



Genomes & Developmental Control

Histone lysine demethylases function as co-repressors of SWI/SNF remodeling activities during *Drosophila* wing developmentBrenda J. Curtis^a, Claudia B. Zraly^b, Daniel R. Marendaz^c, Andrew K. Dingwall^{a,b,d,*}^a Graduate Program in Molecular and Cellular Biochemistry, Loyola University Chicago Stritch School of Medicine, Maywood, IL, 60153, USA^b Oncology Institute, Cardinal Bernardin Cancer Center, Loyola University Chicago Stritch School of Medicine, Maywood, IL, 60153, USA^c Department of Biology, Drexel University, Philadelphia, PA, 19104, USA^d Department of Pathology, Loyola University Chicago Stritch School of Medicine, Maywood, IL, 60153, USA

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ABSTRACT

The conserved SWI/SNF chromatin remodeling complex uses the energy from ATP hydrolysis to alter local chromatin environments through disrupting DNA-histone contacts. These alterations influence transcription activation, as well as repression. The *Drosophila* SWI/SNF counterpart, known as the Brahma or Brm complex, has been shown to have an essential role in regulating the proper expression of many developmentally important genes, including those required for eye and wing tissue morphogenesis. A temperature sensitive mutation in one of the core complex subunits, SNR1 (SNF5/INI1/SMARCB1), results in reproducible wing patterning phenotypes that can be dominantly enhanced and suppressed by extragenic mutations. SNR1 functions as a regulatory subunit to modulate chromatin remodeling activities of the Brahma complex on target genes, including both activation and repression. To help identify gene targets and cofactors of the Brahma complex, we took advantage of the weak dominant nature of the *snr1^{Et}* mutation to carry out an unbiased genetic modifier screen. Using a set of overlapping chromosomal deficiencies that removed the majority of the *Drosophila* genome, we looked for genes that when heterozygous would function to either enhance or suppress the *snr1^{Et}* wing pattern phenotype. Among potential targets of the Brahma complex, we identified components of the Notch, EGFR and DPP signaling pathways important for wing development. Mutations in genes encoding histone demethylase enzymes were identified as cofactors of Brahma complex function. In addition, we found that the *Lysine Specific Demethylase 1* gene (*lsd1*) was important for the proper cell type-specific development of wing patterning.

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Introduction

Within the eukaryotic cell nucleus, chromosomal DNA is first packaged into nucleosomes that are further assembled into a highly structured nucleoprotein complex called chromatin. Chromatin organization is a fundamental constraint involved in regulating the expression of eukaryotic genes, since the presence of a nucleosome around a binding site blocks the accessibility of most transcription factors to their cognate binding sequences. The highly conserved SWI/SNF ATP-dependent chromatin remodeling complex regulates the expression of many genes by remodeling chromatin in response to regulatory signals, thus enabling the binding of transcription factors and activator complexes to regulatory sequences within target genes.

SWI/SNF complexes play critical roles in DNA replication and repair, RNA Polymerase II transcription, as well as metazoan embryonic development and postnatal tissue regeneration by

regulating cell proliferation, differentiation and survival (Mohrmann and Verrijzer, 2005; Wu et al., 2009). Components of the complex were first identified in yeast during two independent genetic screens as mutants that either lost the ability to switch mating type (SWI for Switching Deficient) or displayed an inability to grow in sucrose containing medium (SNF for Sucrose non-fermenting) (Winston and Carlson, 1992). The yeast and metazoan SWI/SNF complexes are composed of 8–11 unique subunits with an approximate molecular mass of 1.2 MDa (Peterson et al., 1994; Smith et al., 2003). The complex has a single ATPase subunit that is required both in vitro and in vivo for ATP-dependent chromatin remodeling activity. However, full in vitro chromatin remodeling activity on mono-nucleosomes and nucleosomal arrays can be achieved with reconstitution of 4 mammalian “core” subunits, BRG1, INI1/BAF47, BAF170, and BAF155 (Phelan et al., 1999), making it likely that the remaining subunits are important for complex stability, regulation, and/or targeting.

The *Drosophila* SWI/SNF counterpart is known as the Brahma (Brm) complex. The chromatin remodeling activities of the Brm complex are important for both the activation and repression of gene transcription during development (Simon and Tamkun, 2002). The BRM protein co-localizes with RNA Polymerase II on salivary gland

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polytene chromosomes within regions undergoing active gene transcription (Armstrong et al., 2002; Zrally et al., 2003). Microarray analyses of Brm complex mutants demonstrated that the complex was likely involved in both transcription activation and repression (Zrally et al., 2006). The role of Brm complex regulation during gene activation has been extensively characterized and often involves recruitment of histone modifying enzymes, such as histone acetyltransferases (HATs), and gene specific transcription factors (Simon and Tamkun, 2002). The role of the Brm complex in gene repression is less well understood, and is generally thought to result from the formation of repressive chromatin within gene promoters or through associations with co-repressor complexes including histone deacetylases and demethylases.

SNF5 is a core component of all purified SWI/SNF complexes and serves an essential function in regulating chromatin remodeling activities. Previous work demonstrated that a dominant negative mutation in the *Drosophila snr1* gene (*snr1^{E1}*) that encodes a core Brm subunit, SNF5-Related-1 (SNR1), leads to differential misregulation of genes required for wing vein and inter-vein cell development (Marenda et al., 2004), suggesting possible regulatory targets for the Brm complex in vivo that include components of the EGFR (Epidermal Growth Factor Receptor), DPP/BMP (Decapapentaplegic/Bone Morphogenetic Protein) and Notch-Delta signaling pathways. These studies revealed tissue-specific differential requirements for Brm complex functions in patterning and allowed us to conclude the following: (i) The ectopic veins associated with *snr1^{E1}* are dependent on BRM ATPase chromatin remodeling activity; (ii) SNR1 functions to regulate BRM ATPase activity on specific gene targets (e.g., *rhomboid*) in intervein cells of the wing through collaborations with transcriptional repressors (e.g., NET) and histone deacetylase (HDAC) activity. These studies revealed that transcription repression by the Brm complex is due in part to restraint of chromatin remodeling activities and that the SNR1 subunit has regulatory role to restrict remodeling activities in a cell-type, tissue-specific manner (Marenda et al., 2004).

We sought to further address how target genes are selectively regulated by the Brm complex through studies of SNR1 function, as this subunit plays a pivotal role in complex dependent gene-specific repression. There are two possible mechanisms of SNR1 mediated complex regulation: (i) physical associations between individual subunits within the complex and (ii) physical association between the Brm complex and other core regulatory proteins. We addressed both of these possibilities and observed an important genetic and physical interaction between two Brm complex core subunits, SNR1 and MOR. Specifically, we found that the highly conserved SNR1 Repeat 2 (R2) and Coiled-coil regions physically associate with the SWIRM domain of MOR. Employing a dominant enhancer-suppressor genetic screen we also identified histone lysine demethylase enzymes as potential coregulators of Brm complex remodeling activities. We found that Lysine Specific Demethylase-1 (LSD1) genetically and physically associates with the Brm complex. Further, *lsd1* appears to genetically interact with a subtype of the Brm complex (Polybromo or PBAP) in the context of wing development. Lastly, we show that LSD1 is expressed throughout the pupal wing, in both vein and intervein cells, and that it likely functions in a cell-type specific fashion to repress highly conserved EGFR and/or DPP signaling in intervein cells.

Materials and methods

Fly stocks and genetic analyses

All *Drosophila* stocks were maintained on standard yeast/cornmeal/dextrose medium at 25 °C, except for *snr1^{E1},e/TM6B,Hu,e* which was maintained at 29 °C. Mutant strains were obtained from private stocks and the Indiana University-Bloomington *Drosophila* Stock Center (BDSC, Bloomington, Indiana). Transgenic RNAi lines were obtained from the Vienna *Drosophila* Resource Center (Vienna,

Austria). All strains and gene mutants are described in detail in Flybase (<http://flybase.bio.indiana.edu>).

Drosophila chromosomal deficiency stocks were obtained from the BDSC. Information on the deficiency stocks tested and predicted/known breakpoints are available upon request. Females of the genotype *snr1^{E1},e/TM6B,Hu,e* were crossed to males harboring the mutation of interest, unless otherwise indicated. Crosses were carried out at 29 °C and progeny scored for enhancement or suppression of the *snr1^{E1}* posterior cross vein and/or L2 and L5 vein wing phenotypes, as described in the text (Marenda et al., 2004, 2003). At least 50 wings were scored for each interaction cross. Genetic analyses involving other Brahma complex components were carried out at 25 °C.

P-element mobilizations were carried out using standard genetic crosses, briefly described as follows. Virgin female *y¹w^{67c23}; P(w⁺, lacW)lid²/CyO* flies were crossed to male *w⁻; wg^{sp1}/CyO; Δ2,3 Sb/TM6B*. Virgin *w⁻; lid²/CyO; Δ2,3 Sb/+* were crossed to male *w⁻; Sco/CyO*. Virgins carrying potential excisions (*w⁻; lid^{2REV}/CyO; +/+*) were selected based on the loss of *mini-white* gene and individually crossed to male *w⁻; Sco/CyO* flies. *w⁻; lid^{2REV}/CyO* siblings were crossed to build stocks. *w⁻; lid^{2REV}/CyO* flies were crossed to *Df(2L)ED354/SM6a*, a deficiency covering *lid*. The *lid²* insertion is lethal, so the presence of *lid^{2REV}/Df(2L)ED354* progeny indicated that the P-element had been precisely excised. In order to excise the P-element affecting the *Jarid2/CG3654* gene, virgin females of the genotype *y¹w^{67c23}; P{w[+mC]y[+mDint2]=EPgy2} Jarid2^{EY02717}* flies were crossed to *w⁻; wg^{sp1}/CyO; Δ2,3 Sb/TM6B*. Male *y¹w^{67c23}/Y; CyO/+; P{w[+mC]y[+mDint2]=EPgy2} Jarid2^{EY02717}/Δ2,3 Sb* were crossed to virgin *w; TM3/TM6B* flies. Virgin female potential excision recombinants were collected based on loss of red eye color, *w⁻[+mC]y[+mDint2]=EPgy2} Jarid2^{EY02717REV}/TM6B*, and were individually crossed to male *w⁻; TM3/TM6B* flies. *w⁻; [+mC]y[+mDint2]=EPgy2} Jarid2^{EY02717REV}/TM6B* siblings were crossed to generate stocks.

Yeast two-hybrid protein interaction studies

Yeast two-hybrid interaction analyses were performed to detect protein–protein interactions using the pRF4-5a and pEG202 vectors (Finley and Brent, 1994). Four SNR1-B42AD fusions, SNR115–370, SNR115–240, SNR1240–370, and SNR115–370 G256D have been previously described (Zrally et al., 2004). The MOR, LSD1, and ADA2 fusions were constructed in the pEG202 vector and tested for protein–protein interaction with the SNR1 fusions. Full-length *mor* and *lsd1* cDNAs were obtained from the DGRC (<http://dgrc.cgb.indiana.edu/>) and regions of interest were amplified using standard PCR procedures (ExTaq; Takara, Inc.). Xho1/BamH1 *mor* fragments and EcoR1/Xho1 *lsd1* and *ada2a* PCR fragments were independently cloned into pSK⁺, screened, and subcloned into the bait LexA pEG202 yeast vector. All constructs were transformed into yeast strains of opposite mating type (RFY231 and Y309), mated to produce diploids, and assayed for protein–protein interaction. A positive protein–protein interaction resulted in binding of the protein complex to an upstream activator sequence (UAS), resulting in expression of either β -galactosidase (*lacZ*), which results in blue colony formation on plates containing X-gal, or *leu2* expression, which allows for growth on plates lacking leucine, as described previously (Marenda et al., 2004). Each pair mating was tested in triplicate. The QuikChangeII Site-Directed Mutagenesis Kit (Stratagene) was utilized to create the specific (I350P) point mutations in *snr1* sequences according to the manufacturer's protocols. Mutations were confirmed by sequencing. Primer sequences are available upon request.

RNAi in cultured S2 cells

Cultured *Drosophila* S2 cells were incubated with dsRNA to knock-down the endogenous *snr1*, *mor* and *brm* transcripts, as well as an unrelated gene *CG10465*, as described previously (Zrally et al., 2006).

Knock-downs were incubated for four days, then whole cell extracts prepared and protein levels assessed by Western blot using antibodies to SNR1, BRM (Zrally et al., 2003), MOR (Mohrmann et al., 2004) and a control protein, CDK9. Untreated S2 cell extracts were used as a control to determine endogenous protein levels.

Co-immunoprecipitation and GST-pull down assays

Co-immunoprecipitation (Co-IP) experiments were carried out as previously described (Zrally et al., 2002) using extracts prepared from 0 to 24 h *Oregon-R* (wild type) embryos. Extracts (500 µg) were pre-cleared with protein G-Sepharose then incubated with primary affinity purified rabbit polyclonal α-SNR1 (Zrally et al., 2002). Protein complexes were precipitated using protein G-Sepharose beads. Bound and unbound proteins were fractionated by SDS-PAGE and analyzed by western blotting using rabbit α-LSD1 (Di Stefano et al., 2007). The extract input (E) lane represents 20% (100 µg) of the cleared lysate used in the IP.

A full-length *lzd1* cDNA was obtained from the Drosophila Genome Resource Center, (DGRC, University of Indiana, Bloomington) and regions of interest were amplified using standard PCR procedures (ExTaq; Takara, Inc.). EcoR1/Xho1 *lzd1* PCR fragments were independently cloned into pSK⁺, screened, and subcloned into pGEX-5X1 (Stratagene). Constructs were generated that fused GST to the LSD1 N-terminal portion (SWIRM) and to the C-terminal portion (Amine Oxidase). These fusion constructs were transformed into *E. coli* BL21 cells, incubated at 37 °C for 2 h, induced with 1 mM IPTG, and harvested 2–3 h later. The N-terminal fusion was grown at 30 °C following induction, the C-terminal fusion and empty vector controls were grown at 37 °C. Fusion proteins were solubilized using standard protocols and equivalent amounts of the fusion proteins as assessed by Coomassie stained gel analyses were bound to glutathione agarose beads. GST-pulldown assays were performed as described previously (Zrally et al., 2004) by incubating wild type *Drosophila Oregon R* embryo extracts (500 µg) with the immobilized fusion proteins or GST alone (Dingwall et al., 1995). Bound proteins were separated by SDS-PAGE and interactions with the Brm complex were assessed by Western immunoblot using antibodies to SNR1. The extract lane contains 20% (100 µg) of the starting material.

Immunohistochemistry

Appropriately staged [stage P7, 34–42 h after pupariation (Bainbridge and Bownes, 1981)] pupal wings were dissected, fixed, and immunostained with α-LSD1 (1:1000) antibody (a kind gift from Nicolas Dyson). Pupae were removed from pupal cases in phosphate buffered saline (PBS, pH 7.4) and fixed in 4% formaldehyde for 30 min. Pupal wings were then dissected in PBST (PBS, 0.1% Triton-X100) and removed from their cuticular envelopes. Pupal wings were washed 3× in PBST and then incubated in 10% normal goat serum (blocking) for 1 h at 4 °C. Blocking solution was removed and wings were incubated with either rabbit α-LSD1 (1:1000) or PBS (negative control) at 4 °C

overnight. Samples were washed 4× in PBST and then incubated with goat α-rabbit-HRP (1:500) (Sigma) for 1–2 h at room temperature. Samples were again washed 3× in PBST and then developed as described previously (Marenda et al., 2004).

Results

The Brm complex subunits SNR1 and MOR directly collaborate to regulate target genes

Several lines of evidence suggest an important functional relationship between two highly conserved BRM complex components, SNR1 and MOIRA (MOR). First, the mammalian SNR1 (SNF5/INI1/BAF47/SMARCB1) and MOR orthologs (BAF155/SGR3/SMARCC1 and BAF170/SMARCC2) are required for full BRG1 remodeling activity in vitro (Phelan et al., 1999). Second, a physical interaction has been observed between murine SNR1 and MOR orthologs (SNF5 and SRG3) via the SRG3 SWIRM domain that is important for protein–protein interactions (Sohn et al., 2007). Third, RNAi-mediated knockdown of *snr1* and *mor* in cultured *Drosophila* S2 cells revealed nearly identical expression profiles (Sohn et al., 2007). Finally, genetic epistasis tests in *Drosophila* using mutant alleles of *snr1* and *mor* confirmed a strong in vivo interaction (Marenda et al., 2003). This evidence suggests that SNR1 and MOR were capable of direct collaboration within the Brm complex.

The *snr1*^{E1} mutant used in these genetic tests is a temperature-sensitive allele that behaves genetically as both a loss-of-function hypomorph and a weak dominant-negative (Marenda et al., 2003). The mutation affects a nearly invariant glycine (G256) residue within the second repeat region, a highly conserved irregular repeat region known to be involved in multiple protein–protein interactions. Heterozygous *snr1*^{E1} flies exhibit ectopic wing vein material perpendicular to the posterior cross vein (PCV) and anterior to longitudinal vein L2 (Fig. 1A). The defects are apparent at the permissive (18 °C) temperature, with increased penetrance at the restrictive (29 °C) temperature. The phenotypes are sensitive to *snr1* gene dosage and are enhanced or suppressed by mutations in other Brm complex genes, revealing that SNR1 directly regulates aspects of Brm complex activity (Marenda et al., 2003). The mutant phenotypes result from reduced or compromised SNR1 function, rather than complete disruption of Brm complex activities (Marenda et al., 2004, 2003) as *snr1*^{E1} produces a stable protein that is assembled into Brm complexes at both temperatures (Marenda et al., 2003).

We previously observed that a large chromosomal deletion that completely removes *mor* (*Df(3R)sbd¹⁰⁵, p^p, Ubx^{bxc-1}, sr¹, e^s*) acted as a very mild enhancer of the dominant negative *snr1*^{E1} wing phenotype, while the hypomorphic *mor*¹ allele acted as a strong enhancer (Marenda et al., 2004). To examine the SNR1–MOR functional relationship in more detail, we performed genetic interaction tests between *snr1*^{E1} and several unique *mor* alleles (Brizuela and Kennison, 1997; Kennison and Tamkun, 1988), as well as a small deficiency (*Df(3R)Exel7327*). While the original deficiency tested, *Df*

Fig. 1. SNR1 interacts directly with the MOR subunit. (A–D) Animals transheterozygous for *snr1*^{E1} and loss of function *mor* alleles display a strong genetic interaction. (A) An *OregonR* (wild type) wing has five longitudinal veins (L1–L5) and two crossveins, Anterior Crossvein (ACV) and Posterior Crossvein (PCV). (B) *snr1*^{E1/+} heterozygous flies have ectopic vein material distal to the PCV (arrow). (C) *snr1*^{E1/Df(3R)Exel7327} transheterozygous flies have an enhanced ectopic wing vein phenotype, with ectopic material distal to the PCV, as well as anterior and posterior to L2 (arrows). (D) *mor*^{3/+} enhances the *snr1*^{E1/+} ectopic vein phenotype. *snr1*^{E1/mor³ transheterozygous flies display ectopic vein material distal to the PCV and anterior to L2. (arrows). All pictures were taken at the same magnification (63×) and crosses were set at 29 °C. (E) The C-terminal region of SNR1, containing the REPEAT REGION 2 (R2) and COILED-COIL, physically interacts with the MOR-SWIRM. SNR1-B42 Activation Domain (AD) fusion proteins were tested for physical interaction with MOR-SWIRM (442–552)–LexA DNA Binding Domain (DBD) fusions in yeast 2-hybrid analyses. The SWIRM, SANT, and LEUCINE-ZIPPER (ZIP) domains of MOR, and REPEAT REGION 1 (R1), REPEAT REGION 2 (R2), and COILED-COIL (CC) domains of SNR1 are indicated. Protein–protein interactions were assessed by two independent assays, the production of β-galactosidase (blue colony formation) and growth on media lacking leucine. Robust interactions were observed between the MOR SWIRM and two SNR1 fusions, SNR115–370 and SNR1240–370, both of which contained the C-terminal region, including R2 and the CC. The structure of the CC is likely not essential for the protein–protein interaction, since the SNR11350P physically associated with the MOR SWIRM. The G256D mutation (as carried in *snr1*^{E1} mutants) strongly interacted with the MOR SWIRM. The LexA-MOR SANT fusion served as a negative control and exhibited no interaction with SNR1 (15–370). (F) Western immunoblot using extracts prepared from cultured embryonic S2 cells were treated with dsRNAs directed against *snr1*, *mor*, *brm* or an unrelated gene control (C) as indicated. Blots were probed with antibodies against MOR, BRM, SNR1 and a control protein, CDK9. Untreated S2 cell extracts (–) were used as a control for endogenous gene expression levels.}

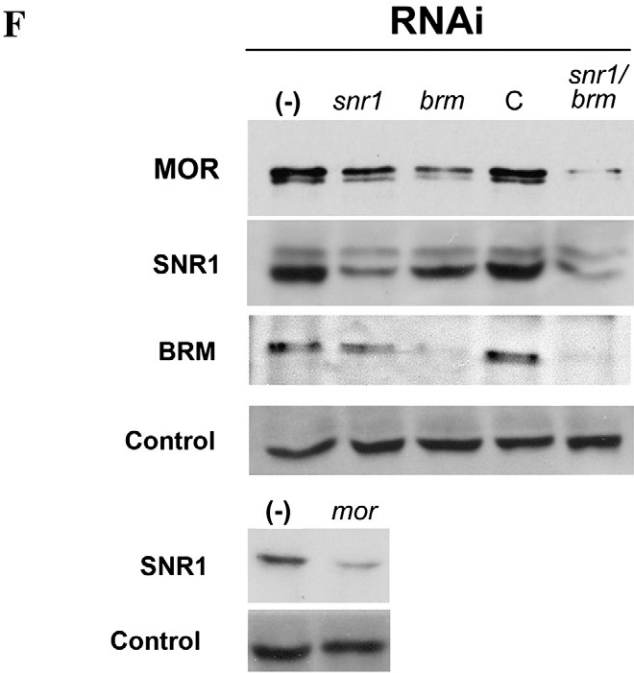
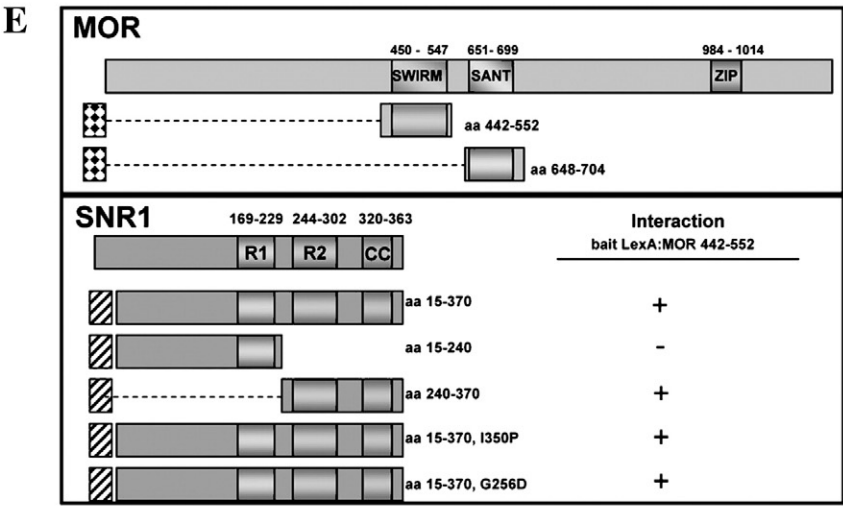
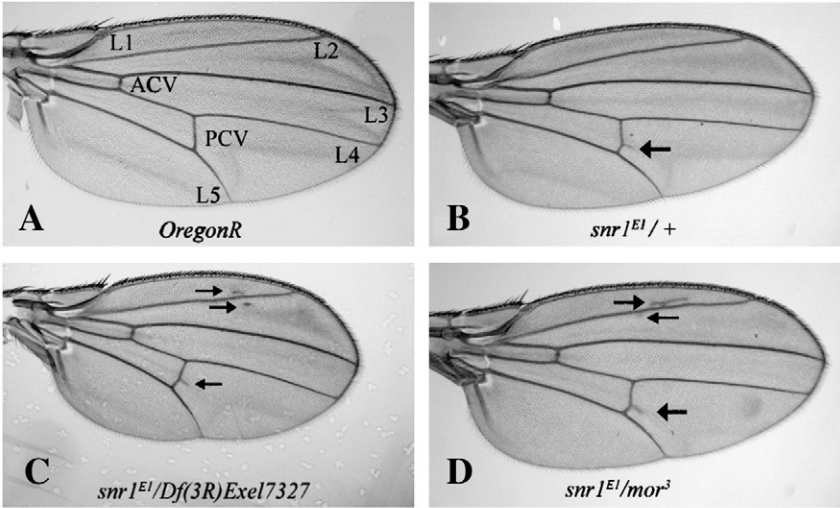


Table 1
Allele specific genetic interaction between *snr1^{E1}* and *mor*.

Allele	Predicted allele class	Genotype	Total wings	Severity of phenotype ^a		
				(–)	(+)	(++)
<i>snr1^{E1}</i>	Weak dominant negative	<i>snr1^{E1}/+</i>	207	67	30	3
<i>mor¹</i>	Hypomorph	<i>mor¹/+</i>	152	94	5	0
		<i>snr1^{E1}/mor¹</i>	116	31	39	30
<i>mor²</i>	Null	<i>mor²/+</i>	267	100	0	0
		<i>snr1^{E1}/mor²</i>	168	97	3	0
<i>mor³</i>	Hypomorph	<i>mor³/+</i>	319	48	38	14
		<i>snr1^{E1}/mor³</i>	100	23	15	62
<i>mor⁴</i>	Hypomorph	<i>mor⁴/+</i>	106	100	0	0
		<i>snr1^{E1}/mor⁴</i>	230	76	23	1
<i>mor⁵</i>	Hypomorph	<i>mor⁵/+</i>	130	66	22	12
		<i>snr1^{E1}/mor⁵</i>	102	46	34	20
<i>mor⁶</i>	C-terminal truncation	<i>mor⁶/+</i>	241	56	40	4
		<i>snr1^{E1}/mor⁶</i>	148	41	23	36
<i>Df(3R)Exel7327</i>	Deficiency	<i>Df(3R)Exel7327/+</i>	59	92	8	0
		<i>snr1^{E1}/Df(3R)Exel7327</i>	118	12	8	80

^a Severity of phenotype was scored as follows: (–) normal vein pattern, (+) single extra vein near posterior cross vein, and (++) two or more wing veins near PCV, anterior to longitudinal vein L2 or posterior to longitudinal L5. Numbers are expressed as percentages of the total wings examined for each genotype.

(3R)*sbd105*, is thought to completely delete *mor*, the endpoints of the chromosomal deletion are not well defined, and the genes predicted to be deleted in the deficiency were based on complementation analyses. Therefore, we chose to test a more accurately described deficiency (*Df(3R)Exel7327*) with defined endpoints that deletes *mor* as well as 18 other surrounding genes.

Strong allele-specific enhancement of the *snr1^{E1}* wing phenotype was observed with the deficiency (*Df(3R)Exel7327*) and several hypomorphic *mor* alleles (*mor¹*, *mor³*, *mor⁵*) and a C-terminal deletion (*mor⁶*) that removes the predicted leucine zipper, a motif important for homo-oligomerization (Table 1). Flies doubly heterozygous for *snr1^{E1}* and the predicted hypomorph, *mor³*, or the deficiency, *Df(3R)Exel7327*, displayed strong enhancement of the *snr1^{E1}* wing phenotype (Fig. 1A). Other *mor* mutant alleles, including a predicted null (*mor²*), and the predicted hypomorph (*mor⁴*) exhibited suppression of the wing phenotype (Table 1). The most likely explanation for the opposite genetic interactions observed between *snr1^{E1}*, the null allele *mor²*, and the larger deficiency is that the deficiency deletes additional gene(s) that may cooperate with the Brm complex to regulate target genes, directly or indirectly, or may have an independent role in tissue patterning. While the exact nature of the *mor* alleles are unknown, our genetic interaction data suggests that *mor⁴* may affect protein expression, similar to the decreased protein expression expected with the *mor²* null allele, leading to an overall decrease in total Brm complex formation. However, *mor¹*, *mor³*, *mor⁵*, and *mor⁶* heterozygotes all display ectopic vein phenotypes, suggesting that these mutations may alter protein folding, altering the ability of MOR to regulate Brm complex activities, perhaps by changing the physical interactions it makes with the other two core complex components, SNR1 and BRM. These data confirm an important functional relationship between SNR1 and MOR in regulating Brm complex target genes.

The BRM, MOR and SNR1 subunits have been proposed to function as the core components of all Brm and metazoan SWI/SNF complexes. MOR (BAF155, BAF170) has been postulated to serve as the bridge between the core subunits (Crosby et al., 1999; Moshkin et al., 2007; Phelan et al., 1999) making it likely that SNR1 regulatory functions are mediated through direct contacts with MOR. We first sought to identify a region of SNR1 essential for interacting with MOR by performing a series of yeast interaction trap analyses (Fig. 1B). The SNR1 protein is composed of three distinct regions, two irregular

repeat regions (Repeat 1 (R1) and Repeat 2 (R2)) both of which are essential for heterotypic protein–protein interactions, and a predicted Coiled-coil (CC) region also possibly involved in protein interactions (Dingwall et al., 1995; Morozov et al., 1998). The MOR protein contains several predicted protein interaction motifs, including a leucine zipper region, SWIRM and SANT domains (Crosby et al., 1999). We constructed several SNR1 deletion derivatives fused to the B42 activation domain and tested them for interaction with portions of MOR fused to the LexA DNA binding domain, including the SWIRM region implicated in mediating interactions between the murine homologs (Sohn et al., 2007). A robust interaction was observed between the C-terminal portion of SNR1 (consisting of R2 and CC regions) and the MOR SWIRM region; however, we did not detect interactions between SNR1 and the SANT domain.

The COILS program (Lupas et al., 1991) predicts that amino acids 338–364 of SNR1 have approximately 50% probability of forming a Coiled-coil structure. Within this region M346, I350, M360, and A364 are likely to be involved in the formation and stability of the Coiled-coil structure, as they are located in the seam joining the two helices. We used the COILS program to predict the effects of substituting a proline for each of these residues as this change could considerably alter the alpha helical structure by forming a kink in the helices. An I350P conversion would theoretically have the most profound effect by decreasing the probability of forming a Coiled-coil to less than 1% (Supplemental Fig. 1). We therefore changed the I350 residue to a proline and tested the interaction with the MOR SWIRM domain using yeast two-hybrid analyses. The I350P conversion in the full-length SNR1 did not disrupt the physical association with the MOR SWIRM domain, suggesting that the formation of a Coiled-coil structure was not necessary for the observed protein interaction (Fig. 1B).

To further ascertain whether physical associations between SNR1 and MOR were important for complex assembly or stability, we depleted SNR1, MOR and BRM in cultured *Drosophila* S2 cells using RNAi and tested for subunit stabilities by Western blot (Fig. 1C). We observed reduced MOR accumulation in cells depleted for either SNR1 or BRM alone, with greater reductions of MOR in cells depleted for both SNR1 and BRM. Similarly, SNR1 levels were reduced following RNAi depletion of MOR. Thus, consistent with the widely held view that protein complex components show decreased stability when partner proteins are deleted or missing and with previous findings (Moshkin et al., 2007; Peterson and Herskowitz, 1992), the stability of the SNR1 and MOR subunits appears to be at least partly dependent on assembly of the remodeling complex core components.

Dominant genetic modifier screens to identify SNR1-interacting coregulators

It is likely that the regulation of BRM complex activities is dependent not only upon physical associations between complex subunits, but also association of the complex with coregulatory proteins. We previously performed candidate genetic screens looking for dominant enhancement or suppression of the *snr1^{E1}*-dependent wing phenotypes (Marenda et al., 2004). In general, we found that loss of function mutations in vein forming genes dominantly suppressed the *snr1^{E1}* phenotypes, while loss of function mutations in vein repressing genes showed enhancement. In order to identify factors that modify SNR1 repression functions in vivo and to identify possible coregulators of Brm complex activity, we carried out an unbiased dominant modifier genetic screen using an ordered set of chromosomal deficiencies from the Bloomington *Drosophila* Stock Center that removed ~85% of the euchromatic genome (both X and autosomes). We looked for deficiencies that enhanced or suppressed the wing patterning phenotype associated with the temperature-sensitive *snr1^{E1}* mutation in doubly heterozygous flies reared at 29 °C. Heterozygous *snr1^{E1}/+* flies display ectopic wing vein material distal to the posterior crossvein in this assay (Fig. 1A) (Marenda et al., 2003).

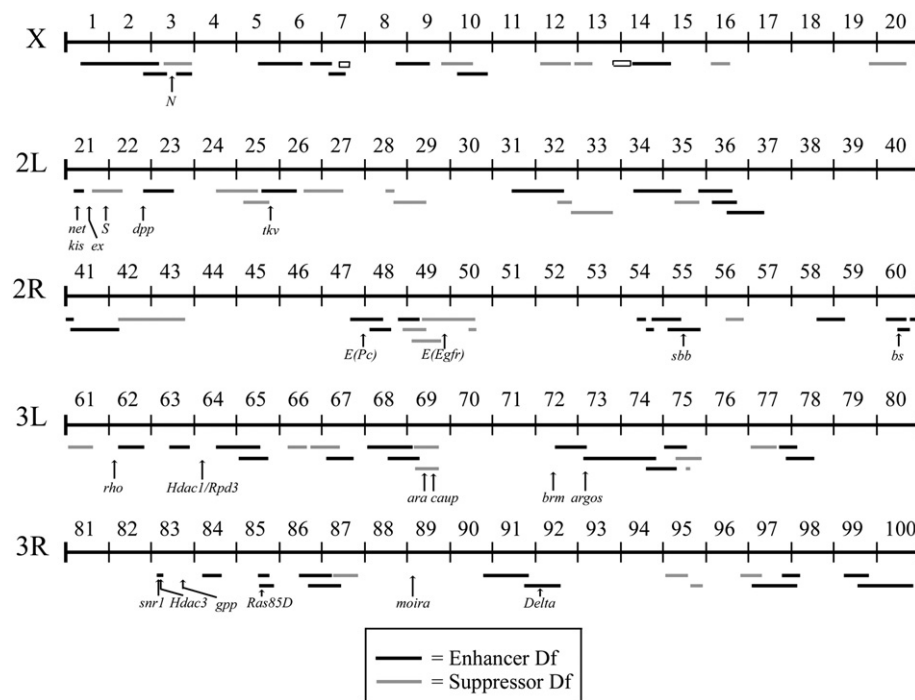


Fig. 2. Dominant modifier screen for enhancers and suppressors of the *snr1^{E1}* wing phenotype. Shown is a cytological map of *Drosophila melanogaster* chromosomes I, II and III and the locations of deficiencies that enhanced and suppressed the *snr1^{E1}* ectopic wing vein phenotype. Each chromosome is subdivided into two arms (L and R), and each arm is further subdivided into 20 cytological markers. Indicated below the cytological map are regions on each chromosome where interacting deficiencies have deleted genomic DNA. Black bars indicate enhancement by the deficiency, and gray bars indicate suppression. Arrows show genes within each deficiency previously identified to interact with *snr1^{E1}*. Open bars represent deficiencies that were synthetically lethal in combination with *snr1^{E1}*.

Suppression was scored as an overall reduction in the penetrance of the ectopic wing vein phenotype (>20% reduction); whereas, enhancement was scored as increased penetrance (>20% increase) and/or the appearance of additional ectopic vein material. While the effects of enhancers of the *snr1^{E1}* vein phenotype could be observed throughout the wing, the effects of suppressors were restricted to the area of the wing being examined (i.e., posterior crossvein and L5 region). In total, 88 of the ~250 deficiency lines tested exhibited strong enhancement (55/88) or suppression (33/88) (Fig. 2; Supplementary Tables 1–3). In addition, two deficiencies removing different portions of the X-chromosome exhibited heterozygous lethality in combination with *snr1^{E1}* (Supplementary Table 1).

As a first step towards determining whether our unbiased approach was sufficiently sensitive to identify novel Brm complex-interacting genes, we correlated the results of the deficiency screen with our earlier candidate genetic screen performed using specific mutant alleles (Marenda et al., 2004). In general, deficiencies that removed genes previously identified as dominant enhancers of the *snr1^{E1}* wing phenotype exhibited similar enhancement (*corto*, *kis*, *DI*, *bs*, *sbb*, *RpII215*). There was also a correspondence between the deficiency screen results and several genes previously identified as suppressors (*S*, *N*, *cycE*). However, many of the enhancer and suppressor deficiencies removed loci not previously identified as interacting with the Brm complex, representing a rich resource for identifying novel coregulators of Brm complex functions.

A second approach used to validate the deficiency screen results was to identify novel interacting genes (Fig. 3). We first narrowed the interacting region(s) using either a partially overlapping deficiency or mutations in candidate genes removed by the deficiency. The heterozygous *Df(3L)81k19* alone does not exhibit a wing patterning defect (Fig. 3D); however, the deficiency was found to dominantly enhance the *snr1^{E1}* phenotype (Fig. 3E). Among the genes predicted to be removed by that deficiency (<http://flybase.org>), the *argos* gene was a likely candidate, as it is a negative regulator of the EGFR pathway (Schweitzer et al., 1995) involved in wing vein specification (Marenda

et al., 2006). Heterozygotes containing a mutant *argos^{Δ7}* allele with the *snr1^{E1}* mutation exhibited ectopic wing veins (Fig. 3F). Similar results were obtained using a second *argos* mutant allele (*argos⁰⁵⁸⁴⁵*; data not shown). The *Df(2R)CX1* deficiency has no dominant heterozygous wing phenotype (Fig. 3G); however, in combination with *snr1^{E1}*, we observed strong suppression of ectopic wing veins (Fig. 3H). The *E(Egfr)* gene was the most likely candidate among the deduced genes removed by the deficiency. We found that a mutation in the *E(Egfr)B56* gene could dominantly suppress the *snr1^{E1}* wing patterning defect (Fig. 3I). We can conclude that the deficiency screen approach is sufficiently sensitive to use as a first step for identifying candidate genes that may interact with the Brm complex to regulate wing patterning. Moreover, our results have indicated that the EGFR pathway may depend in part on Brm complex chromatin remodeling functions to correctly pattern the wing epithelium.

Candidate screen identifies histone demethylase enzymes as potential Brm complex coregulators

The goal of our dominant enhancer/suppressor screen was to identify novel coregulators of the Brm complex in controlling gene expression. Therefore, we looked to see if there was any functional similarity among candidate transcriptional regulators which genetically interacted with *snr1^{E1}*. Several histone lysine demethylase enzymes (KDMs) were identified in our screen as potential mediators of Brm chromatin remodeling activities. There are two 'families' of KDMs (Klose et al., 2006; Klose and Zhang, 2007; Shi and Whetstone, 2007; Swigut and Wysocka, 2007). The Jumonji family has 12 members and is capable of demethylating tri-, di-, and monomethylated lysine residues. A much smaller family of demethylases contains 1 member, LSD1/KDM1, which is only capable of demethylating di-, and monomethylated (H3K4) lysine residues and in mammalian cell culture analysis, di-, and monomethylated (H3K9) lysine residues when in complexes with the Androgen Receptor (AR) (Metzger et al., 2005). We found that 7 deficiencies covering individual histone

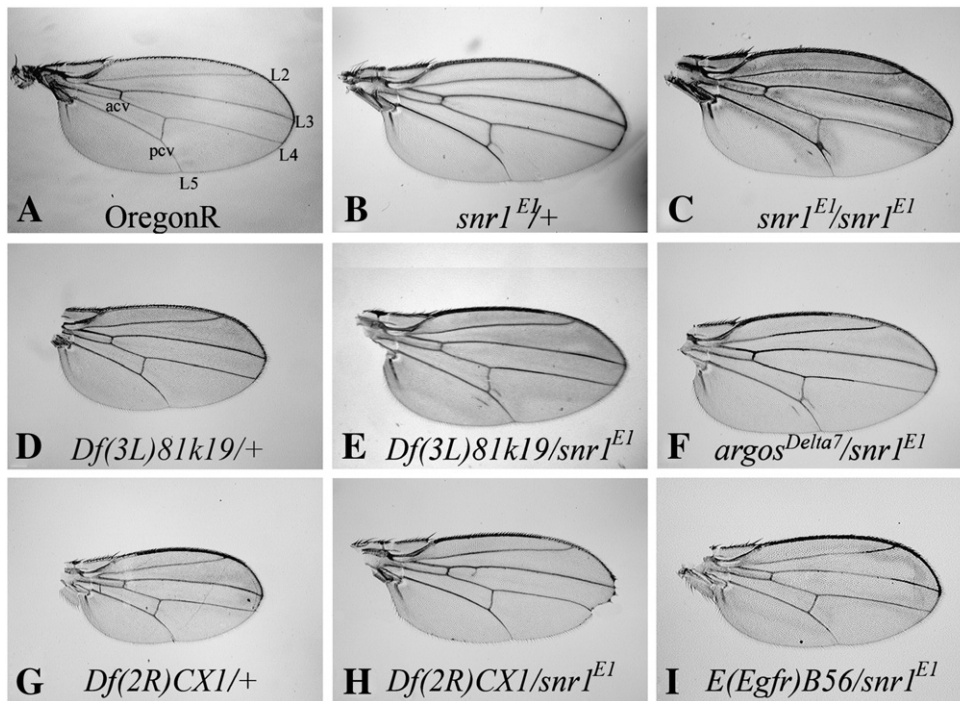


Fig. 3. Validation of the enhancer/suppressor screen. Shown are examples of deficiencies that enhance and suppress the *snr1^{E1}* phenotype. Wings displayed are from adult flies crossed at 29 °C unless otherwise indicated. (A) wild type *OregonR* wing with longitudinal veins L2, L3, L4, L5, and posterior cross vein (PCV) and anterior cross vein (ACV) labeled. (B) *snr1^{E1}/+*, note the weak extra vein present near the PCV. (C) *snr1^{E1}/snr1^{E1}* at 18 °C. (D) *Df(3L)81k19/+*. (E) *Df(3L)81k19/snr1^{E1}*, note the enhancement of the *snr1^{E1}* extra vein phenotype as manifested by additional vein material posterior to L5. (F) *argos^{Delta7}/snr1^{E1}*, the *argos* gene is deleted in *Df(3L)81k19* and shows similar enhancement as the deficiency alone. (G) *Df(2R)CX1/+*. (H) *Df(2R)CX1/snr1^{E1}*, note the minor notching of the posterior and distal portions of the wing blade that is not present in deficiency wings alone as well as the suppression of the weak *snr1^{E1}* vein phenotype. (I) *E(Egfr)B56/snr1^{E1}*, the *E(Egfr)B56* gene is deleted in *Df(2R)CX1* and shows similar suppression as the deficiency alone; however, the wing notching present in (H) is likely due to an unidentified interacting locus removed in the deficiency.

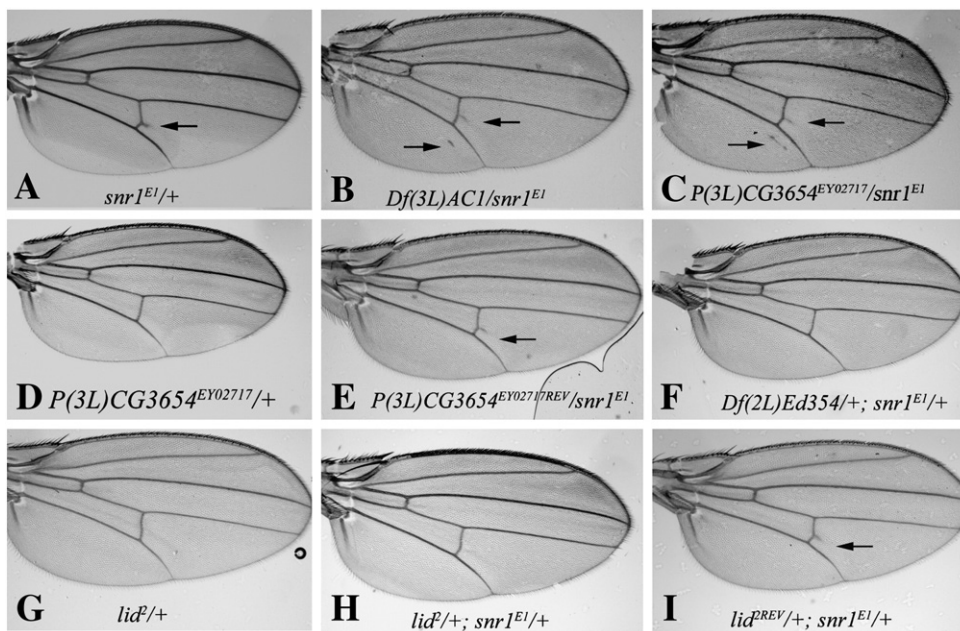


Fig. 4. *snr1* genetically interacts with histone lysine demethylase enzymes. (A) *snr1^{E1}/+* wings display ectopic vein material distal to the PCV (arrow). (B) Wings from *Df(3L)AC1/snr1^{E1}* transheterozygous flies have increased penetrance and expressivity of the ectopic vein phenotype. Note ectopic vein material posterior to L5 and distal to the PCV (arrows). (C) *snr1^{E1}/P(CG3654)^{EY02717}* transheterozygous wings have an enhanced ectopic vein phenotype, with wings displaying ectopic vein material distal to the PCV and anterior to L2 (arrows). (D) The candidate histone demethylase gene covered by the *Df(3L)AC1* deficiency, *P(CG3654)^{EY02717}*, has no heterozygous wing phenotype. (E) *P(CG3654)^{EY02717}* reverts the genetic interaction phenotype observed with *snr1^{E1}*. *snr1^{E1}/P(CG3654)^{EY02717REV}* transheterozygotes have an ectopic vein phenotype similar to *snr1^{E1}/+* siblings [compare (A) to (E)]. (F) The *snr1^{E1}* transheterozygous phenotype is dominantly suppressed by *Df(2L)Ed354*. Note *Df(2L)Ed354/+; snr1^{E1}/+* wings have no ectopic vein material near the PCV. (G) The candidate histone demethylase gene covered by the *Df(2L)Ed354* deficiency, *lid²*, has no heterozygous wing phenotype. (H) Heterozygous *lid²* dominantly suppresses the *snr1^{E1}* ectopic vein phenotype. *lid²/+; snr1^{E1}/+* transheterozygotes have no ectopic veins. (I) Reversion of the *lid²* mutation by *P*-element excision abolishes the suppression of the *snr1^{E1}* phenotype, such that *lid^{2REV}/+; snr1^{E1}/+* transheterozygotes have an ectopic vein phenotype similar to *snr1^{E1}/+* siblings [compare (A) to (I)].

demethylase genes dominantly enhanced or suppressed the *snr1^{E1}* wing patterning phenotype (Supplemental Tables 1–3). To better determine if loss of the particular demethylase gene, and not some other gene covered by the deficiency, was responsible for the observed genetic interaction, we obtained smaller representative deficiencies for each of the 13 predicted histone demethylase genes (Klose et al., 2006; Klose and Zhang, 2007) and carried out a candidate screen by independently testing these deficiencies for a genetic

Table 2
snr1^{E1} genetically interacts with histone lysine demethylase enzymes.

Demethylase	Genotype	Wings	Genetic interaction ^a
LSD1 (CG17149)	<i>snr1^{E1},e/+</i>	267	
	<i>snr1^{E1},e/Df(3L)ED4858</i>	106	Enhancement ^b
	<i>snr1^{E1},e/P(lsd1)^{ΔN}</i>	206	No effect
	<i>snr1^{E1},brm²,e/P(lsd1)^{ΔN}</i>	62	Enhancement ^c
JARID (LID)	<i>Df(2L)ED354/+;snr1^{E1},e/+</i>	128	Suppression
	<i>lid¹/+;snr1^{E1},e/+</i>	144	Suppression
	<i>lid²/+;snr1^{E1}/+</i>	168	Suppression
	<i>lid²(REV)/+;snr1^{E1}/+</i>	60	No effect ^d
JARID2 (CG3654)	<i>Df(3L)AC1/snr1^{E1},e</i>	120	Enhancement ^e
	<i>snr1^{E1},e/CG3654^{EY02717}</i>	152	Enhancement ^f
	<i>CG3654^{EY02717-REV}/+</i>	30	Unique ^g
	<i>snr1^{E1},e/CG3654^{EY02717-REV}</i>	114	No effect ^h
JHDM2 (CG8165)	<i>Df(3R)by10/snr1^{E1},e</i>	66	Enhancement ⁱ
	<i>snr1^{E1},e/P(CG8165)^{EY01319}</i>	116	Enhancement
JHDM3/JMJD2 (CG15835)	<i>Df(2R)Exel6055/+;snr1^{E1},e/+</i>	50	Suppression ^j
	<i>P(2R)(CG15835)^{KGO4636}/+;</i>	136	No effect
JHDM3/JMJD2 (CG33182)	<i>snr1^{E1},e/+</i>	94	Suppression ^h
	<i>P(CG33182)^{aats-val}/+;</i>	136	No effect
JMJD4 (CG7200)	<i>Df(2L)Bsc149/+;snr1^{E1},e/+</i>	118	Suppression
JMJD5 (CG13902)	<i>Df(3L)ED4177/snr1^{E1},e</i>	40	Suppression
	<i>snr1^{E1},e/P(CG13902)^{e01240}</i>	138	Suppression
JMJD5 (CG10133)	<i>snr1^{E1},e/Df(3L)Exel6119</i>	105	Suppression ^h
	<i>snr1^{E1},e/P(CG10133)^{KGO4150}</i>	78	No effect
JmjC domain only (CG5383/PSR)	<i>snr1^{E1},e/Df(3R)Exel6190</i>	162	Suppression
	<i>snr1^{E1},e/P(EPgy2)^{EY07193}</i>	86	Enhancement ^k
JmjC domain only (CG2982)	<i>Df(1)ED6720/X;snr1^{E1},e/+</i>	48	Suppression
	<i>Df(1)ED6720/Y;snr1^{E1},e/+</i>	48	Suppression
	<i>P(CG2982)^{EP1316}/+;snr1^{E1},e/+</i>	64	Suppression
UTX/UTY (CG5640)	<i>Df(2L)Bsc206/+;snr1^{E1},e/+</i>	142	Suppression
JHDM1 (CG11033)	<i>snr1^{E1},e/Df(3R)Exel6151</i>	94	Suppression
	<i>snr1^{E1},e/P(CG11033)^{DG12810}</i>	180	Suppression

^a Genetic interaction was scored as Enhancement, Suppression, No Effect, or Unique. A 20% or greater increase in the number of wings displaying the *snr1^{E1}* ectopic vein phenotype or the presence of ectopic vein material distal to the PCV as well as anterior of L2 or posterior of L5 was classified as enhancement. A 20% or more decrease in the number of wings displaying the *snr1^{E1}* ectopic vein phenotype was considered suppression. A unique phenotype is associated with the demethylase mutant alone.

^b 90% of *Df(3L)ED4858/+* sibling wings displayed an ectopic vein phenotype similar to that of *snr1^{E1},e/+*.

^c 33% of wings display ectopic vein material distal to the PCV as well as posterior to L5.

^d *lid²-REV* is a P-element excision that completely rescues the *lid²* phenotype as well as the genetic interaction phenotype.

^e 24% of wings have ectopic vein material distal to the PCV as well as posterior to L5.

^f 12% of wings show ectopic vein material anterior to L2 or posterior to L5, and ectopic vein material distal to the PCV.

^g *CG3654^{EY02717-REV}* is a P-element excision that rescues the mutant phenotype.

However, 33% of heterozygotes have ectopic vein material distal to the PCV, a phenotype similar to *snr1^{E1}*, complicating the analyses.

^h Approximately 70% of wings display an ectopic vein distal to the PCV. However, multiple ectopic veins were not observed, animals, demonstrating that the genetic interaction observed between *snr1^{E1},e* and *CG3654^{EY02717}* was due to the presence of a P-element insertion within the *CG3654* gene.

ⁱ 23% of wings have ectopic vein material in wing margins, between L3/L4, or anterior to L2, as well as distal to the PCV.

^j Although the deficiency genetically interacted with *snr1^{E1}*, the candidate gene did not, suggesting that the interaction with the deficiency was either due to loss of an unknown gene and not from loss of the histone demethylase, or that the particular demethylase allele tested was weak.

^k Opposite genetic interactions were observed with the deficiency and the candidate gene.

interaction with *snr1^{E1}* (Fig. 4). We found that all 13 deficiencies tested genetically interacted; 3 deficiencies enhanced, while 10 deficiencies suppressed the *snr1^{E1}* ectopic wing vein phenotype (Table 2). To determine if the observed genetic interactions were due to loss of the KDM gene, epistasis tests were performed using P-element insertion lines disrupting several predicted demethylase genes obtained from the Exelixis and Berkeley Drosophila Genome Project (BDGP) collections, as well as a null allele of *lsd1* (*lsd1^{ΔN}*) (Di Stefano et al., 2007). We tested each of these lines for a genetic interaction with *snr1^{E1}* and found that 4 Jumonji family histone demethylase mutants, *little imaginal discs* (*lid*), *CG13902*, *CG2982*, and *CG11033* suppressed the *snr1^{E1}* ectopic vein phenotype and 2 Jumonji members, *CG3654* and *CG8165*, enhanced the phenotype (Table 2; Fig. 4). Transposase mobilized excision of the P-element in two of these lines, one that enhanced (*CG3654^{EY02717}*) and one that suppressed (*lid²*) the *snr1^{E1}* phenotype, abolished the genetic interaction phenotype (Table 2; Fig. 4), confirming that the interactions were not due simply to background effects. Our screen results provide a novel finding that in vivo Brm complex chromatin remodeling functions may be highly dependent on the activity of histone lysine demethylase enzymes.

LSD1 genetically interacts with Brm complex components

The Brm complex has important roles in regulating gene transcription, in part by physically associating with transcription activator proteins (Gutierrez et al., 2007; Hassan et al., 2001; Neely et al., 2002) as well as RNA Polymerase II (Armstrong et al., 2002), gene repressor proteins (Marenda et al., 2004; Sudarsanam et al., 2000), and alternative splicing factors (Batsche et al., 2006; Tyagi et al., 2009). While much work has focused on understanding how

Table 3
lsd1 genetically interacts with mutations in PBAP but not BAP remodeling complexes.

Genotype	# Wings	% Ectopic	Genetic interaction ^a
<i>lsd1^{ΔN}/+</i>	152	1	–
<i>mor¹/+</i>	152	5	Enhancement (+++)
<i>mor¹/lsd1^{ΔN}</i>	188	57	
<i>mor²/+</i>	267	0	No effect
<i>mor²/lsd1^{ΔN}</i>	135	3	
<i>mor³/+</i>	319	52	Enhancement (+++)
<i>mor³/lsd1^{ΔN}</i>	104	100	
<i>mor⁴/+</i>	106	0	No effect
<i>mor⁴/lsd1^{ΔN}</i>	117	6	
<i>mor⁵/+</i>	130	34	Suppression
<i>mor⁵/lsd1^{ΔN}</i>	192	11	
<i>mor⁶/+</i>	241	44	Enhancement (++)
<i>mor⁶/lsd1^{ΔN}</i>	100	61	
<i>brm²/+</i>	134	3	Enhancement (+)
<i>brm²/lsd1^{ΔN}</i>	170	14	
<i>snr1^{R3}/+</i>	90	0	Enhancement (+)
<i>snr1^{R3}/lsd1^{ΔN}</i>	148	9	
<i>osa⁰⁰⁰⁹⁰/+</i>	70	0	No effect
<i>osa⁰⁰⁰⁹⁰/lsd1^{ΔN}</i>	74	5	
<i>osa²/+</i>	84	0	No effect
<i>osa²/lsd1^{ΔN}</i>	64	3	
<i>polybromo^{EY14080}/+</i>	81	31	Enhancement (++)
<i>polybromo^{EY14080}/lsd1^{ΔN}</i>	127	50	
<i>polybromo^{EY14730}/+</i>	28	50	Enhancement (++)
<i>polybromo^{EY14730}/lsd1^{ΔN}</i>	36	81	
<i>Bap180^{Δ86}/+</i>	96	0	Enhancement (++)
<i>Bap180^{Δ86}/lsd1^{ΔN}</i>	101	18	
<i>Bap170^{Δ65}/+</i>	114	2	Enhancement (++)
<i>Bap170^{Δ65}/lsd1^{ΔN}</i>	96	39	

^a Genetic enhancement or suppression was classified based on changes in either expressivity (the degree of ectopic vein material present) or penetrance (changes in the percentage of wings displaying the ectopic vein phenotype). We scored mild enhancement (+) as an approximate 10–20% increase in penetrance, moderate enhancement (++) as 15–40% increase in penetrance, and strong enhancement (+++) as greater than 45% enhancement.

chromatin remodeling complexes contribute to gene activation, far less is known about how remodeling complexes function in gene repression. Therefore, we decided to focus on the group of histone demethylase mutants that enhanced the *snr1^{E1}* wing phenotype. We reasoned that since SNR1 can function to negatively regulate Brm complex activities in intervein cells and prevent differentiation into vein tissue (Marenda et al., 2004), mutations that enhanced the *snr1^{E1}* phenotype may function as corepressors and cooperate with SNR1 to restrict specific target gene transcription.

Although the null mutant of *lsd1* (*lsd1^{ΔN}*) showed little genetic interaction with *snr1^{E1}*, we found that *lsd1^{ΔN}* genetically interacted

with strong loss of function alleles of *mor* and *brm* and *snr1* (Table 3). The *lsd1^{ΔN}* mutation removes the presumptive *lsd1* promoter and N-terminal region, including the SWIRM domain (Di Stefano et al., 2007). The wings of heterozygous *lsd1^{ΔN}/+* flies appear normal (Fig. 5A), and *snr1^{E1}/lsd1^{ΔN}* animals show no change in penetrance or expressivity of the *snr1^{E1}* ectopic vein phenotype (Figs. 5B,C). The wings of *brm²* heterozygotes also appear normal (Fig. 5D), but doubly heterozygous *lsd1^{ΔN}/brm²* wings display ectopic vein material distal to the PCV (Fig. 5E). It was previously demonstrated that a heterozygous *brm²* mutation suppressed the ectopic vein phenotype associated with heterozygous *snr1^{E1}* (Marenda et al., 2003). Therefore,

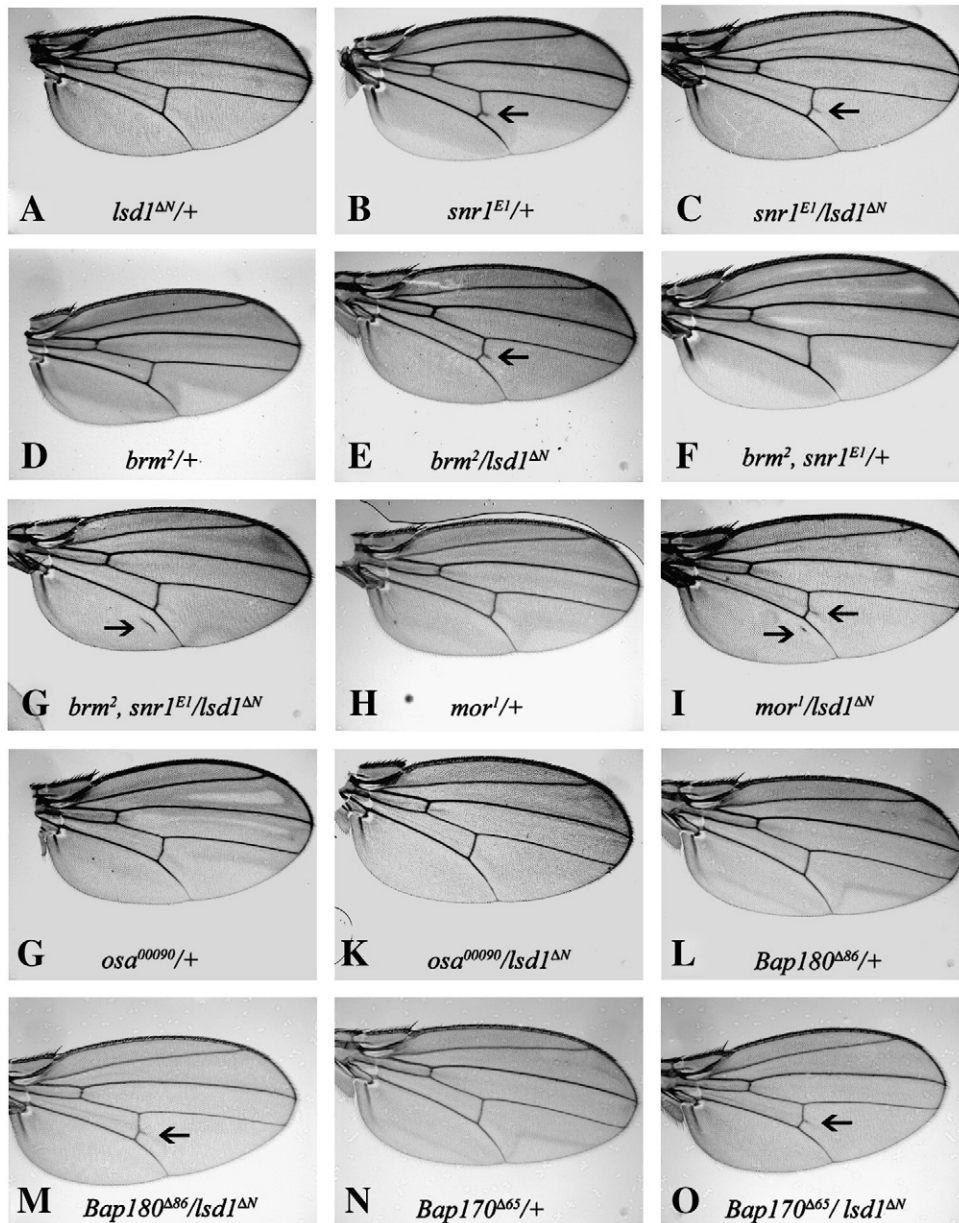


Fig. 5. *lsd1* genetically interacts with PBAP but not BAP complexes. (A) *lsd1^{ΔN}* heterozygotes have normal wings. (B) *snr1^{E1}/+* wings display ectopic vein material distal to the PCV (arrow). (C) *lsd1^{ΔN}* shows no genetic interaction with *snr1^{E1}*. *snr1^{E1}/lsd1^{ΔN}* transheterozygotes have wings similar to *snr1^{E1}/+* animals (arrow) [compare (B) and (C)]. (D) *Drosophila* heterozygous for the *brm²* null allele display normal wing patterning. (E) *lsd1^{ΔN}* genetically interacts with *brm²*. *lsd1^{ΔN}/brm²* transheterozygotes have ectopic vein material distal to the PCV (arrow) [compare (D) and (E)]. (F) *brm²* dominantly suppresses the *snr1^{E1}* ectopic vein phenotype [compare (B) and (F)]. (G) *brm², snr1^{E1}/lsd1^{ΔN}* transheterozygotes show ectopic vein material posterior to L5 (arrow). (H) Flies heterozygous for the point mutant allele *mor¹* display normal wings. (I) *lsd1^{ΔN}* genetically interacts with *mor¹* [compare (H) and (I)]. *lsd1^{ΔN}/mor¹* transheterozygotes have ectopic vein material posterior to L5 and distal to the PCV (arrows). (J) Wings from heterozygous flies carrying a *P*-element insertion in the 5'UTR of the *osa* gene (*osa⁰⁰⁰⁹⁰/+*) appear normal. (K) No genetic interaction observed between *lsd1^{ΔN}* and *osa⁰⁰⁰⁹⁰* [compare (J) and (K)], as wings appear normal in *lsd1^{ΔN}/osa⁰⁰⁰⁹⁰* transheterozygotes. (L) Flies heterozygous for the null allele *Bap180^{Δ86}* have normal wings. (M) *Bap180^{Δ86}* genetically interacts with *lsd1^{ΔN}* [compare (L) and (M)]. Wings from *Bap180^{Δ86}/lsd1^{ΔN}* transheterozygotes have ectopic vein material distal to the PCV (arrow). (N) Flies heterozygous for the *Bap170^{Δ65}* null allele have wings that appear wild type. (O) *Bap170^{Δ65}* genetically interacts with *lsd1^{ΔN}* [compare (N) and (O)]. Wings from *lsd1^{ΔN}/Bap170^{Δ65}* transheterozygotes have ectopic vein material distal to the PCV (arrow).

we predicted that the ectopic vein phenotype observed in *lsd1^{ΔN}/brm²* animals would be suppressed by the additional heterozygous mutation of *snr1^{E1}*. Interestingly, the ectopic vein phenotype observed in *lsd1^{ΔN}/brm²* wings was further enhanced by the *snr1^{E1}* allele (Figs. 5F,G; Table 2). The *lsd1^{ΔN}* allele also demonstrated strong allele-specific interaction with various *mor* mutants (Fig. 5, Table 3). For example, the double heterozygous combination of *lsd1^{ΔN}* with *mor¹*, a loss of function point mutation (Fig. 5H), leads to the appearance of ectopic posterior crossveins by more than 50% (Fig. 5I). These data suggest that some aspects of SNR1-dependent repression of Brm functions in the wing intervein region depend on a fully functional LSD1.

LSD1 associates with Brm complexes containing POLYBROMO/BAP180 and BAP170 (PBAP) but not OSA (BAP)

The SWI/SNF family of remodeling complexes can be divided into two subclasses that have the same constellation of core subunits, with differences in signature subunit composition (Mohrmann and Verrijzer, 2005). In *Drosophila*, BAP complexes contain OSA as a signature subunit while PBAP complexes contain POLYBROMO/BAP180, BAP170 and SAYP but not OSA (Chalkley et al., 2008; Collins and Treisman, 2000; Mohrmann et al., 2004; Moshkin et al., 2007). Genetic studies have revealed pathway-specific regulation of target genes by each of these Brm complex subclasses. Therefore, we tested if *lsd1^{ΔN}* displayed a preferential genetic interaction with mutations in *osa*, *polybromo* (*Bap180*), or *Bap170* (Carrera et al., 2008). We observed no genetic interaction (Fig. 5K) between *lsd1^{ΔN}* and a loss of function *osa* mutant (*osa⁰⁰⁰⁹⁰*) (Marenda et al., 2004; Treisman et al., 1997; Vazquez et al., 1999) in the context of wing pattern development. However, *lsd1^{ΔN}* interacted strongly with null alleles *Bap180^{Δ86}* and *Bap170^{Δ65}* (Figs. 5L–O, Table 3), suggesting that LSD1 corepressor functions may be targeted to PBAP-specific wing development pathways.

LSD1 physically associates with the Brm complex

LSD1 is not a component of purified Brm complexes (Papoulas et al., 1998); therefore, the observed genetic interactions may be either direct or indirect. We addressed whether LSD1 could physically associate with the Brm complex by using *in vivo* coimmunoprecipitation and GST-pulldown assays. *Drosophila* embryo extracts were immunoprecipitated with antibodies directed against the SNR1 subunit, as it is a stable core component of purified Brm complexes (Papoulas et al., 1998; Zrally et al., 2004, 2003). LSD1 was found to reside in the precipitated material (Fig. 6A), indicating that LSD1 could associate with the Brm complex *in vivo*. To help verify the interaction we fused N- and C-terminal portions of *Drosophila* LSD1 to GST and tested for Brm complex associations using an *in vivo* pulldown assay (Fig. 6B) (Zrally et al., 2004). Wild type embryo extracts were incubated with equivalent amounts of purified GST alone, GST:LSD1-N (aa1–255) and GST:LSD1-C (aa258–876) fusions immobilized on glutathione agarose. Complexes were tested for the presence of SNR1 and only the GST:LSD1-N fusion containing the SWIRM domain was found to interact. There was no detectable interaction with the LSD1 amine oxidase domain that is essential for the demethylase activity, suggesting that the LSD1 SWIRM domain is sufficient for interaction with the Brm complex *in vivo*.

LSD1 has an important role in wing development

LSD1 exhibits both genetic and protein interaction with components of the Brm chromatin remodeling complex, suggesting that LSD1 might have an important role in wing pattern formation in cooperation with Brm functions. We previously found that the Brm complex is widely expressed in wing tissues during the early pupal

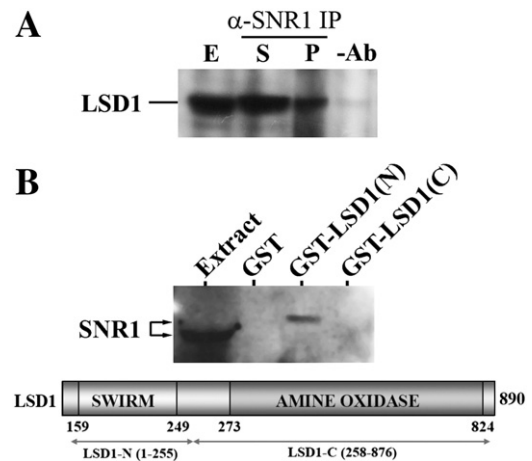


Fig. 6. LSD1 associates with the Brm complex *in vivo*. (A) LSD1 associates with the Brm complex *in vivo*. Wild type embryo extracts (500 μ g) were immunoprecipitated with antibodies to SNR1. Proteins from whole extracts (E, 100 μ g), supernatant (S), and pellet (P) were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane. Western blots were probed with anti-LSD1 antibody. (B) LSD1 SWIRM domain interacts with the Brm complex. GST-LSD1 fusions were generated that contained either the SWIRM domain (LSD1-N) or the amine oxidase domain (LSD1-C) and used for pull-down experiments. Similar amounts of immobilized GST fusions were incubated with whole embryo extracts (500 μ g) and interactions with the Brm complex were assessed by immunoblot analyses using anti-SNR1 antibody. Extract lane represents 100 μ g of embryo lysate.

stage that corresponds to the developmental period when the cross veins are forming (Marenda et al., 2004). Similarly, LSD1 is expressed uniformly throughout the developing pupal wing, in both vein and intervein cells (Figs. 7A,B) which is consistent with a broad role in regulating the expression of genes required for proper wing vein development.

In order to better understand the relationship between LSD1 and the Brm complex, we employed a tissue-specific knockdown of *lsd1* in the developing wing using the GAL4-UAS system (Bernstein et al., 2001). Short hairpin-RNAi (shRNAi) constructs, also known as inverted repeats (IR), were expressed in a tissue specific manner to deplete *lsd1* mRNA. Expression of an *lsd1-IR* using the *GawB69B* GAL4 imaginal disc driver led to ectopic veins anterior to the L2 longitudinal vein and along the posterior crossvein (Fig. 7C), a phenotype similar to the effects of losing Brm complex repressor functions. The penetrance and expressivity of the knockdown phenotype was greater in males as well as flies reared at 29 °C, and enhanced by the addition of a *UAS-Dicer* transgene that amplifies the RNAi effect (Bernstein et al., 2001; Dietzl et al., 2007). *UAS-Dicer; lsd1-IR/GawB69B* males displayed the strongest phenotypes including crumpled, broken and held-out wings (Fig. 7B and data not shown).

Wing veins develop as a consequence of specific signaling pathways that are restricted to subsets of cells within the wing primordium (Bier, 2000). The Brm complex contributes to the development of the veins through activation functions in vein cells and suppression of those pathways in the intervein cells (Marenda et al., 2004). To help determine whether LSD1 cooperates with the Brm complex in restricting vein development in a cell-specific manner, we expressed the *lsd1-IR* in wing intervein cells through the use of a *blistered-GAL4* driver. Blistered (BS) is homologous to mammalian serum response factor and the expression of BS is restricted to intervein cells in the developing *Drosophila* wing (Johannes and Preiss, 2002). We found that loss of *lsd1* in intervein cells resulted in ectopic vein formation including an ectopic anterior cross vein (ACV) in a small percentage of animals (Fig. 7E). This data supports the view that LSD1 functions to repress wing vein formation in intervein cells, possibly by cooperating with the Brm complex to restrict vein-specific gene transcription.

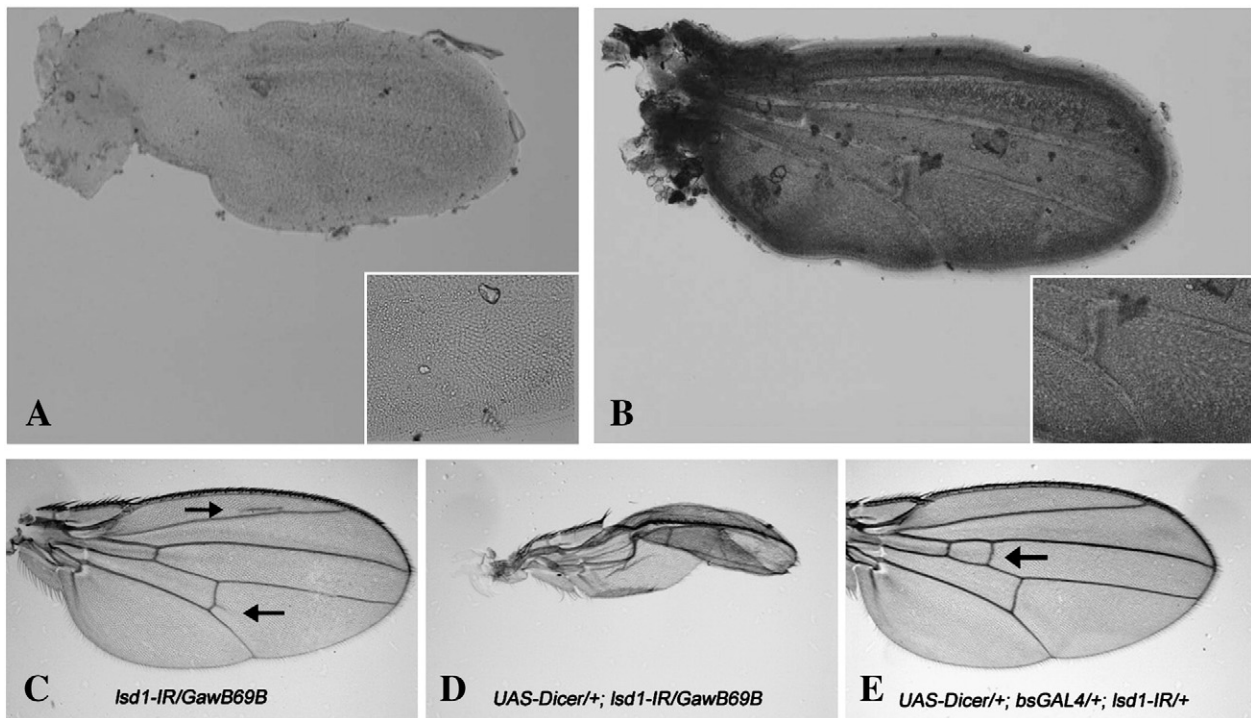


Fig. 7. LSD1 has an important role in wing development. (A and B) LSD1 is uniformly expressed in both vein and intervein cells in wild type *OregonR* Stage P7 (34–50 h APF) pupal wings (Bainbridge and Bownes, 1981). (A) (—) antibody negative control. (B) *Drosophila* LSD1 antibody was used to detect expression in pupal wing cells. Insets in A and B show magnified views of the posterior cross vein region. (C–E) The GAL4-UAS system was utilized to express a short-hairpin RNAi (shRNAi) construct to knockdown *Lsd1* (*Lsd1-IR*) in a cell type or developmental time-specific manner. The *GawB69B* driver leads to GAL4 expression in larval imaginal discs. The *blistered* driver (*bs-GAL4*) leads to GAL4 expression specific to wing intervein cells during pupal development. All knockdown experiments were carried out at 29 °C. (C) Expression of *Lsd1-IR* using the GAL4 driver *GawB69B* causes ectopic vein development (arrows). (D) Overexpression of *Dicer* (*UAS-Dicer*) amplifies the RNAi effects of the *Lsd1-IR*. (E) Expression of *Lsd1-IR* using *bs-GAL4* in intervein cells leads to the formation of an ectopic ACV in a small percentage of flies (arrow).

Discussion

Although chromatin remodeling is an important component of gene activation, its role in gene repression is not as well understood. Our unbiased genetic screen using a weak dominant temperature sensitive mutant allele of a key Brm complex regulatory subunit has provided new insights into the involvement of chromatin remodeling complexes in developmental tissue patterning. We found that mutations in components of several signaling pathways, including Notch, EGFR and DPP/TGF β , genetically interacted in our assay. These results, combined with candidate gene genetic analyses (this study and Marendu et al., 2004), have confirmed our previous hypotheses that the Brm complex participates in both gene activation and gene repression to help coordinate several key signaling pathways that lead to proper animal patterning. Our results are largely concordant with the results of previous limited screens that identified a set of dominant modifiers of *brm*^{K804R} mutant phenotypes (Armstrong et al., 2005; Papoulas et al., 1998). Among 14 chromosomal deficiencies that enhanced the *brm*^{K804R} rough eye phenotype, we found that 6/14 were also dominant enhancers of the *snr1*^{E1} wing phenotype and 3/14 were suppressors (Tables S2, S3), suggesting that dominant modifier screens are effective tools for identifying unknown loci important for Brm complex regulatory functions. Consistent with this view, the Brm complex has been shown to interact the Notch ligand, Delta, in the developing fly eye (Armstrong et al., 2005). Our genetic modifier screen results presented here indicate that Notch signaling functions may also be mediated through the Brm complex in the developing fly wing. Given the strong evolutionary conservation of these pathways, we anticipate that the vertebrate SWI/SNF orthologs will play a similarly important role in patterning the tissues of vertebrate animals.

What are the target genes regulated by the Brm complex in the developing wing? We previously found that loss of *snr1* function

results in ectopic *dpp* and *rhomboid* expression in intervein cells (Marendu et al., 2004). These data are consistent with the genetic interactions shown in this report that were observed using mutants affecting both the DPP and EGFR pathways. Our studies have additionally provided an important insight into gene regulatory factors beyond signaling pathways that contribute to transcription repression in collaboration with chromatin remodeling complexes at key points in the development and differentiation of tissues. In our present analyses, we provide several lines of evidence suggesting that the mechanism of Brm complex-mediated gene repression is not only dependent upon a tight, physical and genetic relationship between two core subunits, SNR1 and MOR, but also on histone lysine demethylase enzymes.

MOR has an important role in regulating Brm complex stability and facilitating SNR1 repressor functions

It has been reported that the full in vitro chromatin remodeling activity of the mammalian BRM/BRG1 complex on reconstituted nucleosomes can be accomplished with a subset of three or four core components, including the SNF5 (SNR1), BAF155/BAF170 (MOR) and BRM/BRG1 ATPase subunits that are highly conserved from yeast to vertebrates (Phelan et al., 1999). Each of these subunits is required for complex stability in vivo as RNAi depletion of the individual components in cultured *Drosophila* cells leads to reduced stability of the other subunits with corresponding changes in target gene expression (this study and Moshkin et al., 2007; Zraly et al., 2006). Loss of BRM function in vivo, using either a dominant negative ATPase deficient mutant (*brm*^{K804R}) or an amorphic allele (*brm*²), can suppress the *snr1*^{E1} wing phenotype (Marendu et al., 2004, 2003) revealing an important role for SNR1 in restraining Brm complex transcription activation functions. In contrast, *mor* mutants enhance

mutant phenotypes associated with reduced *brm* function (Brizuela and Kennison, 1997) and show allele-specific interaction with *snr1^{E1}*, suggesting an important functional relationship between the MOR, BRM and SNR1 subunits. MOR likely serves as a scaffolding protein, since physical associations were observed between SNR1-MOR and MOR-BRM. Two independent domains of MOR, the SWIRM and SANT, domains respectively, are critical for the binding interaction (Crosby et al., 1999; Moshkin et al., 2007; Phelan et al., 1999). Therefore, the contribution of SNR1 regulatory function on Brm complex chromatin remodeling activities may depend on crosstalk through MOR since no direct physical contacts between SNR1 and the BRM subunit have been observed.

Histone demethylase enzymes function as coregulators of the Brm complex

An unbiased dominant modifier genetic screen allowed us to identify histone lysine demethylase enzymes as novel coregulators of the Brm complex in controlling gene expression. Previous screens looking for modifiers of a *brm* dominant negative allele (*brm^{K804R}*) did not uncover mutations in histone-modifying families, such as acetyltransferases, deacetylases, and methyltransferases (Armstrong et al., 2005). However, the wing patterning defect associated with *snr1^{E1}* is highly sensitive, allowing us to observe subtle changes in remodeling activities, and identify a family of epigenetic modifiers as potential Brm regulators. We previously found that histone deacetylases (HDACs) were important corepressors that worked in direct collaboration with the Brm complex (Marenda et al., 2004; Zraly et al., 2004). In the present study, mutations in predicted demethylase genes genetically interacted with *snr1^{E1}* and LSD1 was shown to associate with the Brm complex in vivo, suggesting demethylases are also potential cofactors. While a functional cooperation between histone deacetylation and demethylation activities has been suggested (Lan et al., 2008; Lee et al., 2006), our data implicates at least three chromatin modifying activities—ATP-dependent chromatin remodeling, histone deacetylation and demethylation—cooperating to regulate tissue-specific gene repression through multiple bridging interactions. In this scenario, the commitment of a gene promoter to be repressed in a cell type-specific manner would depend on the collateral influence of several chromatin modifying activities that would serve to help establish a repressed transcriptional environment, refractory to the influence of signaling pathways operational in adjacent cells.

There appears to be no correlation between the predicted demethylase lysine substrate and enhancement/suppression of the *snr1^{E1}* phenotype. This is not surprising, since a high degree of functional redundancy exists amongst demethylase enzymes (Klose and Zhang, 2007; Kouzarides, 2007; Shi and Whetstine, 2007). It is likely that multiple demethylase enzymes cooperate to regulate a variety of target genes. This is supported by experimental evidence showing that knockdown experiments of individual demethylases, for example *lsd1*, in cell culture often showed little or no change in global methylation status, though significant changes were observed on a gene-specific level in vivo (Di Stefano et al., 2007; Rudolph et al., 2007). Independent loss of function mutations in two JARID family members, *lid* and *Jarid2/CG3654*, resulted in an opposite genetic interaction with *snr1^{E1}*. We observed that a loss of function mutation in *lid*, (*lid²*) dominantly suppressed, whereas a loss of function mutation in *Jarid2* (*CG3654^{EY02717}*) enhanced the ectopic vein phenotype associated with *snr1^{E1}*. LID is an H3K4me3/me2 specific demethylase (Lee et al., 2007; Lloret-Llinares et al., 2008; Secombe et al., 2007). JARID2 is predicted to have the same substrate specificity, though overexpression analyses in cell culture experiments showed no global increase in H3K4me3/2 (Lloret-Llinares et al., 2008). The observed opposite genetic interaction with *snr1^{E1}* may reflect differences in target gene regulation by LID and JARID2, either as a consequence of different target genes controlled in the developing

wing or through opposite mechanisms in controlling gene transcription. Importantly, JARID2 homologs in *Xenopus* and mammalian model systems physically associate with the Polycomb Repressor Complex-2 (PRC2) and directly contribute to transcriptional repression by preventing the methylation of the histone lysine residues correlated with transcriptional activation (Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). Therefore, mutation of *JARID2* (*CG3654^{EY02717}*) may enhance the *snr1^{E1}* phenotype if the normal role of *CG3654* is to suppress transcription of a particular gene involved in wing vein development.

The cell-fate decision to become vein or intervein is largely based on cell-type specific expression of transcription factors (Blair, 2007; de Celis, 2003). In vein cells, transcription factors with gene targets that promote vein development are highly expressed, whereas those with gene targets that block vein fate are repressed. In intervein cells, the opposite is observed, with heightened expression intervein-promoting factors and decreased expression of vein promoting factors. The Brm complex has an important role in development of both cell fates, serving a positive role to promote vein development in vein cells, and repress vein development in intervein cells (Marenda et al., 2004). The opposite genetic interaction phenotypes observed with *lid* and *Jarid2* could be partially explained if the Brm complex is coordinating with the each specific demethylase to regulate different target genes. We found that loss of function mutations in vein promoting genes, such as *Egfr*, suppressed the *snr1^{E1}* phenotype (Marenda et al., 2004). Our results suggest that LID and EGFR may regulate the expression of similar target genes and indeed EGFR (as well as other signaling pathways) may function in wing vein development through LID. In this scenario, a loss of function mutation in *lid* would result in a decrease in the expression of vein promoting genes, thereby suppressing the *snr1^{E1}* ectopic vein phenotype. Enhancement of the *snr1^{E1}* phenotype by *Jarid2/CG3654^{EY02717}* can be explained if JARID2 promotes activation of genes required to block vein differentiation, just as loss of function mutations in vein-inhibiting factors, such as *net*, enhanced the *snr1^{E1}* phenotype (Marenda et al., 2004).

LSD1 may be a cofactor specific to the PBAP family of Brm complexes

Our candidate genetic screen results suggest that histone lysine demethylase enzymes are likely cofactors of Brm chromatin remodeling activity. However, it is highly unlikely that stable physical associations are made between the complex and all six demethylases. We cannot eliminate the possibility that the Brm complex and demethylase enzymes are independently regulating genes involved in wing patterning or eliciting their functions on different targets at different times during development to contribute to the final read-out of vein/intervein patterning in the adult wing. However, we did observe a direct physical association between the Brm complex and LSD1 in coimmunoprecipitation and GST-pulldown experiments, implying that LSD1 is a potential cofactor of Brm complex remodeling activities.

Our genetic epistasis experiments demonstrated an important in vivo functional relationship between LSD1 and the core subunits of the Brm complex, SNR1, MOR, and BRM. Brm complexes can be subdivided into two groups: PBAP complexes contain BAP170, POLYBROMO/BAP180, and SAYP, whereas BAP complexes contain OSA (Chalkley et al., 2008; Collins et al., 1999; Kal et al., 2000; Mohrmann et al., 2004; Papoulas et al., 1998). These complexes can regulate target genes in a synergistic, antagonistic, or independent manner (Mohrmann et al., 2004; Moshkin et al., 2007). BAP and PBAP complexes likely have differential regulatory functions, since they have distinct, but overlapping, localization patterns on larval salivary gland polytene chromosomes (Mohrmann et al., 2004) and targeted knockdown of OSA, POLYBROMO, or BAP180 using RNAi in cultured Schneider cells, leads to differential expression profiles on whole

genome arrays (Moshkin et al., 2007). OSA, BAP170, BAP180, and SAYP likely have different roles in development, as mutation of each leads to different abnormalities. For example, BAP180 is required for proper egg shell development, whereas BAP170 is necessary to stabilize BAP180, important for adult viability, and vein cell differentiation (Carrera et al., 2008). OSA is necessary for photoreceptor development, normal embryonic segmentation, and wing patterning (Treisman et al., 1997). BAP, but not PBAP complexes have an important role in regulating cell cycle progression through mitosis (Moshkin et al., 2007).

In mice, knockout of *Baf180* causes misregulation of retinoic acid receptor target genes and heart developmental defects, indicating that PBAP complexes may have a role in nuclear receptor transcriptional regulation (Wang et al., 2004). The LSD1 corepressor complex, including the cofactor proteins, CoREST, and histone deacetylase, HDAC1/2, have also been indicated in nuclear receptor transcriptional regulation. LSD1 association in complexes containing the Estrogen Receptor (ER) or Androgen Receptor (AR) leads to a switch in methylated lysine specificity, and results in demethylation of mono- and dimethylated H3K9 and gene activation (Garcia-Bassets et al., 2007; Metzger et al., 2005).

It is not known how BAP vs. PBAP complexes are differentially recruited to target genes. Recruitment of BAP complexes to specific target genes may depend on the physical associations made by OSA and sequence-specific transcription factors. For example, OSA is required for expression of target genes associated with the transcription factors Pannier and Apterous (Milan et al., 2004) and can promote transcriptional repression of genes regulated by Wnt/Wingless signaling (Collins et al., 1999; Collins and Treisman, 2000). Our genetic epistasis experiments reveal that LSD1 cooperates with PBAP, but not BAP containing complexes in the *Drosophila* wing, suggesting that the physical association we observed between LSD1 and Brm complex may be limited to PBAP complexes and provide a mechanism for selective target gene recruitment and regulation by Brm remodeling complexes. Further analyses, such as GST-pulldown and coimmunoprecipitation experiments using PBAP specific components need to be performed to address this possibility.

LSD1 has cell type-specific role to repress vein cell differentiation

Ectopic vein development within intervein tissue can result from two different possibilities: 1) the loss of a factor necessary to block vein cell development, or 2) the gain of a factor that promotes vein cell differentiation. Our knockdown experiments suggest LSD1/dCoREST functions through the first mechanism. Loss of LSD1/dCoREST throughout the entire developing wing imaginal disc resulted in the development of vein material in intervein tissue, but no changes in vein morphology were observed. If LSD1/dCoREST normally functioned to promote vein development, then loss throughout the entire wing should have led to a loss of vein phenotype.

Several lines of evidence suggest that LSD1 may be capable of regulating gene transcription in a cell-type or stage dependent manner. The affect of homozygous loss of *lzd1* on transcriptional regulation of known target genes, including the *Sodium Channel* and *Nicotinic Acetylcholine Receptor-β* is minimal in embryos and larvae, but significant in pupae (Di Stefano et al., 2007). This implies that LSD1 has an important role in regulating gene transcription during later developmental stages. Moreover, LSD1 negative regulation of the homeobox genes, *Ultrabithorax* (*Ubx*) and *abdominal-B* (*abd-B*) continues into adulthood, as *lzd1* null animals display significantly increased expression of these genes as the animals continue to age (Di Stefano et al., 2007). This stage-dependent requirement appears to be conserved, as the conditional knock-out of LSD1 in the developing mouse pituitary gland causes little or no morphological defects early in pituitary development (E9–E9.5), but significantly alters cell-fate determination choices during later stages (E17.5) (Wang et al., 2007).

Furthermore, LSD1 mediates both gene activation and gene repression of different target genes by associating with several multisubunit complexes (Lan et al., 2008; Wang et al., 2007).

Our knockdown and genetic epistasis experiments further support the idea that LSD1 is important for regulating terminal differentiation, since patterning phenotypes are similar to those observed with defects in DPP and EGFR signaling, the pathways active during pupal development, rather than observed with defects in HH signaling, an early pathway component. Previous work has demonstrated an important role in Brm complex involvement in EGFR, DPP, and Delta/N signaling (Armstrong et al., 2005; Marendza et al., 2004). More recently, it has been demonstrated that OSA, the defining subunit of the BAP complex, is required to activate EGFR targets in the developing wing (Terriente-Félix and de Celis, 2009). In this regard, the Brm complex may be cooperating with LSD1 to regulate several conserved signaling pathways, but this cooperation may be tissue and developmental time-point dependent.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.12.001.

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