An Engineered Mouse to Identify Proliferating Cells and Their Derivatives

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Background: Cell proliferation is a fundamental event during development, disease, and regeneration. Effectively tracking and quantifying proliferating cells and their derivatives is critical for addressing many research questions. Cell cycle expression such as for Ki67, proliferating cell nuclear antigen (PCNA), or aurora kinase B (Aurkb), or measurement of 5-bromo-2′-deoxyuridine (BrdU) or 3H-thymidine incorporation have been widely used to assess and quantify cell proliferation. These are powerful tools for detecting actively proliferating cells, but they do not identify cell populations derived from proliferating progenitors over time.

Aims: We developed a new mouse tool for lineage tracing of proliferating cells by targeting the Aurkb allele.

Results: In quiescent cells or cells arrested at G1/S, little or no Aurkb mRNA is detectable. In cycling cells, Aurkb transcripts are detectable at G2 and become undetectable by telophase. These findings suggest that Aurkb transcription is restricted to proliferating cells and is tightly coupled to cell proliferation. Accordingly, we generated an AurkbER Cre/+ mouse by targeting a tamoxifen inducible Cre cassette into the start codon of Aurkb. We find that the AurkbER Cre/+ mouse faithfully labels proliferating cells in developing embryos and regenerative adult tissues such as intestine but does not label quiescent cells such as post-mitotic neurons.

Conclusion: The AurkbER Cre/+ mouse faithfully labels proliferating cells and their derivatives in developing embryos and regenerative adult tissues. This new mouse tool provides a novel genetic tracing capability for studying tissue proliferation and regeneration.

Keywords: cell proliferation, aurora kinase B, mouse model, lineage tracing, regeneration, development

INTRODUCTION

Cell proliferation is a fundamental biological event in all multicellular organisms (Nasmyth, 2001). Identification or quantification of proliferating cells is essential to understanding organogenesis, morphogenesis, tumorigenesis, and regeneration. Replicating cells can be identified based on expression of cell-cycle markers, such as Ki67, proliferating cell nuclear antigen (PCNA), aurora
kinase B (Aurkb), or the incorporation of thymidine analogs, such as 3H-thymidine, 5-bromo-2′-deoxyuridine (BrdU), or 5-ethynyl-2′-deoxyuridine (EdU) (Mitchison and Salmon, 2001). Incorporation of 5-iodo-2′-deoxyuridine (IdU) has been used to analyze proliferation in human tissue (Pan et al., 2013). These assays are suitable for detecting actively proliferating or label-retaining cells. However, retrospective lineage tracing is often desired when proliferating cells must be tracked for their growth pattern or quantity under certain biological conditions such as tissue morphogenesis or regeneration.

Genetic engineering in the mouse allows lineage tracing mouse models to track the derivatives of proliferating cells. One model is the mosaic analysis with double markers (MADM) mouse model, which labels dividing cells through interchromosomal recombination (Zong et al., 2005), although its application is limited due to low labeling efficiency. More recently, a KI67IRESCreER/+ mouse was generated and used to track proliferating cells in brain or heart (Basak et al., 2018; Kretzschmar et al., 2018). However, KI67 is expressed throughout the cell cycle including G1, and some non-proliferative cells such as adult cardiomyocytes can poise at G1 for an extended period of time without cell division (Alvarez et al., 2019).

Aurkb, a key component of the chromosomal passenger complex, localizes to the centromeres to ensure precise chromosome segregation during mitosis and to the midbody to assist cytoplasmic separation during cytokinesis (van der Waal et al., 2012). Knockdown or inhibition of Aurkb in vitro inhibits cell proliferation (Yu et al., 2015; Helfrich et al., 2016), while knockout of Aurkb in mice results in mitotic defects in the inner cell mass (Fernandez-Miranda et al., 2011). Increased expression of Aurkb is associated with tumorigenesis and inhibition of Aurkb may be an effective cancer therapeutic target (Tang et al., 2017; Tischer and Gergely, 2019). Aurkb has been widely used to identify mitotic cells using immunofluorescence or immunohistochemical methods with anti-Aurkb antibodies (Vader and Lens, 2008; Liu and Lampson, 2009; van der Waal et al., 2012; Tian et al., 2015; Nakada et al., 2017; Yu et al., 2019).

In order to track cell proliferation retrospectively, we have generated AurkbER Cre/+ mice by targeting a tamoxifen inducible Cre cassette into the start codon of Aurkb. By characterizing the AurkbER Cre allele in vitro and in vivo, we show that AurkbER Cre/+ mice faithfully label proliferating cells and their derivatives during development and regeneration.

**MATERIALS AND METHODS**

**Mice**

AurkbER Cre/+ mice were generated by homologous recombination in embryonic stem cells targeting a Cre-Ert2-V2A-tdTomato-Frt-PGK-neo-Frt cassette into the start codon of the Aurkb locus. Thus, the insertion of this cassette will lead to the ablation of endogenous Aurkb expression in the target allele. The PGK-Neo cassette was removed by breeding the initial progeny to mice expressing ubiquitous FlpE recombinase (Rodriguez et al., 2000). Southern blot confirmed the expected homologous recombination and germ line transmission of the targeted allele. The AurkbER Cre allele is detected by PCR using the following primers: Forward: 5′-GTGGGCTCTATGGCTTCTGA-3′, Reverse (common): 5′-CAAATCTTGGAGCCCAAC-3′; product size: 501 bp. The wild-type allele is detected by using the following primers: Forward: 5′-ATGGACCTAGAGCGGGAGAT-3′ and Reverse (common); product size: 264 bp. The V2A-tdTomato included in the targeting construct potentially provides a means to fluorescently label Aurkb-expressing cells without disrupting Cre-Ert2 function. However, although we were able to detect tdTomato protein expression by immunofluorescence using antibodies on fixed intestinal crypts (Supplementary Figure 1), the spontaneous tdTomato fluorescence was below levels of detection. B6.129 × 1-Gt (ROSA) 26SorTM1(EYFP)Cos/+ (abbreviated as R26ER<sup>YFP</sup>) mice were purchased from The Jackson Laboratory (stock number: 006148). All mice were maintained on a mixed genetic background. All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC #: 803396) and the University of Maryland Baltimore Institutional Animal Care and Use Committee (IACUC #: 0118005).

**Administration of Tamoxifen and 5-Bromo-2′-Deoxyuridine (BrdU) in vivo**

Tamoxifen (Sigma-Aldrich, St. Louis, MO, United States) (10 mg/ml) was dissolved in corn oil. Tamoxifen [2 or 100 or 150 mg/kg body weight (BW)] was given to AurkbER Cre/+; R26ER<sup>YFP</sup>/+ mice by either intraperitoneal injection or gavage. BrdU (Sigma-Aldrich, St. Louis, MO, United States) (10 mg/ml) was dissolved in phosphate-buffered saline (PBS) and intraperitoneally delivered to AurkbER Cre/+; R26ER<sup>YFP</sup>/+ mice (100 mg/kg BW).

**Histology, Immunofluorescence and RNAscope**

All specimens for paraffin sections were fixed in 4% (w/v) paraformaldehyde (PFA) overnight, dehydrated through an ethanol series, paraffin embedded, and sectioned (6–7 μm). Primary antibodies (Supplementary Table 1) were incubated at 4°C overnight and secondary antibodies (Alexa 488, 555, or 647, Life Technologies, Grand Island, NY, United States) were incubated at room temperature for 1 h. The Aurkb RNAscope probe (173–1483 bp of the Mus musculus Aurkb mRNA sequence) was designed and provided by Advanced Cell Diagnostics (Hayward, CA, United States). RNAscope in situ hybridizations (Ikpa et al., 2016) were performed according to the protocol provided by manufacturer.

**Image Analysis and Quantification**

ImageJ software was used for quantification of GFP+ and/or BrdU+ cells on histology slides. Samples from 3–6 mice each were counted at any given time point or condition. The reported values represent the mean score.
Live Cell Imaging
Time-lapse phase-contrast and GFP immunofluorescence images of mouse embryo fibroblasts (MEFs) were taken for 22 h after 4-OH tamoxifen induction (final concentration: 1 µg/ml) by using the IncuCyte live-cell culture system (Essen Bioscience). The images were then analyzed and converted to movie format by using IncuCyte software.

Fluorescence-Activated Cell Sorting (FACS) Analyses
MEFs were isolated and cultured as previously described (Li et al., 2011). MEFs were treated with either control vehicles or designated cell cycle inhibitors, then digested and collected as single cell suspensions. The cell suspension was washed with PBS and then fixed with intracellular fixation buffer (eBiosciences). For intracellular FACS analyses, cells were permeabilized with permeabilization buffer (eBiosciences) and then incubated with GFP antibodies (see Supplementary Table 1) for 2 h at room temperature, followed by incubation with secondary antibodies (Alexa fluor, Life Technologies) for 1 h at room temperature. Samples were run and analyzed using a BD FACS Canto II instrument and software (BD Biosciences).

Quantitative Real-Time PCR (qRT-PCR)
Heart, brain, and embryonic tissues were microdissected in cold PBS and snap frozen in liquid nitrogen. TRIzol reagent (Life Technologies, Grand Island, NY, United States) was used to extract total RNA and complementary DNA (cDNA) was generated with the Superscript III kit (Life Technologies, Grand Island, NY, United States). SYBR Green quantitative RT-PCR was performed using the StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). Primers for Aurkb: P1F (forward): 5'-TCGCTGTGTTTCTCCCTCTC-3', P1R (reverse): 5'-TTCAGGCCAGACTGAGACG-3'; P2F (forward): TCGCTGTGTTTCCTCCCTCT, P2R (reverse): TTCAGGCCAGACTGAGACG. Primers for Gapdh: Forward: 5'-TCTTGCTCAGTGTCCTTGCTGG-3', Reverse: 5'-TCCTGGTATGACAATGAATAGGC-3'.

Western Blotting
E12.5 embryos were minced in cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA-Na2, 1 mM EGTA, 1% Triton X-100, 0.5% Sodium Deoxycholate and 0.1% SDS with Protease inhibitor cocktail (Roche); 1 mM phenylmethylsulfonyl fluoride was added before use). Protein samples were resolved on 4–12% SDS-PAGE acrylamide gel before transferring to PVDF membranes. We used primary antibodies to Aurkb (1:1000), Cre (1:1000) and GAPDH (1:5000). Primary antibodies were visualized by chemiluminescence using HRP-conjugated secondary antibodies.

Statistical Analysis
Data are presented as mean ± SEM. Statistical significance between two groups was determined using two-tailed Student's t-test or chi square test. If significance is to be tested between multiple groups, an analysis of variance is performed, followed by Bonferroni post hoc test. P < 0.05 was considered significant.

RESULTS

Aurkb Is Expressed in Proliferating but Not in Quiescent Cells
In cultured MEFs, Aurkb protein is undetectable at G1 phase, but expression becomes prominent at G2 and it is localized to the nucleus. Aurkb reaches and maintains a strong expression level throughout M phase. Aurkb re-localizes to the midbody at telophase (Supplementary Figure 2). These findings are consistent with previous observations (Crosio et al., 2002; Li et al., 2015). Aurkb mRNA is not detectable at G1 but is detectable by G2. Message is present through M phase but becomes undetectable at telophase (Figure 1). These results suggest that Aurkb transcript expression is correlated with the phase of the cell cycle and is largely restricted to mitotic cells. To further test this association, we forced MEFs to arrest at G1/S phase by exposure to hydroxyurea or mimosine (Park et al., 2012) and then assessed the presence of Aurkb transcripts.
Aurkb transcriptional levels were significantly decreased in hydroxyurea- and mimosine-treated groups as compared to the control group (Figure 2A). Both brain and heart experience a proliferation transition from being highly proliferative at embryonic stage to being mostly proliferatively inert in adulthood (Brooks et al., 1998). Aurkb transcription significantly declines from being high at embryonic day (E) 14.5 to being almost undetectable in adult heart and brain (Figure 2B). Altogether, these data suggest that Aurkb transcription is coupled with cell proliferation in vitro and in vivo. Accordingly, we generated AurkbER Cre/+ mice by targeting a Cre-Ert2 cassette into the start codon of the Aurkb locus (Figure 3A). As expected, Aurkb mRNA was reduced to about 50% in AurkbER Cre/+ heterozygous mice as compared to their wildtype littermate controls (Figure 3C). In contrast, Aurkb protein expression was similar between AurkbER Cre/+ heterozygous mice and wildtype mice.
controls (Figure 3D). Aurkb<sup>ER Cre/+</sup> heterozygous mice are phenotypically normal and fertile. The total knockout of Aurkb resulted in early post-implantation lethality by E9.5 (Fernandez-Miranda et al., 2011). Consistently, we did not recover any Aurkb<sup>ER Cre/ER Cre</sup> embryos at E10.5 (0/21).

**Aurkb<sup>ER Cre</sup> Labels Proliferating Cells in vitro**

To characterize the labeling of Aurkb<sup>ER Cre</sup> in vitro and test whether it is associated with cell proliferation, we generated Aurkb<sup>ER Cre/+; R26<sup>2</sup>R<sup>YFP</sup>/+</sup> MEFs and tracked Aurkb<sup>ER Cre</sup> labeling by following the YFP reporter activities after 4-OH tamoxifen induction. YFP signal was detectable in proliferating MEFs about 16 h after 4-OH tamoxifen induction, became strong immediately prior to cell division, and maintained expression in daughter cells (Supplementary Video 1). In contrast, there was no YFP signal in non-dividing Aurkb<sup>ER Cre/+; R26<sup>2</sup>R<sup>YFP</sup>/+</sup> MEFs. Note that YFP signal is well recognized by GFP antibodies. Hereafter, we use GFP antibodies to measure YFP expression when referring to Aurkb<sup>ER Cre/+</sup> fate-mapped cells. There was negligible R26<sup>2</sup>R<sup>YFP</sup>/+ reporter activity in Aurkb<sup>ER Cre/+; R26<sup>2</sup>R<sup>YFP</sup>/+</sup> MEFs without 4-OH tamoxifen induction (Figure 4A), indicating that there is little to no leakiness of the Aurkb<sup>ER Cre/+</sup> allele. According to the expression profile of Aurkb, we expected to see Aurkb<sup>ER Cre/+</sup> labeling in cells as they enter G2 phase. To further analyze the association between Aurkb<sup>ER Cre/+</sup> labeling and cell proliferation, we arrested MEFs at G1/S phase by either hydroxyurea or mimosine treatment, as evidenced by the absence of BrdU incorporation (Figure 4A). R26<sup>2</sup>R<sup>YFP</sup>/+ reporter activities were significantly lower for cell cycle inhibitor-treated Aurkb<sup>ER Cre/+; R26<sup>2</sup>R<sup>YFP</sup>/+</sup> MEFs compared to those under normal culture conditions (Figures 4A,B). This lineage tracing result mirrors the Aurkb transcription profile when wild-type MEFs are arrested at G1/S phase (Figure 2A). These results suggest that Aurkb<sup>ER Cre</sup> labels proliferating but not non-dividing cells in vitro.

**Aurkb<sup>ER Cre</sup> Labels Proliferating Cells During Embryonic Development**

Next, we sought to determine whether Aurkb<sup>ER Cre</sup> labels highly proliferative cells during embryonic development in vivo. We confirmed that Aurkb heterozygosity did not grossly affect embryonic morphogenesis or cellular growth (Supplementary Figure 3), validating the use of Aurkb<sup>ER Cre/+</sup> as a lineage tracing tool during embryonic development. When E8.5 Aurkb<sup>ER Cre/+; R26<sup>2</sup>R<sup>YFP</sup>/+</sup> embryos were induced with tamoxifen, extensive labeling of the embryo was observed. Importantly, there was no leakiness of Aurkb<sup>ER Cre</sup> labeling when corn oil but not tamoxifen was administered (Supplementary Figure 4). When we labeled developing embryos with both Aurkb<sup>ER Cre</sup> and BrdU, we found that about 85% of embryonic cells are labeled by both systems (Figure 5). Further, we performed double immunofluorescence staining of GFP and PCNA on these embryos. We found that nearly 93% of embryonic cells are double positive (Supplementary Figure 5). Altogether, these data indicate that Aurkb<sup>ER Cre</sup> is a sensitive and reliable system for lineage tracking of proliferating embryonic cells.

**Tamoxifen Activation of Aurkb<sup>ER Cre</sup> Labels Proliferating Adult Stem/Progenitor Cells but Not Post-mitotic Cells in vivo**

Next, we assessed Aurkb<sup>ER Cre</sup> labeling in adult regenerative tissues. Two-month-old Aurkb<sup>ER Cre/+; R26<sup>2</sup>R<sup>YFP</sup>/+</sup> mice were given a single dose of tamoxifen (100 mg/kg BW) and we followed the labeling pattern of the YFP reporter over time in the intestine. The labeling displayed a dynamic expansion
from the initial crypt (6 h after tamoxifen administration) to the entire crypt-villus structure over a course of 3 days (Figure 6A), greatly resembling the lineage tracking pattern of intestinal stem cells (ISCs) and progenitors, which are known to be highly proliferative (Barker, 2014). In the crypt zone where Ki67-positive ISCs and progenitors reside, AurkB\textsuperscript{ER} Cre\textsuperscript{+} labeling colocalizes with Ki67. In the villi where derivative, Ki67-negative intestinal epithelial cells reside, AurkB\textsuperscript{ER} Cre\textsuperscript{+} labeling colocalizes with derivatives of the crypt ISCs that appear over time (Figure 6A). Further, administration of low doses of tamoxifen (2 mg/kg BW) in adult AurkB\textsuperscript{ER} Cre\textsuperscript{+}; R26\textsuperscript{eYFP}\textsuperscript{+} mice revealed that single earlier AurkB\textsuperscript{ER} Cre\textsuperscript{+} intestinal progenitor cells (PCNA\textsuperscript{+}) expanded to form clusters of enterocytes after 7 days (Supplementary Figure 6). Altogether, these data indicate that AurkB\textsuperscript{ER} Cre\textsuperscript{+} labels proliferating progenitor cells in the intestine. In brain, neuronal nuclei (NeuN\textsuperscript{+})-positive post-mitotic neurons were not promptly labeled after tamoxifen exposure but eventually became labeled (70 days after the initial tamoxifen induction). This occurred presumably through transit amplifying mini-chromosome maintenance proteins (MCM2\textsuperscript{+})-positive progenitors (Figure 6B). This is consistent with NeuN\textsuperscript{+} neurons as terminally quiescent cells, renewing through neural stem cells and progenitors with prolonged kinetics.

**DISCUSSION**

Assessment of cell proliferation in vivo generally relies on the use of BrdU incorporation or “snapshots” of cell cycle marker expression such as PCNA or Ki67. Both approaches are important complementary methods for detecting actively proliferating...
We found that mouse tool, appears a critical factor in its reliability as a proliferation marker is transcribed broadly throughout the cell cycle, a feature that based on Ki67 expression (Basak et al., 2018). However, Ki67 labeling overlaps well with cell proliferation markers such as BrdU and PCNA but it does not label quiescent cells. We notice that the overlap between AurkbER Cre labeling and BrdU or PCNA seems quite high during development, even though AurkbER Cre labeling was induced by a short pulse of tamoxifen. This is primarily due to the high proliferative characteristics of developing embryos: both earlier proliferating embryonic cells and their later derivatives are all mitotic.

Since AurkbER Cre labeling is based on Aurkb transcription, which turns on at G2 phase, AurkbER Cre labeling should not be interpreted as an absolute cell division or cytokinetic marker. For instance, certain cells such as bi- or multinucleated adult cardiomyocytes and hepatocytes can undergo karyokinesis without cytokinesis under physiological or pathological conditions (Gentic et al., 2012; Derks and Bergmann, 2020). These cells may be labeled by AurkbER Cre in the absence of cell division. In these specific instances, Aurkb protein expression and other cytokinetic events should be analyzed instead.

Since AurkbER Cre/+ is built by a knock-in strategy, the Aurkb transcript is disrupted in the targeted allele. In contrast, Aurkb protein expression level in AurkbER Cre/+ heterozygous mice was quite similar to their wildtype controls (Figure 3D). This is consistent with other reports that heterozygous knockout mice can express similar or more than half of the proteins relative to wildtype control mice in the target genes (Gineste et al., 2013; Zhou et al., 2015; Arkhipov et al., 2019). It is reported that an Aurkb knockout mouse in which exons 2–6 were excised is embryonic lethal, older Aurkb heterozygous mice approximately 12–24 months of age show decreased survival due to susceptibility for tumorigenesis, and a fraction of Aurkb heterozygous males suffer from oligospermia by 12 months of age (Fernandez-Miranda et al., 2011). In our current study, we used much younger mice less than 3-months of age. We did not observe reproductive defects or spontaneous cancer development in young AurkbER Cre/+ mice. Nonetheless, it is possible that AurkbER Cre/+ mice may develop aforementioned pathologies over time, due to genome or chromosomal instability, even though we did not observe such problems in our colony. On the other hand, by intercrossing AurkbER Cre/+ heterozygous mice, the progeny with genotype of AurkbER Cre/ER Cre can be used to study the phenotype of Aurkb depletion or requirement of Aurkb expression in cell division and cytokinesis.

The AurkbER Cre/+ mouse is an important new tool for lineage tracing proliferating cells during embryonic development and adult tissue regeneration. For instance, when crossed to multicolor reporters such as the confetti mouse strain (Snippert et al., 2010), the AurkbER Cre/+ mouse can be used to study the clonogenicity of neurons or other cell types in the developing embryos or tissue regeneration (e.g., whether cells are derived from multiple progenitor cells or from rare dominant clones). Similarly, the AurkbER Cre/+ mouse can be used to track tissue regeneration such as occurring from those rare stem cells whose derivatives forming over long periods of time will be more readily detected with this tool. The AurkbER Cre/+ mouse allows the ability to track how tissues with low rates of proliferation such as brain, lung and heart are regenerated over time in various physiological or pathological conditions. We anticipate the coupling of Aurkb knockout with inducible Cre-mediated

![FIGURE 6](image_url)
capability will be a powerful reagent for the investigation of cell proliferation in the context of Aurkb expression.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

ETHICS STATEMENT

The animal studies were reviewed and approved by the University of Pennsylvania Institutional Animal Care and Use Committee and the University of Maryland Baltimore Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JE and DL conceived and designed the study. JJ, KE, and DL analyzed the experiments and wrote the original draft. JJ, KE, JE, and DL edited and finalized the manuscript. JJ, KE, FL, LL, GS, and DL performed the experiments. All authors reviewed the results and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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