in the osa^{RNAi} midgut as well, Osa might regulate ISC differentiation into ECs by controlling *Dl* expression. To test this, we expressed *UAS-Dl* in osa^{RNAi} posterior midguts (esg^{ts}>osa^{RNAi}+*UAS-Dl*; Fig. 5C) or in GFP-marked wild-type and osa^2 mutant MARCM clones (Fig. 5E; supplementary material Fig. S7E-F′). *UAS-Dl* expression using the esg^{ts} driver rescues the ISC tumor phenotype in osa^{RNAi} midguts to a phenotype resembling wild type (8.5, 43.5 and 10.5 esg^+ cells/5×10^3 μm^2; n=23, 36 and 42, respectively; Fig. 5A-C,F). In osa^2 mutant MARCM clones, all GFP-marked cells were ISC-like diploid cells (Fig. 3B,B′,D, Fig. 5D). *UAS-Dl* expression in osa^2 mutant MARCM clones rescued their phenotypes to that resembling wild-type clones including ISCs (or EBs) and polyploid ECs (Fig. 5E; supplementary material Fig. S7E-F′).

We also quantified the number of Pros$^+$ EE cells in the midguts of wild type (Fig. 5A), osa^{RNAi} (Fig. 5B) and osa^{RNAi} with *UAS-Dl* expression (Fig. 5C). *Dl* expression does not rescue the EE cell-loss phenotype in osa^{RNAi} midguts (3.8, 1.8 and 1.7 EE cells/5×10^3 μm^2; n=23, 36 and 42, respectively; Fig. 5G).

These data suggest that Osa regulates ISC self-renewal and differentiation into ECs by controlling Dl expression and regulates EE cell formation by controlling other gene(s).

The Osa-containing SWI/SNF complex functions downstream of N in regulating EE cell formation

In addition to regulating *Dl* expression to control EC fates, the Osa-containing SWI/SNF complex might control EE cell formation by regulating other signal(s). We examined the epistatic relationship of N signaling and the SWI/SNF complex in regulating EE cell fate determination. Expressing a dominant-negative form of *N* in the posterior midgut (esg^{ts}>N^{DN}; 50.9 EE cells/5×10^3 μm^2, n=33; Fig. 5H,K) resulted in the formation of

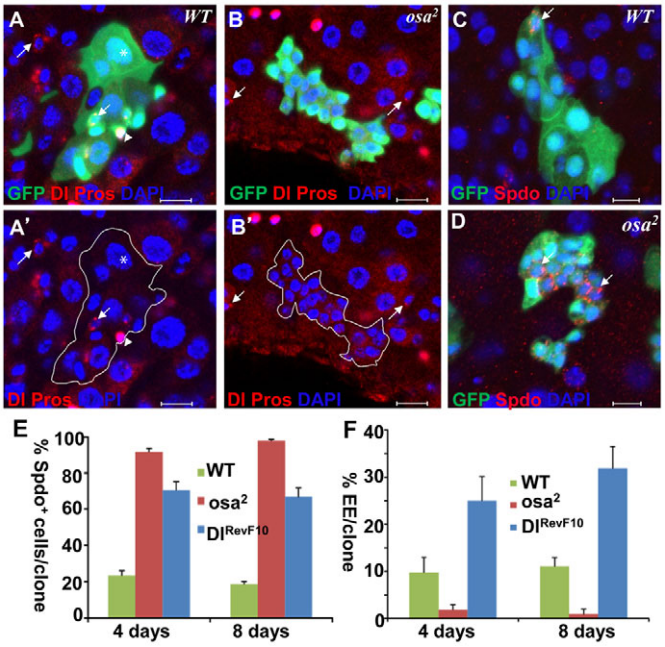

Fig. 3. Osa cell-autonomously regulates ISC self-renewal and differentiation into EE cells. GFP$^+$ clones were generated in the posterior midguts of flies of the indicated genotypes using the MARCM technique. They were stained on the eighth day after clone induction (ACI) with the indicated antibodies. DAPI, blue or white. (**A**,**A′**) The *FRT*^{82B}-*piM* wild-type clone at 8 days ACI. There are ISCs labeled by Dl (cytoplasmic red, arrows), EE cells labeled by Pros (nuclear red, arrowhead), and polyploid ECs (asterisk) in the clone. (**B**,**B′**) The *FRT*^{82B}-*osa*^{2} clone at 8 days ACI. All cells in the clone are diploid and do not express Dl, whereas neighboring wild-type ISCs express Dl (arrow). (A′,B′) GFP-labeled MARCM clone is outlined. (**C**,**D**) In the wild-type clone (C), Spdo is only expressed in ISCs (arrow), whereas all cells in the *osa*^{2} clone (D) express the ISC-specific marker Spdo. (**E**) Analysis of the percentage of Spdo$^+$ ISC-like cells in wild-type, *osa*^{2} and *Dl* clones at 4 days and 8 days ACI. Almost all cells in the *osa*^{2} clone are Spdo$^+$ ISC-like cells. (**F**) Analysis of the percentage of Pros$^+$ EE cells in wild-type, *osa*^{2} and *Dl* clones at 4 and 8 days ACI. *osa*^{2} clones contain significantly fewer Pros$^+$ EE cells than *Dl* and wild-type clones. Data are mean ± s.e.m. Scale bars: 10 μm.

excess EE cells. The expression of *osa*^{RNAi} (*esg*^{ts}>*N*^{DN}+*osa*^{RNAi}; 3.2 EE cells/5×10^3 μm^2, *n*=45; Fig. 5I,K) and *Snr1*^{RNAi} (*esg*^{ts}>*N*^{DN}+*osa*^{RNAi}; 3.2 EE cells/5×10^3 μm^2, *n*=25; Fig. 5J,K) in *N*^{DN} midguts completely suppressed the phenotype of excess EE cells to wild-type midgut levels (3.7 EE cells/5×10^3 μm^2; *n*=30; Fig. 5A,K).

In *N* null (*N*^{55e11}) mutant MARCM clones, excess Pros$^+$ EE cells were found (38% of the cells are Pros$^+$ EE cells, *n*=30; supplementary material Fig. S7G,I). As in *osa*^{2} mutant MARCM clones (Fig. 3B,B′,F), very few Pros$^+$ EE cells were detected in *N*^{55e11}; *osa*^{2} double-mutant MARCM clones (only 1.2% of the cells are Pros$^+$ EE cells, *n*=40; supplementary material Fig. S7H,I). Consistently, we also noted that *osa*^{2} repressed *Dl* expression in *N*^{55e11} clones (supplementary material Fig. S7H). To exclude the possibility that Osa directly regulates expression of the EE cell marker Pros in contexts other than in EE cell formation, we identified another EE cell-specific marker, nc82 (Bruchpilot – FlyBase), which labels the synaptic active zone in the *Drosophila* neuromuscular junction (Wagh et al., 2006). nc82 exhibits punctate staining at the membrane of Pros$^+$ EE cells (supplementary material Fig. S8A-B′); however, unlike in the *N*^{DN} midgut where there are many nc82$^+$ EE cell clusters (supplementary material Fig. S8D,D′), we could not detect the expansion of any nc82$^+$ EE cells in *osa*^{RNAi} midguts (*esg*^{ts}>*osa*^{RNAi}; supplementary material Fig. S8C,C′). Together, these data suggest that the Osa-containing SWI/SNF complex functions downstream of N in regulating EE cell formation.

Osa regulates EE cell formation through Ase

Two transcription factors, Sc and Ase, have been shown to play a major role in EE cell fate determination and to be upregulated in the *N*^{DN} midgut by mRNA profiling (Bardin et al., 2010). Expression of Sc and Ase in the posterior midgut was below the detection level using specific antibodies (Brand et al., 1993; Stagg et al., 2011) (supplementary material Fig. S9A,A′,C,C′). Nevertheless, Sc and Ase were readily detected in the midgut with ectopic *sc* and *ase* expression driven by *esg*^{ts} (*esg*^{ts}>*UAS-sc* and *esg*^{ts}>*UAS-ase*) for 24 hours at 29°C (supplementary material Fig. S9B,B′,D,D′). In addition, consistent with previous observations by mRNA profiling (Bardin et al., 2010), both Sc and Ase were upregulated in the *N*^{DN} midgut as assessed by qPCR (Fig. 6A,B) and antibody immunofluorescence (compare supplementary material Fig. S9G with S9A,A′ and S9I with S9C,C′). Using the more sensitive *ase-Gal4* (*ase-Gal4*>*UAS-mCD8-GFP*) transgene (Zhu et al., 2006), we detected that *ase* is weakly expressed in ISCs and EBs but not in Pros$^+$ EE cells (supplementary material Fig. S9E-F′).

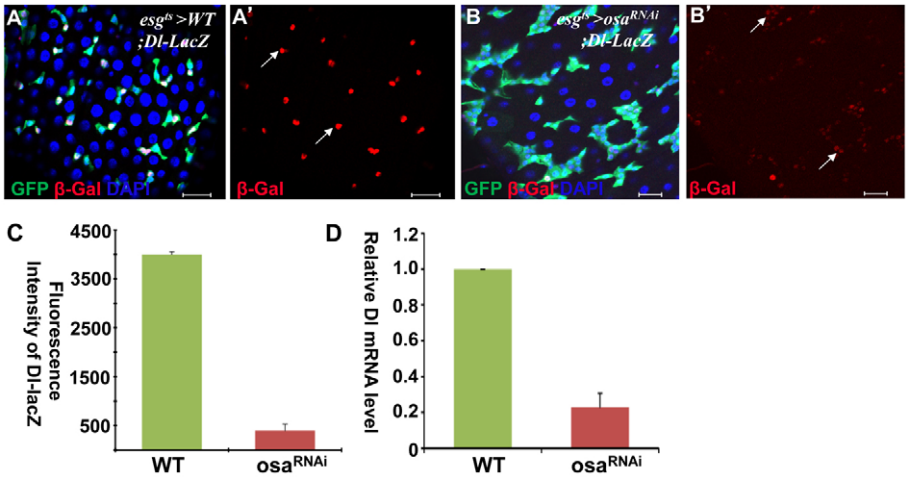

Fig. 4. Osa regulates *Dl* expression at the transcriptional level in ISCs. (**A-B′**) The transcriptional reporter *Dl-lacZ* is highly expressed in ISCs in the wild-type midgut (arrows in A,A′). No, or very weak, expression of *Dl-lacZ* was detected in ISCs that express *osa*^{RNAi} (arrows in B,B′). The wild-type control and *osa*^{RNAi} were driven by *esg*^{ts} and stained with the antibodies indicated. (**C**) Analysis of the fluorescence intensity of *Dl-lacZ* in wild-type ISCs and ISC-like cells in the *osa*^{RNAi} midgut indicates that the latter have significantly less *Dl-lacZ* expression. (**D**) Quantification of *Dl* mRNA in wild-type and *osa*^{RNAi} midguts by qPCR. Compared with wild-type, *osa*^{RNAi} midguts have significantly lower *Dl* mRNA levels. Data are mean ± s.e.m. Scale bars: 10 μm.

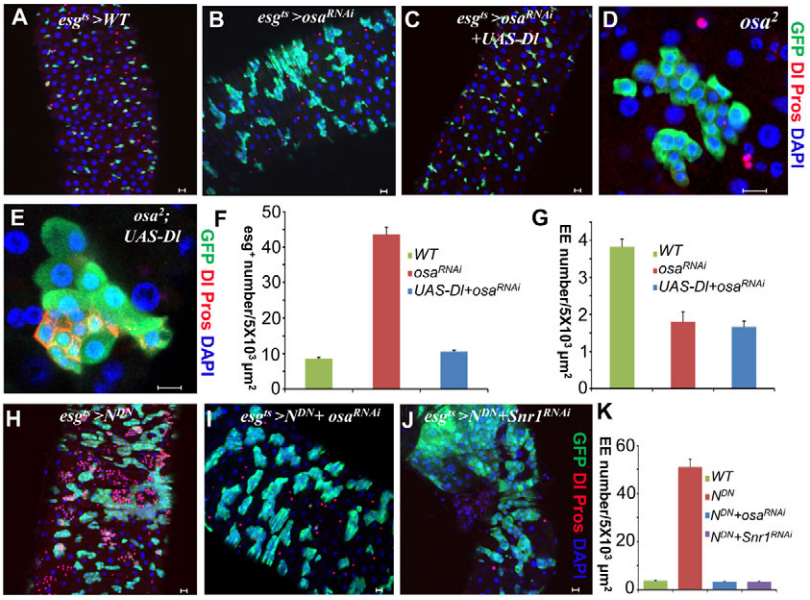

Fig. 5. Repression of ISC expansion by expression of Dl in *osa* mutants. (**A-C**) The wild-type control (A), osa^{RNAi} (B) and osa^{RNAi}+*UAS-Dl* (C) are expressed in the posterior midgut using esg^{ts}. The expression of osa^{RNAi} results in ISC-like cell expansion (B), and the expression of *UAS-Dl* rescues the ISC-like cell expansion phenotype caused by *osa* depletion (compare C with B and A). (**D**,**E**) GFP$^+$ clones were generated in the posterior midguts of flies of the indicated genotypes by the MARCM technique, and were stained with the indicated antibodies at 6 days ACI. (D) osa^2 mutant MARCM clones form ISC-like tumors without Dl expression. (E) The expression of *UAS-Dl* in osa^2 mutant MARCM clones rescues the ISC-like tumor phenotype. (**F**) Analysis of the percentage of esg^+ cells in wild type, in those expressing osa^{RNAi}, and in those expressing osa^{RNAi}+*UAS-Dl*. (**G**) Analysis of the percentage of Pros$^+$ EE cells in wild type, in those expressing osa^{RNAi}, and in those expressing osa^{RNAi}+*UAS-Dl*. The expression of *Dl* in osa^{RNAi} midguts does not increase the number of Pros$^+$ EE cells to the wild-type level. (**H-K**) Osa and Snr1 function downstream of N in regulating EE cell formation. *UAS-*N^{DN} (H), *UAS-*N^{DN}+osa^{RNAi} (I) and *UAS-*N^{DN}+$Snr1^{RNAi}$ (J) were expressed in the posterior midgut using esg^{ts}. The expression of osa^{RNAi} in the N^{DN} midgut suppresses the phenotype of excess EE cells. (K) Quantification of EE cell number in the indicated genotypes. Data are mean ± s.e.m. Scale bars: 10 μm.

To examine the relationship of Osa to Sc and Ase, we first compared mRNA levels of *sc* and *ase* in wild-type and osa^{RNAi} midguts by qPCR. Although *sc* mRNA levels were lower in osa^{RNAi} than in N^{DN} midgut, *sc* mRNA levels were upregulated in osa^{RNAi} compared with wild-type midgut (Fig. 6A). However, unlike in the N^{DN} midgut (Bardin et al., 2010), the *ase* mRNA levels were significantly lower in osa^{RNAi} than in wild-type control midgut (Fig. 6B). We further confirmed the expression levels of Sc and Ase in osa^{RNAi} midgut by antibody immunofluorescence. osa^{RNAi} midguts had much higher expression of *sc* than wild-type midguts (compare supplementary material Fig. S9H with S9A,A′), whereas *ase* expression was undetectable in osa^{RNAi} as in wild-type midguts (compare supplementary material Fig. S9J with S9C,C′).

These data suggest that the Osa-containing SWI/SNF complex might control EE cell formation through regulating the expression of *ase*. Indeed, expression of ase^{RNAi} in the N^{DN} midgut (Fig. 6D; esg^{ts}>N^{DN}+ase^{RNAi}) suppressed the excess EE cell phenotype of N^{DN} (compare Fig. 6D with 6C). When we tried to overexpress *ase* in the osa^{RNAi} midgut (esg^{ts}>osa^{RNAi}+*ase*) it suppressed the EE cell-loss phenotype of osa^{RNAi} (only 1.8 EE cells/5×10^3 μm^2 in osa^{RNAi} midgut but 39.7 EE cells/5×10^3 μm^2 in the osa^{RNAi}+*ase* midgut; *n*=36 and 33, respectively; Fig. 6E-G). These data together suggest that Osa regulates EE cell formation through Ase.

Osa and Snr1 bind to the promoters of *Dl* and *ase*

We further examined the interaction of Osa and Snr1 proteins with the promoters of *Dl*, *ase* and *spdo* in a ChIP assay using Osa- and

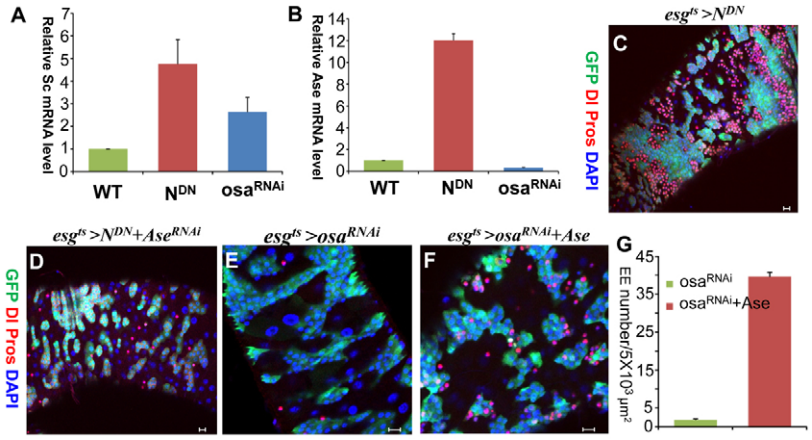

Fig. 6. Osa regulates EE cell fate specification by controlling *ase* expression. (**A**,**B**) *sc* (A) and *ase* (B) mRNA levels in wild-type, N^{DN}, and osa^{RNAi} midguts by qPCR. Both *ase* and *sc* are upregulated in the N^{DN} midgut. Compared with the wild-type control, *sc* mRNA was upregulated but *ase* mRNA was downregulated in the osa^{RNAi} midgut. (**C**,**D**) Knockdown of *ase* in the N^{DN} midgut (D) suppresses the phenotype of excess EE cells associated with expressing N^{DN} (C, compare D with C). (**E**,**F**) The expression of *ase* in the osa^{RNAi} midgut (F) results in a dramatic increase in EE cells (compare F with E). All flies were driven by esg^{ts} and stained by indicated antibodies. Scale bars: 10 μm. (**G**) Quantification of EE cell number in osa^{RNAi} and osa^{RNAi}+*ase* midgut. Data are represented as mean ± s.e.m.

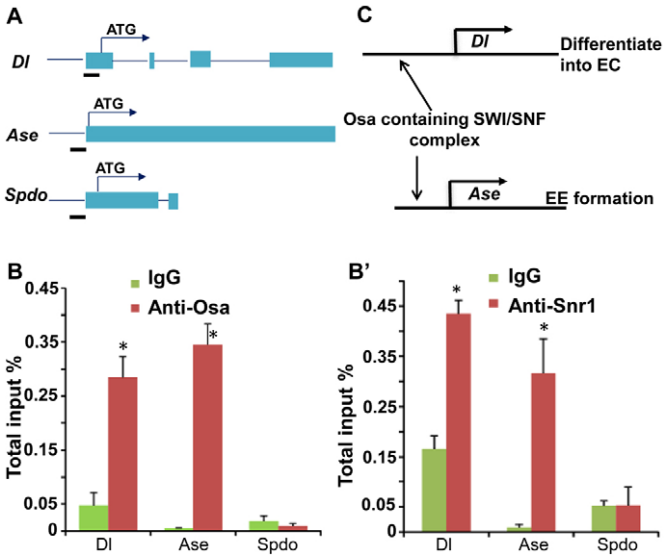

Fig. 7. Osa and Snr1 bind to the promoters of *Dl* and *ase*. (**A**) The *Drosophila* genomic locus of *Dl*, *ase* and *spdo*. The black bar under the locus indicates the region used for ChIP assay. (**B**,**B′**) Osa- and Snr1-specific antibodies or unrelated IgG (control) were used in the ChIP assay. Osa and Snr1 are associated with the *Dl* and *ase* promoters at the chromatin level, but neither binds to the *spdo* promoter. **P*<0.01 (Student's *t*-test). Data (qPCR) are mean ± s.d. (**C**) The Osa-containing SWI/SNF (Brahma) chromatin remodeling complex regulates the fate of ISCs by controlling the expression of *Dl* and *ase*.

Snr1-specific antibodies or an unrelated IgG control (Fig. 7A-B′). We found that Osa and Snr1 associated with the *Dl* and *ase* promoters at the chromatin level, whereas neither bound to the *spdo* promoter, which suggests that Osa and Snr1 might regulate *Dl* and *ase* expression directly. However, we could not exclude the possibility that *Dl* and *ase* are indirectly regulated by another transcriptional target of Osa.

In summary, these data suggest that Osa regulates ISC differentiation into ECs by controlling *Dl* expression in ISCs, and regulates EE cell formation by controlling *ase* expression (Fig. 7C).

DISCUSSION

Previous studies have demonstrated that N signaling regulates ISC fate specification in the *Drosophila* posterior midgut (Ohlstein and Spradling, 2007). The Dl ligand is specifically expressed in ISCs to activate N signaling in neighboring EBs to promote EB to EC or inhibit EB to EE cell differentiation. However, it is still not fully understood how ISCs are coordinately controlled to differentiate into EC or EE cells, thus maintaining tissue homeostasis. Here, we found that the commitment of stem cells to discrete differentiated cells is coordinately regulated by the Osa-containing SWI/SNF chromatin-remodeling complex. Osa regulates ISC differentiation into ECs by controlling the expression of *Dl* to elaborate N signaling, and regulates EE cell formation by controlling the expression of *ase* transcription.

The role of the Osa-containing SWI/SNF complex in ISC self-renewal and differentiation into ECs

Our results suggest that Osa, a component of the SWI/SNF complex, is essential for ISC differentiation into EC and EE cells in the *Drosophila* posterior midgut. The SWI/SNF chromatin-remodeling complex performs a crucial function in gene regulation by using energy derived from ATP hydrolysis to alter the contacts between histones and DNA in nucleosomes, resulting in increased DNA accessibility to transcription factors and other regulatory proteins. Activating the N signal in EBs with the ISC-originated N ligand Dl is essential for EB differentiation into ECs. Osa promotes the expression of *Dl* in ISCs and maintains activation of the N signal in EBs to promote EB to EC differentiation. Repressing Dl expression by loss of function of Osa enhances ISC self-renewal and blocks ISC commitment to ECs, resulting in ISC expansion/tumors. Dl expression alone is sufficient to rescue the ISC expansion/tumor phenotype in *osa* mutants. SWI/SNF components have been shown to frequently occupy transcription start sites, enhancers and CTCF-binding regions (Euskirchen et al., 2011). Indeed, Osa and Snr1 bind to the promoter region of *Dl* at the chromatin level. Consistently, genetic interaction between the SWI/SNF complex and Dl were found during a genetic screen (Armstrong et al., 2005), and the expression of a dominant-negative form of Brm, a core component of the SWI/SNF complex, in the adult sense organ precursor lineage causes phenotypes similar to those resulting from impaired Dl-N signaling.

Why does the Osa-containing SWI/SNF complex regulate *Dl* expression only in ISCs and not in EBs? One possible explanation is that the complex in ISCs and EBs has different associated factors, and only that in ISCs is able to regulate *Dl* expression. Another possibility is that some transcription factors recruit the complex to regulate *Dl* expression, and these transcription factors exist only in ISCs.

The role of the Osa-containing SWI/SNF complex in ISC differentiation into EE cells

Disrupting the Dl-N signal in ISCs results in both ISC and EE cell expansion. Given that Osa regulates Dl expression in ISCs, *osa* mutant ISCs should have phenotypes similar to those that result from impaired Dl-N signaling; however, *osa* mutation only causes ISC expansion, and EE cell formation is repressed. Proneural bHLH factors of the achaete-scute complex (AS-C), Sc and Ase, have been reported to play a crucial role in EE cell fate specification (Bardin et al., 2010). *sc* or *ase* overexpression in midgut progenitors leads to dramatically increased EE cell numbers, whereas mutant ISC clones of Df(1)scB57, which lack all four AS-C genes, i.e. *achaete* (*ac*), *sc*, *lethal of scute* [*l(1)sc*] and *ase*, are devoid of EE cells (Bardin et al., 2010). *sc* and *ase* expression is upregulated when the N signal is blocked in the intestinal midgut. Similarly, we also found that the expression of *sc* is upregulated in the osa^{RNAi} midgut compared with the wild-type control, whereas *ase* expression is significantly decreased in the osa^{RNAi} midgut. Furthermore, knockdown of *ase* alone blocks EE cell formation in the N^{DN} midgut. Thus, our data suggest that Osa controls EE cell formation by regulating the expression of *ase*. Terminal differentiation of EBs into EC or EE cells has been proposed to be regulated by differential N signaling, whereby a high level of N activity induces EBs to differentiate into ECs, whereas a low level of N activity promotes the EE cell fate (Ohlstein and Spradling, 2007). However, EE cells can still be generated even when N activity is suppressed by either the expression of N^{DN} or the clonal deletion of *Dl* or *N*, suggesting that the N signal is not required for EE cells at all.

Both Sc and Ase may play important roles in regulating EE cell fate in the midgut. A high level of N activity might suppress the expression of *sc* and *ase* and thereby promote EC fate. By contrast, a low level of N activity might activate the expression of *sc* and *ase*,

inducing EB cells to differentiate into EE cells. Excess EE cells in the N^{DN} midgut might be the result of upregulation of *ase* and *sc* caused by disruption of the N signal. Indeed, previous studies have shown that N signaling activates the expression of the Enhancer of split complex [E(spl)-C], which can, in turn, repress the expression of the proneural genes *ac*, *sc*, *l(1)sc* and *ase* (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). In addition to *sc* and *ase*, the overexpression of *ac* and *l(1)sc* in the midgut also results in dramatically increased EE cell numbers, but only Sc and Ase are required for EE cell formation (data not shown). Why does Osa regulate *ase* and not other AS-C members? Even though Ase belongs to the AS-C proneural family, it differs from the other AS-C members in its expression pattern, regulation, mutant phenotype and in some DNA-binding properties (Jarman et al., 1993). These characteristics suggest that Ase might be associated with some specific transcription factors that bind to *ase* DNA at particular chromatin states controlled by the Osa-containing SWI/SNF chromatin-remodeling complex.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
X.Z. and S.X.H. conceived the project, designed and performed the experiments and wrote the manuscript. X.L. provided reagents and edited the manuscript.

Supplementary material
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