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Histone demethylases Kdm6ba and Kdm6bb redundantly promote cardiomyocyte proliferation during zebrafish heart ventricle maturation

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ABSTRACT

Trimethylation of lysine 27 on histone 3 (H3K27me3) by the Polycomb repressive complex 2 (PRC2) contributes to localized and inherited transcriptional repression. Kdm6b (Jmjd3) is a H3K27me3 demethylase that can relieve repression-associated H3K27me3 marks, thereby supporting activation of previously silenced genes. Kdm6b is proposed to contribute to early developmental cell fate specification, cardiovascular differentiation, and/or later steps of organogenesis, including endochondral bone formation and lung development. We pursued loss-of-function studies in zebrafish to define the conserved developmental roles of Kdm6b. *kdm6ba* and *kdm6bb* homozygous deficient zebrafish are each viable and fertile. However, loss of both *kdm6ba* and *kdm6bb* shows Kdm6b proteins share redundant and pleiotropic roles in organogenesis without impacting initial cell fate specification. In the developing heart, co-expressed Kdm6b proteins promote cardiomyocyte proliferation coupled with the initial stages of cardiac trabeculation. While newly formed trabecular cardiomyocytes display a striking transient decrease in bulk cellular H3K27me3 levels, this demethylation is independent of collective Kdm6b. Our results indicate a restricted and likely locus-specific role for Kdm6b demethylases during heart ventricle maturation rather than initial cardiogenesis.

1. Introduction

Covalent histone modifications establish chromatin landscapes that influence gene expression programs by promoting active or silenced transcriptional states. For instance, localized trimethylation of lysine 27 on histone 3 (H3K27me3) in vertebrates is deposited by Enhancer of zeste homolog 1 and 2 (Ezh1 and 2), the catalytic subunits of the Polycomb repressive complex 2 (PRC2), and is associated with gene silencing (Cao et al., 2002; Margueron and Reinberg, 2011; Müller et al., 2002). H3K27me3 marks can be removed by the lysine-specific demethylases Kdm6a (Utx) and Kdm6b (Jmjd3) (Cloos et al., 2008; Klose and Zhang, 2007; Mosammaparast and Shi, 2010). Therefore, H3K27me3 modifications likely provide an epigenetically inherited but reversible layer of transcriptional control. In vertebrates, H3K27me3 dynamics are implicated in cell fate specification (Alder et al., 2010; Dahl et al., 2010; Rugg-Gunn et al., 2010) and cell reprogramming associated with acquired pluripotency (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007). Further, vertebrate H3K27me3 demethylases directly support cell signal or transcription factor-induced gene expression programs (Jiang et al., 2013; Kartikasari et al., 2013; Ramadoss et al., 2012) and de-differentiation during organ regeneration (Stewart et al., 2009).

Studies using embryonic stem cells (ES cells) suggest Kdm6b enables cell specification of all three germ layers (Burgold et al., 2008; Kartikasari et al., 2013; Ohtani et al., 2011, 2013). These results predict that Kdm6b is responsible for relieving H3K27me3 marks at fate specifying genes as pluripotent cells adopt distinct cell lineage identities (Bernstein et al., 2006; Mikkelsen et al., 2007). However, in vivo tests of this model using mouse *Kdm6b* reverse genetics have yielded conflicting results. Mice homozygous for a *Kdm6b* allele that deletes exons encoding N-terminal regions of the protein are peri-implantation lethal at embryonic day 6.5 (E6.5) (Ohtani et al., 2013). In contrast, mice homozygous for several independently generated *Kdm6b* mutant alleles that delete the catalytic JmjC domain die perinatally with lung defects (Q. Li et al., 2014; Satoh et al., 2010; Shpargel et al., 2014; Zhang et al., 2015). These latter studies suggest that Kdm6b-driven H3K27me3 demethylation does not instructively derepress key cell fate regulatory genes during early embryonic development.

Kdm6b-deficient mouse embryos are smaller and edematous (Q. Li et al., 2014b), implicating Kdm6b in cardiovascular development. In further support, H3K27me3 marks are conspicuously lost from key

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cardiogenic genes during in vitro cardiac differentiation of ES cells (Paige et al., 2012; Wamstad et al., 2012) and PRC2 is required to maintain cardiomyocyte identity (Delgado-Olguín et al., 2012; He et al., 2012; San et al., 2016). Additionally, *Kdm6b*$^{-/-}$ ESCs induced to undergo cardiomyocyte differentiation misexpress many genes implicated in early heart development, including decreased levels of the cardiac progenitor factors *Mesp1*, *Pdgfra*, and *Mef2c* (Ohtani et al., 2011). Correspondingly, *Kdm6b*-deficient ES cells exhibit reduced ability to form differentiated cardiomyocytes and contractile embryoid bodies (EBs). Although in vivo mouse and zebrafish studies demonstrate Kdm6a contributes to the activation of core cardiac transcription factors (Lee et al., 2012; Van Laarhoven et al., 2015; Welstead et al., 2012), the in vivo contributions of Kdm6b to cardiovascular development have not been examined.

We pursued in vivo loss-of-function studies in zebrafish to define the evolutionarily conserved contributions of *Kdm6b* to vertebrate development. Due to an ancestral whole genome duplication in the teleost lineage (Amores, 1998; Jaillon et al., 2004; Meyer and Schartl, 1999), the zebrafish genome contains two *Kdm6b* orthologs, *kdm6ba* and *kdm6bb*. In such instances of duplicated genes, the "ohnologs" frequently acquire distinct roles or one ohnolog becomes non-functional due to relaxed selective pressure (Force et al., 1999; Lynch and Force, 2000; Postlethwait, 2007; Steinke et al., 2006). However, redundant functions, with or without compensatory networks, are also possible. While *kdm6bb* may support the reactivation of embryonic developmental gene programs during fin regeneration (Stewart et al., 2009), *kdm6ba* has not been studied in any context.

We generated *kdm6ba* and *kdm6bb* loss-of-function alleles using CRISPR/Cas9 mutagenesis. Zebrafish homozygous for either individual allele are viable and fertile. However, *kdm6ba*$^{-/-}$; *kdm6bb*$^{-/-}$ zebrafish larvae have pleiotropic yet specific organogenesis defects, including a small heart ventricle, consistent with coinciding heart expression. The small ventricle originates from insufficient proliferation of both endocardial and myocardial cells associated with the initiation of cardiac trabeculation at larval hatching. This burst of trabecular cardiomyocyte proliferation correlates with transient bulk cellular H3K27me3 demethylation, implying a global relaxing of gene repression enables the cell cycle entry transition. However, collective Kdm6b does not appear to promote this observed cell-wide demethylation and therefore likely has locus-specific roles. Our data provide an example of straightforward non-compensatory redundancy between duplicated genes in zebrafish. Further, we show that Kdm6b's unique functions (those not shared with Kdm6a) are largely restricted to later stages of organ growth and morphogenesis rather than early cell fate specification or progenitor cell activity.

2. Materials and methods

2.1. Zebrafish

The University of Oregon Institutional Animal Care and Use Committee (IACUC) approved and monitored all zebrafish procedures following the guidelines and recommendations outlined by the Guide for the Care and Use of Laboratory Animals (National Academic Press). Wildtype AB, *Tg(myl7:dsRedExpress-nuc)* (Takeuchi et al., 2011), and *Tg(kdrl:EGFP)* (Jin et al., 2005) lines were used in this study.

2.2. CRISPR-Cas9 generation of mutant alleles

CRISPR-Cas9-mediated mutagenesis was used to generate targeted deletions within the JmjC domains of both *kdm6ba* and *kdm6bb*. Guide RNAs (gRNAs) were generated following methods adapted from (Bassett et al., 2013). Guide oligonucleotides were obtained from Integrated DNA Technologies and had the following core sequence: 5'-AATTAATACGACTCACTATA-NNNNNNNNNNNNNNNNNNN-GTTTTAGAGCTAGAAATAGC-3', with a T7 promoter sequence followed by a series of Ns indicating the targeting sequence. The gRNA targeting sequences for *kdm6ba* and *kdm6bb* were 5'-GCTTAGCATATTCCCACTGG-3' and 5'-CAGGTGGAAGGC-GCAGTTGC-3', respectively. Guide oligonucleotides were annealed to a generic gRNA scaffold (5'-GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACT-AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC-3') and extended using Phusion polymerase (New England Biolabs) followed by column-based purification.

In vitro transcription of gRNAs was performed using the Megascript T7 Transcription Kit (Thermo Fisher) and purified via RNA Clean & Concentrator columns (Zymo Research). Cas9 mRNA was in vitro transcribed from Addgene plasmid 46757:pT3TS-nCas9n (Jao et al., 2013) using the T3 mMessage mMachine kit (Thermo Fisher). Cas9 mRNA and gRNA were combined and diluted to a final concentration of 100 ng/μl in Danieau's buffer containing phenol red. We injected 1–3 nl of the Cas9/gRNA mix into the developing embryo at the one-cell stage. Fish raised from injected embryos (see below) were screened for mutations by amplicon sequencing. Founders carrying isolated mutant alleles were outcrossed to wildtype fish to isolate stable germline transmitting lines used for subsequent crosses and phenotypic analyses.

2.3. Genotyping kdm6ba and kdm6bb mutant alleles

The *kdm6ba* allele was assessed by PCR using primers 5'-TGGCACAAACATTGACCTGT-3' and 5'-CATCATGGACAGCAAACCAC-3' followed by restriction digest with NcoI (New England Biolabs). The *kdm6bb* mutant allele was assessed by PCR using primers 5'-CTCAAAGCAGAAAGCTGTTGG-3' and 5'-GTGTGGCCAACATGACTCAG-3'.

2.4. In situ hybridization

In situ hybridization probes were synthesized from PCRII-based plasmids (Life Technologies) containing cloned cDNA amplicons. PCR products were obtained from pooled samples of embryonic zebrafish cDNA using the following primers: *kdm6bb* 5'-CCACTTGACCAACTGCCTTGCAAAAC-3', 5'-CTGAAAACACACTCCGAGAGGTATCGC-3'; *kdm6ba* 5'-TGGCACAAACATTGACCTGT-3', 5'- GTGTGAGGGAAAGGGATGAG-3'. Plasmids were linearized by digestion with NotI and BamHI, respectively. Digoxigenin-11-dUTP (Roche) labeled RNA synthesis was performed using Sp6 (for *kdm6bb* probe) or T7 (for *kdm6ba* probe). DIG-labeled probes were DNase treated prior to LiCl precipitation and then resuspended in RNase/DNase free water. Whole-mount in situ hybridizations were performed as described (Thisse and Thisse, 2008). Hybridized embryos were blocked with 5% normal goat serum in phosphate-buffered saline (PBS) and then incubated overnight with alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted 1:1000 in blocking buffer. Embryos were developed at room temperature using NBT/BCIP (Promega) and then dehydrated into 100% methanol and stored at −20 °C. Embryos were then rehydrated and allowed to equilibrate in 100% glycerol at 4 °C prior to imaging on a Leica M165 FC stereomicroscope.

2.5. Whole mount immunofluorescence and imaging

Anti-GFP (AVES) immunostaining was performed as described (Akerberg et al., 2014). Staining for cardiac troponin (CT3, Developmental Studies Hybridoma Bank), DsRed (Clontech) and S46 (Developmental Studies Hybridoma Bank) was performed using a protocol adapted from (Zhou et al., 2011). Embryonic hearts were arrested in diastole with 0.5 M KCl and fixed overnight at 4 °C in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. The following day, embryos were washed in PBS and dehydrated through a methanol series. Embryos in 100% methanol were stored at −20 °C for at least 24 h prior to rehydration into PBS containing 0.1% Tween-20 (PBST). Embryos were then blocked with PBS containing 1% BSA, 1%