Comparative expression analysis of the H3K27 demethylases, JMJD3 and UTX, with the H3K27 methylase, EZH2, in Xenopus

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ABSTRACT The regulated removal of the gene-silencing epigenetic mark, trimethylation of lysine 27 of histone H3 (H3K27me3), has been shown to be critical for tissue-specific activation of developmental genes; however, the extent of embryonic expression of its demethylases, JMJD3 and UTX, has remained unclear. In this study, we investigated the expression of jmjd3 and utx genes in Xenopus embryos in parallel with that of the H3K27 methylase gene, ezh2. At the blastula stage, jmjd3, utx and ezh2 showed similar expression patterns in the animal cap and marginal zone that give rise to the ectoderm and mesoderm, respectively. The three genes maintained similar expression patterns in the neural plate, preplacodal ectoderm and axial mesoderm during the gastrula and neurula stages. Later, expression was maintained in the developing brain and cranial sensory tissues, such as the eye and ear, of tailbud embryos. These findings suggest that the H3K27 demethylases and methylase may function continuously for progressive switching of genetic programs during neural development, a model involving the simultaneous action of both of the demethylases for the de-repression of silent genes and the methylase for the silencing of active genes.

KEY WORDS: H3K27, demethylase, methylase, Xenopus

Introduction

Epigenetic modifications, such as DNA methylation, and histone methylation and acetylation, determine chromatin conformation states and modulate the accessibility of DNA target genes to their transcriptional regulators and/or other co-factors (Beisel and Paro, 2011). The trimethylation of lysine 27 of histone H3 (H3K27me3) is one such epigenetic modification, which is associated with repressed, transcriptionally inactive, chromatin. In mouse embryonic stem (ES) cells, H3K27me3 marks the loci of key developmental regulators, such as Sox and Nkx genes (Bernstein et al., 2006). When the ES cells differentiate into neural stem cells, removal of the H3K27me3 marks from these gene loci precedes their transcriptional up-regulation.

The methylation of H3K27 is catalyzed by Polycomb Repressive Complex 2 (PRC2), a multi-protein complex containing three core subunits: SUZ12, EED and EZH1/2, in vertebrates (Margueron and Reinberg, 2011). The demethylation of H3K27 is catalyzed by two independent enzymes, JMJD3 and UTX, both of which are members of the JUMONJI family proteins that possess the characteristic metalloenzyme-like domain, JmjC (Hubner and Spec- tor, 2011). Previous studies identified crucial roles for the PRC2 methylase complex and the demethylases in various aspects of development. For example, antagonistic functions of PRC2 and UTX regulate the collinear expression of Hox genes (Lan et al., 2007). PRC2 is also necessary for the maintenance of myogenic and epidermal precursor cells, whereas JMJD3 is required for macrophage differentiation (De Santa et al., 2007; Margueron and Reinberg, 2011). These studies suggest that dynamic regulation of the H3K27 methylation state continues throughout development; not only during the early stages as suggested by ES cell studies, but also during the later stages of body axis formation and terminal cell differentiation. However, analysis of the embryonic expression of these epigenetic regulators is very limited, especially in early developmental stages.

Xenopus is an ideal model system for studying epigenetic regulation in vertebrate development, as its large, synchronized,

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externally developing embryos facilitate not only gene manipulations but also biochemical analysis of chromatin states at early embryonic stages. While the traditionally used species, *Xenopus laevis*, is not suitable for genomic analysis due to its large allo-tetraploid genome, the recent development of its close relative, *Xenopus tropicalis*, as an alternative model system has largely resolved this issue (Hellsten et al., 2010). In this study, we compare the embryonic expression of *jmjd3*, *utm* and *ezh2* throughout development in *X. tropicalis* (hereafter referred to as *xtjmjd3*, *xutm* and *xezh2*). Expression of *ezh2* was previously characterized in *X. laevis*, but histological analysis was limited to the neurula and tailbud stages (Barnett et al., 2001; Aldiri and Vetter, 2009). We found that the H3K27 demethylase and methylase genes, which have antagonistic functions, show quite similar tissue-specific expression patterns in mesoderm and ectoderm lineages.

**Results**

**Identification of *X. tropicalis jmjd3*, *utm* and *ezh2* genes**

A search of the NCBI database resulted in the identification of a *X. tropicalis* cDNA clone (GenBank accession no. BC167994) exhibiting high sequence similarity to the previously identified mouse *Jmjd3* (De Santa et al., 2007). This cDNA is largely homologous to an Ensembl *X. tropicalis* gene model, ENSXETT00000034109.

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**Fig. 1. Identification of *X. tropicalis* JMJD3 and UTX proteins.**

(A) Schematic comparison between mouse and *X. tropicalis* JMJD3, and between mouse and *X. tropicalis* UTX. Numbers in each domain of *X. tropicalis* JMJD3 and UTX indicate percent identity of the amino acid sequence to the corresponding domain of their mouse orthologs. Amino acid positions are numbered, and JmjC domain and TPR are indicated. Scale bar represents distance calculated on the basis of amino acid substitution rates. Numbers on each node indicate percentage bootstraping values.

GenBank accession numbers of the protein sequences used for this analysis are as follows: zebrafish JMJD3, XP001343575; human JMJD3, NP001073893; mouse JMJD3, NP001017426; zebrafish UTX, XP697746; human UTX, NP066963; mouse UTX, NP033509; human JMJD1a, NP060903; human JMJD2a, NP0565478. (C) Synteny analysis of *Jmjd3*, *Utx* and *Ezh2* in mouse and *X. tropicalis*. Genes were identified on the UCSC Genome Browser (http://genome.ucsc.edu/). Arrows indicate transcription start sites and the transcriptional orientation of the genes. Only part of the chromosome or scaffold around the *Ezh2* loci is shown. *xtjmjd3* and *xutm* locate close to the 3’ end of scaffold 1278 and scaffold 35, respectively (left most and middle panels). Although a synteny break resides between the *Ezh2* and *Rn4.5s* or *zdhhc3* loci, the synergy from *Ctnnap2* to *Ezh2* is conserved (right most panel). (D) Alignment of *Jmjd3* domain sequences of vertebrate JMJD3 and UTX with that of human JMJD1a and JMJD2a. Amino acids that are identical in more than five proteins are highlighted with a gray background. Black and white circles mark the 2-OG- and Fe (II)-binding residues, respectively. These residues are thought to be critical for enzymatic activity of the JUMONJI family proteins (Hubner and Spector, 2011).
However, open reading frame analysis of the BC167994 led to the identification of a single adenine insertion in the middle of its coding region, which does not exist in the *X. tropicalis* genome sequence and causes a frame shift error at the 1018th amino acid (data not shown). A partial cDNA fragment that contains the middle part of this gene was isolated from *X. tropicalis* tailbud embryos by reverse transcription-polymerase chain reaction (RT-PCR), and sequencing confirmed that the isolated cDNA did not contain the adenine insertion. Therefore we concluded that the inserted adenine in BC167994 was likely a cloning artifact that occurred during the library construction, and thus we reconstructed the full-length coding region (5220 bp) without the adenine using our newly isolated cDNA. We named this reconstructed clone *xtjmjd3*, and deposited its nucleotide sequence to GenBank (accession no. JN107757). Amino acid sequence comparison between the mouse JMJD3 and the putative XIJMJD3 demonstrates that the catalytic JmjC domain, responsible for demethylation, and the C-terminal domain are both highly conserved (91 and 75% identity, respectively, Fig. 1A), while the N-terminal domain is largely divergent (39% identity, Fig. 1A).

We also found *X. tropicalis* cDNA clones in the NCBI database that showed high sequence similarity to the previously identified mouse *Utx* and *Ezh2* (Laible et al., 1997; Greenfield et al., 1998). The clone identified as *xtutx* (NM001130335) encodes a protein whose N-terminal domain with a tetratricopeptide repeat (TPR), a JmjC domain, and a C-terminal domain share 87, 95 and 69% amino acid sequence identity, respectively, with their homologous domains in the mouse UTx protein (Fig. 1A). The TPR is thought to mediate protein-protein interactions (Hubner and Spector, 2011). The protein encoded by the clone identified as *xtezh2* (NM001017293) shares 94% amino acid sequence identity with the mouse EZH2 protein and 100% identity with the previously reported *X. laevis* EZH2 protein (Barnett et al., 2001).

A phylogenetic tree analysis confirmed that XIJMJD3 and XIUTX proteins are more closely related to the previously identified JMJD3 and UTX proteins of other vertebrates, respectively, than to the other members of the JMJD family proteins, such as JMJD1a and JMJD2a (Fig. 1B). Synteny analysis also suggested that *xtjmjd3*, *xtutx* and *xtezh2* are orthologous of the mouse *Jmjd3*, *Utx* and *Ezh2* genes, respectively (Fig. 1C). Amino acid sequence alignment shows that the JmjC domains of JMJD3 and UTX are conserved in vertebrates, and are distinct from the JmjC domains of JMJD1a and JMJD2a (Fig. 1D).

**Overexpression of xtjmjd3 and xtutx reduces H3K27me3 levels in cell culture**

As described above, the amino acid sequences of XI EZH2 and mouse EZH2 are highly conserved, whereas the sequences of XIJMJD3 and XIUTX are somewhat diverged from those of their mouse orthologs. Hence, we examined the demethylase activity of XIJMJD3 and XIUTX by transient transfection of their expression vectors into COS-7 cells followed by fluorescent immunostaining of H3K27me3, as previously performed with mouse JMJD3 (Hong et al., 2007). The transfected cells were distinguished by antibody staining and anti-GFP antibody staining, respectively. White arrows indicate nuclei of transfected cells.

![Fig. 2. Overexpression of XIJMJD3 or XIUTX reduced H3K27me3 levels in cell culture. COS-7 cells were transfected with an empty expression vector, pCS2+ (A-C), an XIJMJD3 expression vector, pCS2+xtjmjd3 (D-F), an XIUTX expression vector, pCMVSPORT6-xtutx (G-I), or a mouse JMJD3 expression vector, pCS2+mJmjd3 (J-L). A GFP expression vector, pCAGGS-GFP, was co-transfected in each case to identify the transfected cells. The blue, red and green are nuclear DAPI staining, anti-H3K27me3 antibody staining and anti-GFP antibody staining, respectively.](image-url)
The expression level of xtezh was fairly constant by the gastrula stage, and increased slightly thereafter.

We then analyzed the spatio-temporal expression patterns of xtjmjd3, xtutx and xtezh2 by in situ hybridization. At the blastula stage (stage 8), expression of all three genes was detected in the animal hemisphere (Fig. 3 B,G,K). Sections of the embryos showed that both xtutx and xtezh2 transcripts mostly localize in the animal cap and marginal zone (Fig. 3 H,L), whereas xtjmjd3 transcripts are distributed more broadly through the upper part of the vegetal region (Fig. 3C). In the gastrula embryos, the three genes show similar expression patterns in the animal cap and involuting marginal zone (Fig. 3 D,E,E',I,J,J',M,N,N'). This expression domain in the marginal zone appears to correspond to the part of the Spemann organizer (Spemann and Mangold, 1924). After gastrulation, expression of the three genes persists together in the developing neural plate and underlying mesoderm (Fig. 3 O,P,S,T,W,X). Their expression was also evident in the preplacodal ectoderm, which surrounds the anterior margin of the neural plate and later gives rise to the cranial sensory tissues, including the lens, nasal epithelium and inner ear (Schlosser, 2006).

High magnification views of the parasagittal section of the anterior region revealed that the anterior endoderm beneath the preplacodal ectoderm also expresses the three genes (Fig. 3 P',T',X'). Transverse sections show that the notochord appears to be the center of xtjmjd3 and xtutx expression, and their diffuse expression was detected in the ventral part of the neural plate, proximal part of the paraxial mesoderm and the archenteron roof beneath the notochord (Fig. 3 Q,U). In contrast, xtezh2 shows stronger expression in the neural plate than in the underlying tissues including the notochord, and the expression patterns are uniform in each tissue (Fig. 3Y).

In the late neurula, expression of the three genes continues in the developing neural tissues, including the eye, brain, and neural crest cells migrating into the branchial arch (Fig. 3 R,V,Z). At the tailbud stage, expression patterns of all three genes appear very similar from the outside (Fig. 4 A,G,M). Transverse sections of the head region of tailbud embryos show that the three genes are expressed in the optic cup, mesencephalon, diencephalon, rhombencephalon, otic vesicle, and migrated neural crest cells, but are no longer expressed in the notochord (Fig. 4 B,D,D',H,J, J',N,P,P'). In the eye, the three genes show broad expression in the optic cup, except in its outer layer (Fig. 4 B,H,N'). In the lens, expression of xtjmjd3 is evident, but expression of other genes is faint. Transverse sections of the trunk region show that xtjmjd3 and
xtezh2, but not xtxtx, are expressed in the spinal cord (Fig. 4 E,K,Q). We also examined the expression of xtjmjd3, xtxtx and xtezh2 in tadpole eyes in which differentiated retinal layers were established. At this stage, xtezh2 expression was distinct in the ganglion cell layer and inner nuclear layer, but appeared to be absent from the photoreceptor layer (Fig. 4R). Low expression of xtjmjd3 was detected in the ganglion cell layer and inner nuclear layer (Fig. 4F), but xtxtx expression was not detected in any of the retinal layers (Fig. 4L).

Discussion

Our expression analysis reveals that the transcripts of xtjmjd3, xtxtx and xtezh2 preferentially localize to the animal hemisphere by the early gastrula stage. It is not known whether the transcripts are translated into proteins at these stages. However, this localized expression of the H3K27 methylase, xtezh2, may explain the previous findings that genes preferentially expressed in the vegetal hemisphere, such as vegtand sox17a, are marked with H3K27me3 in the animal hemisphere whereas the genes preferentially expressed in the animal hemisphere do not have such marks in whole Xenopus embryos (Akkers et al., 2009). Constitutive expression of xtjmjd3 and/or xtxtx may be responsible for maintaining the demethylated state of the active genes in the animal hemisphere, and their continuous expression in the developing neuroectoderm in the early neurula is consistent with the requirement of Jmjd3 in the neural commitment of ES cells (Burgold et al., 2008). Expression of xtjmjd3 was also shown to continue from the marginal zone to the notochord lineage, where a T-box gene for mesoderm formation, brachyury/T, is expressed (Fig. 3F) (Smith et al., 1991). As shown in ES cells, XUJMJD3 may be directly involved in the activation of the brachyury/T gene by interacting with NODAL-activated SMAD2/3 in these tissues (Dahle et al., 2010).

The details of our expression analysis suggest many unexplored roles for jmjd3, utx and ezh2 in the development of specific tissues, such as the retina, lens and inner ear. It is also noteworthy that their expression patterns are very similar throughout development, from early body axis formation to late organogenesis, even though demethylases and methylases are thought to antagonize each other. Thus, we speculate that xtjmjd3, xtxtx and xtezh2 may be, in part, co-regulated to allow for their cooperation. The progressive changes of gene expression during development likely require the simultaneous action of both the demethylase and methylase; the former de-represses silent genes to activate new genetic programs while the latter silences active genes to shutdown old programs. This possibility will be explored in future analyses of the regulatory mechanisms controlling these epigenetic regulators and the mechanisms governing their recruitment to target gene loci.

Materials and Methods

Plasmid construction

The xtjmjd3 cDNA clone, BC167994, was obtained from Open Biosystems. The 1656 bp partial cDNA fragment of xtjmjd3 was amplified by PCR from a cDNA pool of X. tropicalis tailbud embryos (stages 27-28) using the primers 5'-ATGCCCGGATGGAGGATCTTCG-3' and 5'-GGTCGAGTGTCCGAGAAGGCATTG-3'. The 1420 bp Xba-I-Sac region from this cDNA fragment was inserted into pCS2+ in place of the corresponding Xba-I-Sac region containing the adenine insertion to reconstruct the 5220 bp full-length coding region of xtjmjd3 without the frame shift mutation. The resulting xtjmjd3 cDNA was subcloned into an expression vector, pCS2+, to generate pCS2+xtjmjd3. pGEM-Txtutx was generated by subcloning part of the xtjmjd3 cDNA into the pGEM-T Easy vector (Promega). The cDNA fragment used for this subcloning was amplified from pCS2+xtjmjd3 using the primers 5'-TGACCGGATCTTGGTGGCC-3' and 5'-TCACCGGATGTTCGGGGGT-3'. pCS2+mJmjd3 was constructed by introducing the full coding region of the mouse Jmjd3 gene by interacting with NODAL-activated SMAD2/3 (Promega). The cDNA fragment used for this subcloning was amplified from pCS2+xtjmjd3 using the primers 5'-TGACCGGATCTTGGTGGCC-3' and 5'-TCACCGGATGTTCGGGGGT-3'. pcS2+mJmjd3 was constructed by introducing the full coding region of the mouse Jmjd3 cDNA that was excised from BCO75632 (Open Biosystems) into pCS2+.

For the cloning of xtxtx and xtezh2 cDNAs, we designed the following primer pairs according to the sequences of NM001130335 and NM001017293: xtxtx, 5'-GGTAGCGGAGATTGTCAATGC-3' and 5'-TTCGAGTATGGGGAGACATT-3'; xtezh2, 5'-GGTGATGGTTGTCGAAGACAT-3' and 5'-TCAGGGATTTCCATTCCTC-3'. Using these primer pairs, a 729 bp cDNA fragment of xtxtx and an 881 bp cDNA fragment of xtezh2 were amplified from the cDNA pool of X. tropicalis tailbud embryos and cloned into a pGEM-T Easy vector. The resulting plasmids, pGEM-xtxtx and pGEM-xtezh2, were verified by sequencing. The expression vector containing the complete coding sequence for xtxtx, pCMVSPORT6-xttx (BC167610), was obtained from Open Biosystems and verified by sequencing.
Cell culture and fluorescent immunostaining

Transient transfection into COS-7 cells was performed using FuGene6 (Promega) with 1.5 μg of pCS2+, pCS2+xtjmjd3, pCMVSPORT6-xtutx, or pCS2+mJmjd3, along with 0.5 μg of a tracer plasmid, pCAGGS-GFP (Ogino and Yasuda, 1998), in a 35-mm culture dish. After a 36-hr incubation, the cells were fixed for fluorescent immunostaining as previously described (Hong et al., 2007). A 1/500 diluted rabbit anti-H3K27me3 antibody (Millipore, 07449) and a 1/1000 diluted mouse anti-GFP antibody (Santa Cruz Biotechnology, sc-9996) were used as primary antibodies in conjunction with 1/1000 diluted Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen, A11001) and 1/1000 diluted Alexa 568-conjugated goat anti-mouse IgG (Invitrogen, A11011) secondary antibodies, respectively. After the antibody reactions, the cells were mounted with VECTASHIELD Mount Medium with DAPI (Vector). The images were acquired using a laser-scanning confocal microscope (Carl Zeiss, LSM710).

RT-PCR analysis and in situ hybridization

The mRNAs were extracted from X. tropicalis embryos using the RNeasy kit (Qiagen). cDNA synthesis from these mRNAs and subsequent PCR reactions were performed using the PrimeScript 1st strand cDNA Synthesis kit (Takara) and AmpliTaq Gold 360 Master Mix (Applied Biosystems). Primer pairs and corresponding numbers of PCR cycles were as follows: xtjmjd3, 5’-GCTCTCCAGACCTTACCTT-3’ and 5’-TAGACTCTGACAGCT-GCTCC-3’, 25 cycles; xtutx, 5’-GTTCCGGGAGATTGTGAATGG-3’ and 5’-TCAGGATGATGGTGAAGGCATT-3’, 30 cycles; xtezh2, 5’-CCTGAAAAT-CAGAAGAGG-3’ and 5’-CCCCAACTTAAACACAG-3’, 30 cycles; X. tropicalis odc (xtodc), 5’-GTGGAGCAGAAAGATCAAAGTTGCC-3’ and 5’-CAGTGCCTGTCCATTGTC-3’, 25 cycles. Each primer pair was designed to hybridize to exons separated by an intron(s) in order to distinguish whether the resulting amplicons were derived from the cDNA or contamination with genomic DNA. The digoxigenin-labeled antisense probes for in situ hybridization analyses of xtjmjd3, xtutx and xtezh2 were generated using pGEM-xtjmjd3, pGEM-xtutx and pGEM-xtezh2, respectively. The probe for brachyury was generated using pSP73-Xbra (Smith et al., 1991). In situ hybridization was performed as previously described (Sive et al., 2000). The blastula, gastrula and early neurula embryos were subjected to whole-mount in situ hybridization and then sectioned using a microslicer (DTK-1000, Dosaka EM Co.). The tailbud embryos were subjected to both whole-mount and hybridization and then sectioned using a microslicer (DTK-1000, Dosaka EM Co.). The tailbud embryos were subjected to both whole-mount and hybridization, and tadpoles were subjected to section in situ hybridization.

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