Jarid2 (Jumonji, AT Rich Interactive Domain 2) Regulates NOTCH1 Expression via Histone Modification in the Developing Heart*

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Background: Jarid2 regulates Notch1 expression in the developing heart through an unidentified mechanism.

Results: Regulation of Notch1 by Jarid2 is through recruitment of SETDB1, resulting in increased methylation of histone H3 lysine 9.

Conclusion: Jarid2 regulation of a subset of genes during cardiac development involves histone methylation through SETDB1 recruitment.

Significance: This is a novel mechanism of epigenetic regulation by Jarid2 during cardiac development.

Jarid2, Jumonji, the founding member of the Jmj factor family, critically regulates various developmental processes, including cardiovascular development. The Jmj family was identified as histone demethylases, indicating epigenetic regulation by Jmj proteins. Deletion of Jarid2 in mice resulted in cardiac malformation and increased endocardial Notch1 expression during development. Although Jarid2 has been shown to occupy the Notch1 locus in the developing heart, the precise molecular role of Jarid2 remains unknown. Here we show that deletion of Jarid2 results in reduced methylation of lysine 9 on histone H3 (H3K9) at the Notch1 genomic locus in embryonic hearts. Interestingly, SETDB1, a histone H3K9 methyltransferase, was identified as a putative cofactor of Jarid2 by yeast two-hybrid screening, and the physical interaction between Jarid2 and SETDB1 was confirmed by coimmunoprecipitation experiments. Concurrently, accumulation of SETDB1 at the site of Jarid2 occupancy was significantly reduced in Jarid2 knockout (KO) hearts. Employing genome-wide approaches, putative Jarid2 target genes regulated by SETDB1 via H3K9 methylation were identified in the developing heart by ChIP-chip. These targets are involved in biological processes that, when dysregulated, could manifest in the phenotypic defects observed in Jarid2 KO mice. Our data demonstrate that Jarid2 functions as a transcriptional repressor of target genes, including Notch1, through a novel process involving the modification of H3K9 methylation via specific interaction with SETDB1 during heart development. Therefore, our study provides new mechanistic insights into epigenetic regulation by Jarid2, which will enhance our understanding of the molecular basis of other organ development and biological processes.

Jarid2 is required for normal cardiac development, and all mice harboring a homozygous Jarid2 deletion (Jarid2 KO) die in the uterus or right after birth (1–3). We have previously reported that whole body or endothelial-specific deletion of Jarid2 (Jarid2enKO) results in cardiac defects mimicking human congenital cardiac defects, including ventricular septal defects, double outlet right ventricle, and hypertrabeculation associated with noncompaction of the ventricular wall resulting in a thin compact layer (1, 4, 5). Notch1 signaling is critical for normal cardiac development. Whole body or endothelial deletion of Notch1 in the mouse results in embryonic lethality at embryonic day 10.5 (E10.5)2 with hearts showing little or no trabeculation (6, 7). We identified Notch1 as a potential target of Jarid2 and observed elevated Notch1 expression in the endocardium and elevated Notch1 signaling to the underlying myocardium in Jarid2 KO and Jarid2enKO embryonic hearts (5). This dysregulation of the Notch1 pathway is a potential cause for the defects observed. However, the precise mechanistic function of Jarid2 in regulation of Notch1 expression in the developing heart remains to be elucidated.

Histone methylation was once considered to be static and an enzymatically irreversible chromatin modification. However, recent reports have shown that both methylation and demethylation of histones is a highly regulated process that allows for fine epigenetic regulation of many cellular processes including transcriptional regulation, regulation of cell fate, and cell proliferation (8). For example, methylation of H3K9 and H3K27 is generally associated with gene silencing (9–11). Jarid2 has been reported to function as a transcriptional repressor and to interact with other nuclear factors (4, 5, 12–17). Jarid2 is the founding member of the Jumonji family of proteins, all of which contain the JmjC domain that generally confers histone demethylase activities. The recent discovery of Jmj family factors as histone demethylases has ushered in a new era of investigating the role of histone methylation status in regulating

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The abbreviations used are: E10.5, embryonic day 10.5; H3K27, histone H3 lysine 27; PRC, polycomb repressor complex; H3K9me2, dimethyl H3K9; H3K9me3, trimethyl H3K9; aa, amino acids; DBD, DNA binding domain.
gene expression. Intriguingly, Jarid2 contains substitutions at key amino acids necessary for enzymatic function and is highly likely enzymatically inactive (18–20). Therefore, transcriptional regulation by Jarid2 may be dependent on binding partners that function as histone modifiers. Recent work suggests that Jarid2 is involved in methylation of histone H3 lysine 27 (H3K27) through interaction with members of the polycomb repressor complex (PRC) in embryonic stem cells, induced pluripotent stem cells, and in epidermal stem cells (20–25). Although Jarid2 is uniformly agreed to interact with the PRC complex and to be crucial for normal differentiation of embryonic stem cells, the precise role of Jarid2 in regulation of histone methylation status is conflicting. Most importantly, it remains to be determined whether Jarid2 interacts with any histone-modifying enzymes to regulate cardiac morphogenesis in the developing heart. Therefore, it is imperative to delineate whether dysregulation of gene expression in Jarid2 KO mice is due to improper epigenetic regulation via defective histone modification.

To identify the molecular mechanisms by which Jarid2 regulates target gene expression in the developing heart, we investigated the regulation of Notch1 by Jarid2, focusing on the methylation status of lysine residues of histone H3 at the Notch1 locus. We provide evidence that Jarid2 directly regulates Notch1 expression through interaction with a specific enhancer region of the Notch1 locus. Our study indicates that deletion of Jarid2 results in decreased dimethyl and trimethyl H3K9 (H3K9me2 and H3K9me3) at the same region occupied by Jarid2 on the Notch1 locus, which correlates well with aberrant continued Notch1 expression in the Jarid2 KO hearts. We show that Jarid2 interacts with the H3K9 methylase SET domain, bifurcated 1 protein (SETDB1) (26). Further, Jarid2 is required for the recruitment of SETDB1, which confers H3K9me2 and H3K9me3 at the Notch1 enhancer region. This defect in histone modification likely causes failure to regulate Notch1 expression, contributing to the defects observed in Jarid2 mutant hearts. Finally, we have performed ChIP-chip experiments on the developing heart for Jarid2, SETDB1, and H3K9me3, and identified a critical subset of genes regulated by Jarid2 and SETDB1 whose dysregulation may be involved in the phenotypic defects observed in Jarid2 KO hearts. Therefore, our current study provides new insights into epigenetic regulation of cardiac development by Jarid2, which will form a basis to investigate other organ development and broad biological processes.

**EXPERIMENTAL PROCEDURES**

**Quantitative ChIP**—Quantitative ChIP was performed as described (5, 27) at least three times using two pooled E17.5 hearts for each experiment. Antibodies used were H3K27me1 (Upstate, catalog no. 07-448), H3K27me2 (Abcam, catalog no. ab24684), H3K27me3 (Abcam, catalog no. ab6002), H3K9me1 (Upstate, catalog no. 05-1248), H3K9me2 (Upstate, catalog no. 07-441), H3K9me3 (Upstate, catalog no. 07-442), SETDB1 (Abcam, catalog no. ab12317, and Santa Cruz Biotechnology, Inc., catalog no. sc66884). Recovered DNA was used for quantitative real-time PCR in triplicate using the standard curve method with primers described previously (5).
**Regulation of Notch1 by Jarid2**

**RESULTS**

**Jarid2 Deletion Results in Decreased Methylation of H3K9**—We have shown previously that Jarid2 occupies a specific region of the Notch1 genomic locus in E17.5 wild-type (WT) embryonic hearts, likely resulting in Notch1 repression (5). However, the molecular mechanism of Jarid2 in regulation of Notch1 expression has not been elucidated. Histone methylation status is a crucial determinant of gene regulation. H3K9me2 and H3K9me3 are commonly associated with heterochromatin formation and gene silencing. Interestingly, we have identified SETDB1, which functions as a H3K9 methylase as a Jarid2 binding protein by yeast two-hybrid screening. We therefore investigated whether Jarid2 occupancy at the Notch1 locus is associated with regulation of H3K9 methylation, specifically comparing WT and Jarid2 KO mouse hearts at E17.5 when Notch1 is normally repressed. The levels of methylation were examined on the Notch1 locus at both Jarid2-occupied and Jarid2-unoccupied regions by ChIP assays using antibodies against H3K9me1, H3K9me2, and H3K9me3 (5). There was no significant difference in the levels of methylation of H3K9me1 between the WT and Jarid2 KO mouse (Fig. 1A). In contrast, both H3K9me2 (Fig. 1B) and H3K9me3 (C) were enriched at the +1150 bp site in the WT but significantly reduced in the Jarid2 KO heart. This suggests that Notch1 is repressed in the WT mouse because of accumulation of methylation of H3K9 and that loss of Jarid2 disrupts this epigenetic mechanism, resulting in a failure of Notch1 down-regulation in the developing heart.

Jarid2 has recently been shown to recruit the PRC in embryonic stem cells, which functions to methylate H3K27 that act as a repressive mark. Therefore, we investigated whether the deletion of Jarid2 also resulted in a change of methylation at H3K27 on the Notch1 locus. ChIP on WT or Jarid2 KO E17.5 hearts for
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H3K27me1 (Fig. 1D), H3K27me2 (E), or H3K27me3 (F) showed no significant difference at the Notch1 locus. This result suggests that H3K27 methylation is not involved in the regulation of Notch1 expression by Jarid2 in late-stage developing hearts.

Jarid2 Physically Associates with SETDB1 in Vivo—In an effort to determine physical interactors of Jarid2, we performed yeast two hybrid screening (15) and identified two putative cofactors of Jarid2 (Fig. 2A). The recovered cDNA from yeast encoded the second bifurcation of the SET domain (aa 979–1307), suggesting that this region of SETDB1 is sufficient to interact with Jarid2 in yeast.

It is important to examine whether both Jarid2 and SETDB1 are expressed in the developing heart, as the expression pattern of SETDB1 is unknown in the mouse embryonic heart. We have confirmed that both factors are expressed in the embryonic hearts by Western blotting (Fig. 2B, lanes 1 and 4). To determine whether Jarid2 and SETDB1 interact in vivo, coimmunoprecipitation experiments were performed on heart extracts. When heart extract was immunoprecipitated with a Jarid2 antibody, SETDB1 protein was detected (Fig. 2B, lane 3). SETDB1 was not detected when a nonspecific mouse IgG was used for immunoprecipitation (Fig. 2B, lane 2), indicating the specific interaction of Jarid2 with SETDB1. The converse experiment employing a SETDB1 antibody for immunoprecipitation also revealed the specific interactions of Jarid2 with SETDB1 (Fig. 2B, lane 6). Jarid2 was not detected when a nonspecific IgG was used for immunoprecipitation (Fig. 2B, lane 5). These data clearly demonstrate that Jarid2 physically associates with SETDB1 in vivo in the developing hearts. To confirm that Jarid2 and SETDB1 are both expressed in the same cell lineages within the developing heart, immunostaining experiments were performed. Jarid2 (Fig. 2, C and F) and SETDB1 (D and G) are expressed in the endocardium as well as the myocardium in WT E17.5 hearts.

The DBD of Jarid2 Interacts with the SET Domain of SETDB1—To investigate the regions involved in the protein–protein interaction between Jarid2 and SETDB1, GST pull-down assays were performed (Fig. 3A and B). Various 35S-labeled Jarid2 proteins (Fig. 3A, lanes 1–4) were incubated with GST (lanes 5–8) or GST-SETDB1;SET domain only (lanes 9–12) coupled to agarose beads. Both the Jarid2 Ct (Fig. 3A, lane 9) and Jarid2 DBD (lane 11) were able to bind the GST-SETDB1;SET domain only but not to the GST alone. To perform the reciprocal experiment (Fig. 3B), 35S-labeled Jarid2 Ct was incubated with GST (Fig. 3B, lane 1) or various GST-SETDB1 proteins (lanes 2–4) coupled to agarose beads. Our data show that GST-SETDB1 FL and GST-SETDB1;SET only associated with Jarid2 Ct. These results indicate that the DBD of Jarid2 interacts with the SET domain of SETDB1 in vitro, which is consistent with the results of yeast two-hybrid screening.

Jarid2 Recruits SETDB1 to the Notch1 Locus—We have shown that Jarid2 is required for accumulation of H3K9me2 and H3K9me3 at the +1150bp Notch1 locus. However, because Jarid2 is likely enzymatically inactive (18–20), we sought to

**FIGURE 2.** Jarid2 physically interacts with SETDB1. A, diagram depicting the protein structures of Jarid2 and SETDB1. Jarid2 contains a JmjN, AT-rich interacting domain (ARID), JmjC, and zinc finger (ZF) domain. SETDB1 contains a Tudor, Methyl CpG (MBD) binding domain and a bifurcated SET domain. A yeast two-hybrid using Jarid2 as bait identified cDNA encoding aa 979–1307 of SETDB1 as the region mediating physical interaction. B, coimmunoprecipitation experiments were performed on heart extracts. When heart extract was immunoprecipitated with a Jarid2 antibody, SETDB1 protein was detected (Fig. 2B, lane 3). SETDB1 was not detected when a nonspecific mouse IgG was used for immunoprecipitation (Fig. 2B, lane 2), indicating the specific interaction of Jarid2 with SETDB1. The converse experiment employing a SETDB1 antibody for immunoprecipitation also revealed the specific interactions of Jarid2 with SETDB1 (Fig. 2B, lane 6). Jarid2 was not detected when a nonspecific IgG was used for immunoprecipitation (Fig. 2B, lane 5). These data clearly demonstrate that Jarid2 physically associates with SETDB1 in vivo in the developing hearts. To confirm that Jarid2 and SETDB1 are both expressed in the same cell lineages within the developing heart, immunostaining experiments were performed. Jarid2 (Fig. 2, C and F) and SETDB1 (D and G) are expressed in the endocardium as well as the myocardium in WT E17.5 hearts.

**FIGURE 3.** GST pull-down assays determine domains mediating interaction between Jarid2 and SETDB1. A, various 35S-labeled Jarid2 mutant proteins (lanes 1–4) were incubated with GST alone (lanes 5–8) or with GST-SETDB1 (lanes 9–12) containing only the bifurcated SET domain. Jarid2 Ct and Jarid2 DBD bound to GST-SETDB1 SET only. B, [35S]methionine-labeled Jarid2 Ct was incubated with GST (lane 1), GST-SETDB1 FL (lane 2), GST-SET only (lane 3), and GST-no SET (lane 4). GST-SETDB1 FL and GST-SET only bound to Jarid2 Ct. +, binding; -, no binding.
investigate how Jarid2 regulates histone methyl marks on the Notch1 locus. SETDB1, a histone methylase that specifically converts H3K9me1 to H3K9me2 and H3K9me3 (26), was identified as a putative physical interactor of Jarid2 (Figs. 2B and 3A and B). As shown in Fig. 4A, SETDB1 is enriched at the same +1150 bp region of the Notch1 locus where Jarid2 accumulates in the WT heart, but enrichment is significantly reduced in the Jarid2 KO mouse heart. This strongly supports our model (Fig. 4B) that Jarid2 is necessary for the recruitment of SETDB1 to the +1150 bp region of the Notch1 locus, resulting in methylation (indicated by the asterisk) of H3K9 and Notch1 silencing. Deletion of Jarid2 results in failed recruitment of SETDB1 and no methylation of H3K9.

Global Determination of Putative Jarid2 Targets in the Developing Heart—To determine genes regulated through H3K9me that are putative targets of both Jarid2 and SETDB1, we have conducted genome-wide searches by ChIP-chip using E17.5 mouse hearts. As shown in Fig. 5A, SETDB1 is enriched at the same +1150 bp Notch1 locus where Jarid2 accumulates in the WT heart, but enrichment is significantly reduced in the Jarid2 KO mouse heart. This strongly supports our model (Fig. 4B) that Jarid2 is necessary for the recruitment of SETDB1 to the Notch1 locus where SETDB1 is required for H3K9me2 and H3K9me3 accumulation, leading to gene silencing. Further, it reveals a novel molecular function for target gene regulation by Jarid2.

for the identification of a subset of Jarid2 targets that are regulated in a SETDB1 independent manner. We have previously performed microarray analyses on E17.5 WT versus Jarid2 KO hearts to identify dysregulated genes and molecular pathways (5). When the 594 ChIP-chip genes are overlapped with the microarray data set, 172 genes are represented with a change of greater than 1.2-fold in the mutant heart (Fig. 5B). Of these, 107 are up-regulated, whereas only 65 are down-regulated.

Next, we further define potential targets that are repressed by Jarid2. Additional analysis by comparing this subset of up-regulated genes to those contained in the top 11 significantly up-regulated pathways in Jarid2 KO hearts by microarray at E17.5 (5) results in only nine genes that are potentially repressed by Jarid2 through H3K9me3 by SETDB1 (Fig. 5D). These genes are involved in Notch signaling, vasculature development and morphogenesis, regulation of cell proliferation and death, cellular adhesion, and heart development. Notch1 is represented in four of these five processes, including all of the pathways that could account for the phenotypic defects observed in Jarid2 KO hearts, strongly supporting Notch1 as an endogenous target of Jarid2 that is critical for cardiac development.
DISCUSSION

We have previously shown that Notch1 expression is elevated in Jarid2 KO or Jarid2<sup>+</sup> mouse hearts at later stages of development, which in part causes cardiac developmental defects including hypertrabeculation and a thin ventricular myocardium. Moreover, Jarid2 occupied a specific conserved region of the Notch1 genomic locus (5). Although recent work has demonstrated that Jarid2 interacts with members of the PRC to modulate methylation of H3K27 in embryonic stem cells, induced pluripotent stem cells, and epidermal stem cells (20–25), the molecular basis of Notch1 regulation by Jarid2 in the developing heart remains unknown. Therefore, we set out to determine the precise molecular consequence of Jarid2 binding to the Notch1 locus in the developing heart. Here, we demonstrate a novel mechanism whereby the proper epigenetic methylation of H3K9 by Jarid2 is required for proper Notch1 regulation in the developing heart. Although it has been reported that overexpression of Jarid2 results in recruitment of the histone methylases G9a and GLP and accumulation of H3K9me1 and H3K9me2 at the CyclinD1 promoter (16), G9a and GLP do not catalyze H3K9me2 to H3K9me3 and are therefore unlikely to be involved in Notch1 regulation via H3K9 trimethylation. In addition, these results were obtained using an overexpression system in cultured fibroblast cells, which may not reflect physiologically relevant mechanisms. Our studies identified SETDB1 as a direct binding partner of Jarid2 and demonstrated that in the developing heart tissue, Jarid2 is essential for the recruitment of SETDB1 to the Notch1 locus where trimethylation takes place, resulting in gene silencing. The decreased levels of H3K9me2 and H3K9me3 exhibited in Jarid2 KO mice at later stages of development likely account for the persistent Notch1 expression.

We also determined global genomic targets for Jarid2, SETDB1, and H3K9me3 by performing ChIP-chip analyses on embryonic hearts. By overlapping all three data sets, we have identified 594 putative target genes that are regulated at the epigenetic level by the same mechanisms as Notch1, which warrants further investigation into other potential targets regulated by Jarid2 and SETDB1. This represents 15% of all Jarid2 targets identified by ChIP-chip, suggesting that Jarid2 critically regulates a subset of genes through SETDB1 recruitment and H3K9 trimethylation at later stages of the developing heart. It is possible that Jarid2 may function through PRC recruitment in the heart, as demonstrated in stem cells (20–25) or interact with yet unidentified factors that are involved in epigenetic regulation of other genes.

Further examination of the 594 genes identified as putative targets revealed that 172 (33%) are differentially expressed greater than 1.2-fold in the hearts of Jarid2 KO versus WT mice at E17.5 by microarray analyses (5). Further, 62% (107) of these genes are up-regulated more than 1.2-fold, whereas only 38% (65) are down-regulated more than 1.2-fold. This is consistent with Jarid2 functioning primarily as a transcriptional repressor. However, it is plausible that Jarid2 may in some cases function as an activator of transcription. This data set represents a powerful and manageable database for identifying putative targets regulated by Jarid2 through recruitment of SETDB1 and trimethylation of H3K9. It should be noted that the Notch1 gene is among nine putative target genes that are occupied by Jarid2, SETDB1, and H3K9me3, and up-regulated more than 1.2-fold by microarray. Notch1 is also highly represented in the 11 most significantly up-regulated biological processes by microarray, strongly correlating it as a <i>bona fide</i> target of Jarid2 regulated through H3K9me3 by SETDB1. We cannot rule out the possibility that Jarid2 regulates other genes by different mechanisms in the developing heart, including H3K27 methylation. Altogether we provide strong evidence for determining endogenous targets of Jarid2 in the developing heart by employing such combinatorial approaches.

It is intriguing that Jarid2 interacts with a subset of proteins that are involved in histone modifications. In addition to the interaction with the SET domain containing proteins of the PRC, we have shown that Jarid2 interacts with Zkscan17 (15), which interacts with the SET domain containing histone methylase NSD1 (21). Additionally, Jarid2 interacts with the SET domain containing histone methylases GLP and G9a (16). In our current study, we identified SETDB1 as a direct binding partner of Jarid2. Therefore, the involvement of Jarid2 with various SET domain containing proteins or complexes raises the interesting possibility that Jarid2 acts as a “pan-SET domain” interacting protein. Jarid2 may regulate many different cellular mechanisms that are dependent on the specific SET cofactor, which warrants further investigation to reveal new mechanisms of epigenetic regulation involving Jarid2.

Here we show a novel mechanism by which Jarid2 silences Notch1 in the late stages of embryonic cardiac development through recruiting SETDB1 to facilitate enrichment of H3K9me2 and H3K9me3 at that locus. This mechanistic insight coupled with the identification of a subset of genes regulated in a similar process using ChIP-chip and microarray experiments allows for a greater understanding of the molecular processes regulated by Jarid2 in the later stages of embryonic cardiac development and other likely developmental processes.

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