Published in final edited form as:

Genesis. 2014 December ; 52(12): 959–966. doi:10.1002/dvg.22827.

Genome Editing a Mouse Locus Encoding a Variant Histone, H3.3B, to Report on its Expression in Live Animals

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Summary

Chromatin remodeling via incorporation of histone variants plays a key role in the regulation of embryonic development. The histone variant H3.3 has been associated with a number of early events including formation of the paternal pronucleus upon fertilization. The small number of amino acid differences between H3.3 and its canonical counterparts (H3.1 and H3.2) has limited studies of the developmental significance of H3.3 deposition into chromatin due to difficulties in distinguishing the H3 isoforms. To this end, we used zinc-finger nuclease (ZFN) mediated gene editing to introduce a small C-terminal hemagglutinin (HA) tag to the endogenous *H3.3B* locus in mouse embryonic stem cells (ESCs), along with an internal ribosome entry site (IRES) and a separately translated fluorescent reporter of expression. This system will allow detection of expression driven by the reporter in cells, animals, and embryos, and will facilitate investigation of differential roles of paternal and maternal H3.3 protein during embryogenesis that would not be possible using variant-specific antibodies. Further, the ability to monitor endogenous H3.3 protein in various cell lineages will enhance our understanding of the dynamics of this histone variant over the course of development. *genesis*

Keywords

histone variants; H3.3; targeted gene editing; epigenetics; chromatin

In mammals, the histone variant H3.3 is encoded by two different genes (*h3f3a* and *h3f3b*) whose translation results in an identical protein product. These genes contain unique regulatory elements and exhibit differential transcript usage in different tissue types (Frank *et al.*, 2003; Wellman *et al.*, 1987). Disruption of *H3.3A* in mice results in partial neonatal

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lethality, neuromuscular impairment, and reduced fertility (Couldrey *et al.*, 1999). Individual *H3.3B* knockout mice are reported to be semilethal and infertile, linked to defective cell division resulting in abnormalities in specific germ cell populations (Bush *et al.*, 2013; Yuen *et al.*, 2014). A number of roles have been ascribed to H3.3 related to developmental processes. H3.3 is critical for male pronucleus and nuclear pore complex formation (Inoue and Zhang, 2014; Loppin *et al.*, 2005; Santenard and Torres-Padilla, 2009; Santenard *et al.*, 2010; van der Heijden *et al.*, 2005) and early embryonic development during embryogenesis (Lin *et al.*, 2013; Wen *et al.*, 2014a). Additionally, our recent studies have shown that H3.3 is important for maintenance of chromatin landscapes in ESCs that facilitate differentiation (Banaszynski *et al.*, 2013), and that H3.3 is an essential maternal factor for oocyte reprogramming required for reactivation of many key pluripotency genes in nuclear transfer embryos (Wen *et al.*, 2014a,b).

H3.3 differs from canonical H3 isoforms H3.1 and H3.2 by only 4–5 amino acids, yet displays unique features in its regulation and expression pattern, chaperone-mediated deposition pathways, genome localization, and post-translational modification (Akhmanova *et al.*, 1995; Frank *et al.*, 2003; Szenker *et al.*, 2011; Wellman *et al.*, 1987). While canonical H3 expression and incorporation is limited to replication-coupled deposition during S phase, H3.3 is expressed and incorporated into chromatin throughout the cell cycle independent of replication. Recent studies have demonstrated that H3.3 incorporation into chromatin is mediated by two independent chaperone systems, with Hira responsible for most if not all genic H3.3 deposition (Goldberg *et al.*, 2010; Tagami *et al.*, 2004), and the ATRX/DAXX complex facilitating deposition at repeat elements such as telomeres and pericentric heterochromatin (Drane *et al.*, 2010; Goldberg *et al.*, 2010).

In addition to a role in early development, recent studies have suggested that H3.3 can also play a role in disease states. For example, mutations in ATRX/DAXX are linked to pancreatic neuroendocrine tumors (pan-NETs) (Jiao *et al.*, 2011) and acute myeloid leukemia (AML) (Ding *et al.*, 2012), suggesting an important physiological role for H3.3 deposition at heterochromatic regions. In addition, mutations in the histone variant itself, as well as canonical H3, at or near sites of posttranslational modification have also been discovered in a number of pediatric cancers, providing evidence for the hypothesis that posttranslational modification of histones has downstream biological consequences and suggesting that their misregulation might act as a “driver” for oncogenesis (Behjati *et al.*, 2013; Schwartzenruber *et al.*, 2012; Wu *et al.*, 2012).

The ability to monitor H3.3 expression and deposition into chromatin during embryogenesis and development would greatly enhance our understanding of its functional relevance during this time frame, as well as in the adult organism and disease states. Highly specific and ChIP quality H3.3 antibodies have been difficult to generate, likely due to the small differences in protein sequences among the H3 isoforms. We therefore considered an alternative approach to generate a knock-in allele fusing the small peptide hemagglutinin (HA) to the last exon of the endogenous *H3.3B* (*h3f3b*) gene along with a fluorescence reporter. We used the highly efficient technique of genome editing with engineered zinc-finger nucleases (ZFNs) (Carroll, 2014; Urnov *et al.*, 2010). Using a ZFN specific to the last coding exon of the *H3.3B* gene and a promoter-less donor construct (Goldberg *et al.*, 2010), we introduced the HA tag to the

C-terminus of endogenous *H3.3B*, along with an internal ribosomal entry site (IRES) and separately translated EYFP or mCherry tag to provide a reporter of *H3.3B* gene expression (Fig. 1a, H3.3B-HA-iEYFP/H3.3B-HA-imCherry). Our strategy retained the non-coding sequences of *H3.3B*, including the 5' and 3' UTRs and all introns, in an attempt to retain regulatory information encoded by these sequences.

We used a confirmed germline transmission competent ESC line (129xC57Bl/6 background) for ZFN-mediated gene editing to obtain heterozygous cell lines containing an *H3.3B-HA* knock-in at a frequency of ~1% (Goldberg *et al.*, 2010). Correct targeted addition of the donor-specified cassette in single cell clones carrying either the EYFP or mCherry reporter was validated by Southern blot (Fig. 1b,c) and Sanger sequencing of both the wild type and edited allele (~70% of clones were correctly edited). ChIP-seq confirmed a high degree of concordance between enrichment profiles generated in ESCs for H3.3B-HA and wild type H3.3 protein (KMN, CDA, and LAB, unpublished results), suggesting that H3.3B deposition is similar to the overall H3.3 profile and that the epitope tag does not greatly alter patterns of H3.3 deposition. Validated clones were then injected into wild type diploid blastocysts for chimera production and germline transmission. Established mouse lines heterozygous for H3.3B-HA-iEYFP or H3.3B-HA-imCherry mice were obtained and backcrossed with C57Bl/6. A small colony was established and maintained by breeding between EYFP positive and mCherry positive mice (Fig. 1d). The presence of EYFP and mCherry allowed us to monitor the colony by fluorescence, with additional genotyping by PCR confirming the presence or absence of the HA epitope (Fig. 1e,f).

Heterozygous H3.3B-HA mice are viable, fertile, and histologically normal. However, homozygous H3.3B-HA pups display significantly low birth weight (Fig. 1g), lower than expected Mendelian ratio, and postnatal lethality. Homozygous pups usually die within a week after birth, but occasionally pups are found to survive for longer periods of time; we have yet to determine whether these mice are fertile. As we did not observe any overt histological defects or lesions for the homozygous pups aside from stunted growth, the cause of lethality remains unclear. Semilethality has also been reported for *H3.3B* knockout mice, linked to cell cycle defects and karyotypic abnormalities (Bush *et al.*, 2013). Comparative analysis of these two models may allow additional insights into *h3f3b* gene function, and our homozygous mice may serve useful as a hypomorph in these studies.

H3.3 is abundant in mature oocytes and is critical for male pronucleus formation and subsequent embryonic development during early embryogenesis (Lin *et al.*, 2013; Loppin *et al.*, 2005; Santenard and Torres-Padilla, 2009; Santenard *et al.*, 2010; van der Heijden *et al.*, 2005; Wen *et al.*, 2014a). Using our H3.3B-HA-iEYFP and H3.3B-HA-imCherry reporter mice, we monitored fluorescence as a proxy for H3.3B expression in oocytes and pre-implantation embryos. All oocytes recovered from heterozygous females show strong fluorescence of maternal origin, regardless of genotype (Fig. 2a). We continue to detect fluorescence of maternal origin in all embryos from the zygote to the late eight-cell and early morula stage (Fig. 2a–d). By the blastocyst stage, we observe maternal origin fluorescence in 48% (15/31) of embryos; we hypothesize that these embryos have activated zygotic transcription of the reporter from the maternal genome, and that we no longer observe fluorescence of oocyte origin in the remaining wild type embryos (Fig. 2e,

mCherry-maternal). Likewise, we do not observe fluorescence expression from the paternal genome until the eight-cell and early morula stage, indicating accumulation of fluorescence due to zygotic transcription (Fig. 2d, EYFP-paternal). At the blastocyst stage, zygotic fluorescence expression from both paternal and maternal alleles is detectable, allowing a non-invasive approach to genotype living embryos (Fig. 2e). Of note, we observe expected Mendelian ratios at the blastocyst stage (Fig. 2e), suggesting that any defects resulting from gene disruption or protein tagging occur post-implantation.

As reporter fluorescence is uncoupled from stability of the histone variant itself, we next took advantage of the introduced epitope tag to monitor H3.3 protein directly in germ cells and in the early developing embryos. Using HA antibodies, we detected H3.3B-HA protein in the nuclei of all mature sperm from heterozygous H3.3B-HA mice (sperm-derived H3.3, sH3.3) (Fig. 3a). This data supports published observations (Brykczynska *et al.*, 2010; Hammoud *et al.*, 2009) indicating that nucleosomes still exist in mature sperm nuclei, even though the majority of sperm DNA is packaged with protamines. H3.3B-HA protein is detected in the genomic DNA and the first polar body in all the oocytes from heterozygous H3.3B-HA mice (oocyte nucleus-derived H3.3, oH3.3) (Fig. 3b). H3.3B-HA protein is also detected in the cytoplasm of activated oocytes (3 h after activation) (Fig. 3c), but not in the cytoplasm of unactivated oocytes (maternal mRNA-derived H3.3, mH3.3) (Fig. 3b), suggesting that this H3.3 protein is produced from maternal H3.3B mRNA stored in the mature oocyte cytoplasm after activation (Wen *et al.*, 2014a). Paternally expressed H3.3B-HA protein is detected in embryos as early as the two-cell stage (paternally expressed H3.3, pH3.3) (Fig. 3d), demonstrating that antibody detection is more sensitive than our fluorescent reporter that is detectable only after the eight-cell stage. Interestingly, we observe that the paternally expressed H3.3B-HA protein is detected in only one of the blastomere nuclei in early two-cell stage embryos (Fig. 3e), while in the later two-cell stage, paternally expressed H3.3B-HA protein is present in the nuclei of both blastomeres (Fig. 3d), suggesting a delay of paternal genome activation in one of the blastomeres at this stage. Whether this phenomenon is associated later with cell lineage specification during embryogenesis remains an intriguing question.

Our H3.3B-HA mouse can be used for the study of endogenous H3.3B protein in different tissues and organs during development without the use of specific H3.3 antibodies to address a number of important questions. For example, ChIP-seq of H3.3B-HA sperm would determine whether remaining H3.3 protein in the sperm genome might be enriched at early developmentally poised genes, perhaps playing a role in their activation in the developing embryo. This mouse model will also be very useful in evaluating differential roles of maternal and paternal H3.3 in early embryonic development (e.g., tracking sperm-derived H3.3, oocyte nucleus-derived H3.3 and maternal mRNA-derived H3.3 protein during fertilization and early embryonic development) which would not be possible using an antibody specific to H3.3. Additionally, the presence of an epitope tag on H3.3 will facilitate biochemical analysis of H3.3-containing chromatin from differentiated adult tissues. It remains that H3.3A and H3.3B may play possibly non-redundant roles during specific biological processes. The advent of new gene targeting technologies such as CRISPR/Cas9 (Yang *et al.*, 2013) will facilitate specific targeting of *H3.3A* for the introduction of a unique epitope tag that, coupled with our H3.3B-HA mice and the use of currently available

individual genetic *H3.3A* and *H3.3B* knockout models (Tang *et al.*, 2013), will allow insights into the conserved genetic redundancy encoding an identical protein product.

METHODS

ZFN Design, Genome Editing, and Genotyping of Edited Alleles

ZFN design, genome editing, and validation were described previously (Goldberg *et al.*, 2010). Briefly, we knocked-in a small C-terminal hemagglutinin (HA) sequence (~1 kDa) to the last exon of the endogenous *H3.3B* gene, along with an internal ribosomal entry site (IRES) and separately translated EYFP or mCherry tag to provide a reporter of the gene expression. We used an inhouse derived hybrid ES cell line (129S1xC57Bl/6 background) for targeted addition that had been previously confirmed to be germline transmission competent. After transfection with ZFNs and the appropriate donor construct, ES cells were cultured in standard conditions for 3–5 days prior to manual isolation of the fluorescent colonies. Following clonal isolation and expansion, we prepared genomic DNA using the Qiagen DNeasy Blood and Tissue Kit (Qiagen 69504). Individual clones were screened by PCR using a forward *H3.3B* primer (TTGGTGGAGTATCTGCCCGTTCTG) outside of the donor construct and reverse primer (GTTCTTCTGCTTGTCGGCCATGATA) within EYFP. To verify that the wild-type *H3.3B* allele remained intact, we also amplified both *H3.3B* alleles by using the same forward primer above, and a reverse primer (TGAAGCCAACCTGCTGCTCTACAGT) outside the donor construct, performed gel electrophoresis, and isolated the smaller band corresponding to the wild-type *H3.3B* allele. PCR products from both wild-type and modified *H3.3B* alleles were sequenced using standard methods. For PCR genotyping of mice, primers recognizing the wild type allele resulted in a 383 bp product, while the *H3.3B*-HA allele resulted in a 203 bp product. Common primer (forward)—GGACAAAAGGCTTGGTGGCACTC; wild type (reverse)—CACTGAGGTCTGTGAACAGTCAGTCAC; *H3.3B*-HA (reverse)—GTAGTCGGGCACGTCGTAG.

Production of Chimeras by Blastocyst Injection

Animals were housed and prepared according to the protocol approved by the IACUC of Weill Cornell Medical College (Protocol number: 2009-0061). ICR mice for production of the recipient blastocysts were purchased from Taconic Farms (Germantown, NY). Females were superovulated at 6–8 weeks with 5 IU PMSG (Pregnant mare serum gonadotrophin, Sigma–Aldrich, St. Louis, MO) and 5 IU hCG (Human chorionic gonadotrophin, Sigma–Aldrich) at intervals of 48 h. The females were mated individually to males, and checked for the presence of a vaginal plug the following morning. Plugged females were sacrificed by cervical dislocation at 1.5 days after hCG injection for the collection of two-cell embryos. These embryos were flushed from the oviducts with KSOM+AA (Specialty Media, Cat# MR-106-D), and cultured in KSOM for 2.5 days *in vitro* at 37°C under 5% CO₂ in air to the blastocyst stage.

Both *H3.3B*-HA-iEYFP and *H3.3B*-HA-imCherry ESC lines were used for blastocyst injection and making chimeras. For blastocyst injection, ESCs were trypsinized, resuspended in DMEM without LIF, and kept on ice. A flat tip microinjection pipette was

used for ESC injection. ESCs were picked up in the end of the injection pipette and 10–15 ESCs were injected into each blastocyst. The injection pipette was used to collect ESCs as a clump and to place them close to the ICM of the blastocyst. The injected blastocysts were kept in KSOM+AA until embryo transfer. Ten injected blastocysts were transferred into each uterine horn of 2.5 dpc pseudo-pregnant ICR females. Mouse lines are available upon request.

Fluorescence Microscopy

Expression of EYFP and mCherry were detected in live embryos using a fluorescence inverted microscope, with a yellow fluorescent protein (EYFP) filter set and red fluorescent protein (mCherry) filter set (Nikon, TE2000-U). Images were captured with a digital camera and merged in NIS-Elements D software (Nikon).

Immunohistochemistry and Confocal Imaging

For immunohistochemistry staining, oocytes or embryos were fixed (4% paraformaldehyde), permeabilized (0.5% Triton X-100 in PBS), blocked (10% Normal donkey serum and 0.5% Triton in PBS) and incubated in working dilutions of the antibodies. As primary antibodies, anti-HA goat IgG (Abcam, ab9134, 1:100), anti- α -tubulin-FITC (Sigma, F2168, 1:300) were used. We used anti-goat IgG conjugated with Alexa Fluor 647 (Invitrogen, A-21245) as a secondary antibody. Imaging was performed with a Zeiss 710 confocal imaging system. Z-stack images with 20 sequential sections for each embryo were taken.

Acknowledgments

Contract grant sponsors: Tri-Institutional Stem Cell Initiative; the Rockefeller University Fund; Damon Runyon Cancer Research Foundation; Rockefeller University Women and Science Initiative

The authors thank F. Urnov for advice and critical reading of this manuscript.

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FIG. 1. Production of H3.3B-HA-IRES-EYFP/mCherry knock-in mice. **(a)** Schematic of zinc finger nucleases (ZFNs) recognizing the endogenous H3.3B (*h3f3b*) sequence near the end of the last coding H3.3B exon on chromosome 11. Exon coding sequences are thick rectangles, while non-coding untranslated sequences are thin rectangles. ZFN facilitate double-strand break (DSB) formation at *h3f3b*, and donor homologous sequences allow gene editing to generate a new H3.3B allele with a C-terminal HA tag (red) and an internal ribosomal entry site (IRES, blue) followed by a fluorescent reporter. **(b,c)** Southern blot schematic (b) and validation (c) of *h3f3b* gene targeting. Genomic DNA from targeted mESCs and tail tips of mice generated from the targeted mESCs was digested with BsrBI, resulting in DNA fragmentation as indicated. Southern blotting was performed using a 638 bp *AvaII* fragment purified from the *h3f3b* 3' UTR. **(d,e)** Pups at day 4 (d) and fluorescence analysis of tail tips (e) from an H3.3B-HA-IRES-EYFP heterozygous female crossed with an H3.3B-HA-IRES-mCherry heterozygous male. Bar scale for panel e: 500 μ m. **(f)** PCR genotyping of tail tips and control ESCs. Primers recognizing the wild type allele resulted in a 383 bp product, while the H3.3B-HA allele resulted in a 203 bp product. **(g)** Birth weight (gram) of pups with different genotypes. The average birth weight of heterozygous pups is similar to that of the wild-type pups ($P=0.430$, student t test); homozygous pups are significantly smaller than both heterozygous and wild-type pups ($P<0.01$, student t test).

FIG. 2.

Expression of H3.3B-HA-IRES-EYFP/mCherry fluorescent reporters in oocytes and pre-implantation embryos. (a) Oocytes from H3.3B-HA-imCherry heterozygous females all express mCherry fluorescence, regardless of genotype. (b) All 2-cell stage embryos from H3.3B-HA-iYFP heterozygous females crossed with wild-type males express EYFP fluorescence. (c) All the morula stage embryos from H3.3B-HA-imCherry heterozygous females crossed with wild-type males express mCherry. Note for panels a–c, half of these embryos are wild type, but still express maternal fluorescent reporters. (d) Morula stage embryos from H3.3B-HA-imCherry females crossed with H3.3B-HA-iEYFP males. Seventeen of 31 embryos are EYFP positive, showing that the paternal H3.3B-HA-iEYFP is expressed. All embryos are still expressing maternal mCherry. (e) Blastocyst stage embryos from H3.3B-HA-imCherry females crossed with H3.3B-HA-iEYFP males. Fluorescence of maternal origin (mCherry) becomes undetectable in 7 of 31 wild-type embryos (both EYFP and mCherry negative). Zygotic expression in heterozygous and homozygous embryos allows for genotyping of living embryos. Bar scale: 200 μm.

FIG. 3.

Detection of H3.3B-HA protein in gametes and early embryos using an HA antibody. **(a)** Mature sperm from heterozygous H3.3B-HA male mouse showing HA positive staining (red) in the nucleus (sperm-derived H3.3, sH3.3). **(b)** A metaphase II oocyte from H3.3B-HA mouse stained with HA antibody (red) and α -tubulin (green) showing HA positive staining in the genomic DNA and the first polar body (oocyte nucleus-derived H3.3, oH3.3). Sp: spindle; PB: the first polar body. **(c)** A somatic cell nuclear transfer embryo (wild-type cumulus nucleus) 3h after activation showing HA positive staining in the cytoplasm (red) (maternal mRNA-derived H3.3, mH3.3). **(d)** and **(e)** A two-cell stage embryo from a wild-type female crossed with an H3.3B-HA male, showing HA positive staining in both the nuclei in a later two-cell embryo **(d)** and in only one blastomere in an early two-cell stage embryo **(e)** (paternally expressed H3.3, pH3.3). Bar scale: 20 μ m.