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Histone lysine demethylases function as co-repressors of SWI/SNF remodeling activities during *Drosophila* wing development

Brenda J. Curtis [a], Claudia B. Zraly [b], Daniel R. Marenda [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 $snr1^{E1}$ 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 $snr1^{E1}$ 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.

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 *Swi*tching Deficient) or displayed an inability to grow in sucrose containing medium (SNF for *S*ucrose *n*on-*f*ermenting) (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

* Corresponding author. Cardinal Bernardin Cancer Center, Room 334, Loyola University Chicago Stritch School of Medicine, 2160 S. First Avenue, Maywood, IL 60153, USA. Fax: +1 708 327 3342.
E-mail address: adingwall@lumc.edu (A.K. Dingwall).

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polytene chromosomes within regions undergoing active gene transcription (Armstrong et al., 2002; Zraly et al., 2003). Microarray analyses of Brm complex mutants demonstrated that the complex was likely involved in both transcription activation and repression (Zraly 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 (*E*pidermal *G*rowth *F*actor *R*eceptor), DPP/BMP (*D*ecapa*p*enta*p*legic/Bone *M*orphogenetic *P*rotein) 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 coregulatory 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 y^1w^{67c23}*; P(w*$^+$*, lacW)lid*^{2}*/CyO* flies were crossed to male w^-*; wg*^{sp1}*/CyO; Δ2,3 Sb/TM6B*. Virgin w^-*; lid*^{2}*/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 *lid*^{2} 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 y^1w^{67c23}*; P{w[+mC]y[+mDint2]=EPgy2} Jarid2*^{EY02717} flies were crossed to w^-*; wg*^{sp1}*/CyO; Δ2,3 Sb/TM6B*. Male y^1w^{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 pRF4-5o 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 (Zraly 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 knockdown the endogenous *snr1*, *mor* and *brm* transcripts, as well as an unrelated gene *CG10465*, as described previously (Zraly et al., 2006).