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(71) Applicant: **REGENTS OF THE UNIVERSITY OF MINNESOTA** [US/US]; 600 McNamara Alumni Center, 200 Oak Street SE, Minneapolis, MN 55455-2020 (US).

(72) Inventors; and
(71) Applicants : **HARRIS, Reuben, S.** [CA/US]; 1396 Canfield Avenue, St. Paul, MN 55108 (US). **LAW, Emily** [US/US]; 830 Laurel Avenue; Apt. 6, St. Paul, MN 55104 (US).

(74) Agent: **GRAM, Christopher, D.**; Mueting, Raasch & Gebhardt, P.A., P.O. Box 581336, Minneapolis, MN 55458-1336 (US).

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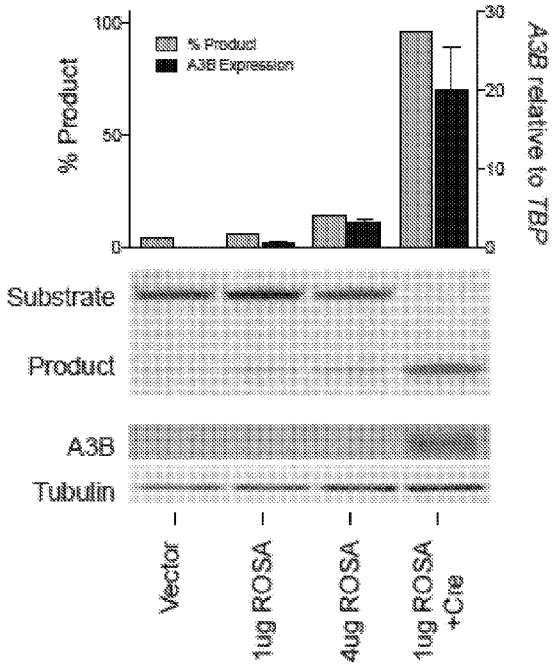
(54) Title: TRANSGENIC MOUSE FOR EXPRESSING APOBEC3B

FIG. 3



(57) Abstract: In one aspect, a vector for expressing human APOBEC3B generally includes a first region having homology to a mouse gene, a /ox-STOP-*lox* (LSL) cassette, and a human *APOBEC3B* gene. The LSL cassette includes two *loxP* sites, a first promoter, a drug resistance gene, and a first poly-A region. In another aspect, a vector for expressing human APOBEC3B generally includes a *Rosa26* promoter, a human *APOBEC3B* gene, and a polyadenylation region. In another aspect, a transgenic mouse includes a vector that includes a human *APOBEC3B* gene so that the mouse expresses the human *APOBEC3B* gene.

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TRANSGENIC MOUSE FOR EXPRESSING APOBEC3B

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Patent Application No. 62/186,824, filed June 30, 2015, which is incorporated herein by reference.

SEQUENCE LISTING

This application contains a Sequence Listing electronically submitted via EFS-Web to the United States Patent and Trademark Office as an ASCII text file entitled "2016-06-23-SequenceListing_471_ST25.txt" having a size of 9 kilobytes and created on June 23, 2016. The information contained in the Sequence Listing is incorporated by reference herein.

SUMMARY

In one aspect, this disclosure describes a vector for expressing human APOBEC3B. Generally, the vector includes a first region having homology to a mouse gene, a *lox*-STOP-*lox* (LSL) cassette, and a human *APOBEC3B* gene. The LSL cassette includes two *loxP* sites, a first promoter, a drug resistance gene, and a first poly-A region.

In some embodiments, the first region having homology to a mouse gene includes a constitutive low-mid-copy promoter.

In some embodiments, the mouse gene includes at least a portion of *Rosa26*.

In some embodiments, the first region having homology a mouse gene includes a *Rosa26* promoter.

In some embodiments, the vector further includes a second region having homology to the mouse gene. In some of these embodiments, the first region having homology to a mouse gene and the second region having homology to a mouse gene flank the LSL cassette and the human *APOBEC3B* gene.

In some embodiments, the first poly-A region includes a *PGK* poly-A region or an SV40 poly-A region. In some embodiments, the first poly-A region includes a *PGK* poly-A region and an SV40 poly-A region.

In some embodiments, the first poly-A region includes a poly-A region from more than one gene.

In some embodiments, the drug resistance gene encodes a protein that provides resistance to a drug comprising G418, gentamycin, neomycin, puromycin, blasticidin, histidiol, hygromycin, or zeocin.

In some embodiments, the first promoter includes a *PGK* promoter.

In some embodiments, the *APOBEC3B* gene includes an intron.

In some embodiments, the *APOBEC3B* gene includes a sequence having at least 90% homology to SEQ ID NO:1. In some of these embodiments, the *APOBEC3B* gene includes SEQ ID NO:1.

In some embodiments, the *APOBEC3B* gene includes a naturally occurring minor allele in the human population. In other embodiments, the *APOBEC3B* gene includes a naturally occurring major allele in the human population.

In some embodiments, the *APOBEC3B* gene is flanked on the 3' side by a second poly-A region. In some of these embodiments, the second poly-A region includes a bovine growth hormone polyadenylation signal (BGHpA).

In another aspect, this disclosure describes a vector for expressing human APOBEC3B. Generally, the vector includes comprising a *Rosa26* promoter, a human *APOBEC3B* gene, and a polyadenylation region.

In some embodiments, the polyadenylation region includes a bovine growth hormone polyadenylation signal (BGHpA).

In some embodiments, the *APOBEC3B* gene is an intron-containing *APOBEC3B* gene.

In some embodiments, the *APOBEC3B* gene includes a sequence having at least 90% homology to SEQ ID NO:1. In some of these embodiments, the *APOBEC3B* gene includes SEQ ID NO:1.

In some embodiments, the *APOBEC3B* gene includes a naturally occurring minor allele in the human population. In other embodiments, the *APOBEC3B* gene includes a naturally occurring major allele in the human population.

In some embodiments, the *Rosa26* promoter, the *APOBEC3B* gene, and the polyadenylation region are operably linked to each other.

In some embodiments, the vector further includes a site-directed recombination cassette. In some of these embodiments, the site-directed recombination cassette is a *lox*-STOP-*lox* (LSL)

cassette. In other embodiments, the site-directed recombination cassette is a *FRT*-STOP-*FRT* cassette.

In some embodiments, the site-directed recombination cassette includes a *PGK* promoter and a poly-A region.

In some embodiments, the site-directed recombination cassette further includes a drug resistance gene. In some of these embodiments, the drug resistance gene encodes a protein that provides resistance to a drug comprising G418, gentamycin, neomycin, puromycin, blasticidin, histidiol, hygromycin, or zeocin.

In another aspect, this disclosure describes a transgenic mouse, or a cell or tissue isolated therefrom, that includes any embodiment the vectors summarized above.

In another aspect, this disclosure describes a method for generating a transgenic mouse. Generally, the method includes transfecting an embryonic stem cell with any embodiments of the vectors summarized above, injecting the embryonic stem cell into a blastocyst, and allowing the blastocyst to grow into a transgenic mouse.

In some embodiments, the method further includes breeding the transgenic mouse with a mouse the includes one or more tissues expressing Cre or Flp.

In another aspect, this disclosure describes a transgenic mouse that includes a *Rosa26* promoter operably linked to an intron-containing human *APOBEC3B* gene.

In another aspect, this disclosure describes a transgenic mouse that includes a *Rosa26* promoter, an intron-containing human *APOBEC3B* gene, and a *lox*-STOP-*lox* cassette, wherein the *Rosa26* promoter is operably linked to the the *lox*-STOP-*lox* cassette.

As used herein, the term "gene" refers to a region of a deoxyribonucleic acid that encodes a protein. A gene can include certain non-coding sequences (e.g., one or more introns) or may be intronless, whether natively intronless or derived from cDNA. The transfer of a gene from one organism to another can include the transfer of expression control sequences native to the gene being transferred. Alternatively, the gene may be placed under the control of heterologous expression control sequences.

As used herein, "operably linked" refers to a functional linkage between a first nucleic acid sequence and second nucleic acid sequence in such a manner as to allow general functions. For example, a nucleic acid sequence encoding a protein or RNA may be operably linked to a nucleic acid expression control sequence, in such a manner that the expression control sequence affects expression of the coding nucleic acid sequence.

As used herein, the term "transgene" refers to a nucleic acid sequence that is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can be operably linked to one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

The term "transgenic" is used herein as an adjective to describe the property, for example, of an animal or a construct, of harboring a transgene.

As used herein, "transformation" refers to a process in which an organism's genotype is changed as a result of the uptake of exogenous DNA.

The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful, and is not intended to exclude other embodiments from the scope of the invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

Also herein, the recitations of numerical ranges by endpoints include all numbers subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.).

For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

The above summary of the present invention is not intended to describe each disclosed embodiment or every implementation of the present invention. The description that follows more particularly exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. MluI-A3Bi-MluI nucleotide sequence (SEQ ID NO:1). The intron-containing APOBEC3B (A3Bi) coding sequence is shown in CAPITAL letters, intronic sequence in lower case letters, and MluI restriction sites are shown in bold type. This cDNA sequence encodes an APOBEC3B (A3B) protein that matches GenBank NM_004900.4, with the exception of a single Thr-to-Lys amino acid substitution at position 146 (ACG-to-AAG at the codon level). This Lys146 variant is a naturally occurring minor allele in the human population.

FIG. 2. Schematic of the *Rosa26::LSL-A3Bi* construct. The targeted *Rosa26::LSL-A3Bi* allele produces a neomycin/G418-resistance protein from both the *Rosa26* promoter and the *PGK* promoter. Both of these transcripts terminate within one of three transcription termination/poly-adenylation regions (PGK or 2x SV40 poly-A regions), which prevent *A3Bi* from being expressed. This construct is collectively called a *lox*-STOP-*lox* (LSL) cassette. Upon whole body or tissue specific expression of the Cre recombinase, the *loxP* sites are fused, the LSL cassette deleted, and the A3Bi transcript is expressed from the *Rosa26* promoter. The resulting mRNA, containing A3B N-terminal domain ($A3B_{NTD}$) and A3B C-terminal domain ($A3B_{CTD}$) is processed to remove the beta-globin (bGLO) intron and to produce a mature mRNA that encodes the full-length A3B enzyme. This protein (SEQ ID NO:2) matches GenBank NM_004900.4, with the exception of a single Thr-to-Lys amino acid substitution at position 146 (ACG-to-AAG at the codon level):

FIG. 3. A functional test of the *Rosa26::LSL-A3Bi* construct. The upper panel shows a histogram that reports the percentage of deaminated product (gray bars; left Y-axis) and the level of *A3B* mRNA relative to *TATA-binding protein (TBP)* mRNA (black bars; right Y-axis) with vector alone, 1 μg *Rosa26::LSL-A3Bi*, 4 μg *Rosa26::LSL-A3Bi*, and 1 μg *Rosa26::LSL-A3Bi* +CRE. The middle panel shows a representative image of C-containing ssDNA substrate and product (after deamination, uracil excision, and backbone cleavage). The ssDNA substrates and products are visualized by virtue of a fluorescent DNA end label (for example, 3' 6FAM (fluorescein)). The bottom panel shows representative anti-A3B and anti-tubulin immunoblots. A3B induction by Cre recombinase is evidenced by increased A3B mRNA and protein levels and correspondingly higher DNA deaminase activity in cell extracts. The anti-A3B antibody used here was the rabbit anti-APOBEC3B monoclonal antibody 5210-87-13 (described in U.S. Provisional Patent Application No. 62/186,109, filed June 29, 2015).

FIG. 4. Representative genotyping data. The wild-type (WT) *Rosa26* amplicon is 300 bp, and the knock-in *Rosa26::LSL-A3Bi* amplicon is 500 bp. Animal 177 is homozygous WT, Animal 178 is heterozygous, and Animal 179 is homozygous for *Rosa26::LSL-A3Bi.*

FIG. 5. Representative Cre-mediated recombination data. Cre recombination was confirmed by PCR analysis of genomic DNA 5'-AGCACTTGCTCTCCCAAAGTC-3' (SEQ ID NO:3) (common wild-type forward primer) and 5'-GCACATTTCTGCGTGGTACTGAGG-3' (SEQ ID NO:4), which yields a 590 bp amplicon. Animal 194 has the full *Rosa26::LSL-A3Bi* cassette, and Animal 195 has a collapsed cassette and a single *loxP* site, *Rosa26::L-A3Bi*, which encodes an active A3B enzyme.

FIG. 6. A3B activity *in vivo*. The tissues listed above were harvested from mice expressing full body A3B or controls, processed into soluble extracts, and assayed neat or as a 1:10 dilution for ssDNA deaminase activity. A3B activity is evident in most tissues, with exceptions likely due to poor lysis (heart) or contaminating nucleases (spleen). Negative and positive controls were generated by transfecting the LSL-A3Bi construct into 293 cells, without and with a Cre-expressing construct, respectively, converted into soluble extracts, and assayed in parallel.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The antiviral DNA cytosine deaminase APOBEC3B (A3B) causes mutations in cancer (Burns et al., 2013, *Nature* 494(7437):366-370; Burns et al., 2013, *Nat Genet.* 45(9):977-983; Leonard et al., 2013, *Cancer Res.* 73(24):7222-7231; Sieuwerts et al., 2014, *Horm. Cancer* 5(6):405-13), but although A3B is expressed in higher primates, including humans, it is not found in non-primate species such as rodents. This disclosure describes a transgenic mouse that can conditionally express human A3B. The knock-in mouse provides a murine model for A3B mutagenesis and carcinogenesis. This disclosure also describes a vector construct for expressing A3B. In addition, this disclosure describes methods of making a transgenic mouse that can express A3B.

Previous attempts to make an A3B transgenic mouse have been unsuccessful. For example, a classical multicopy transgenic approach with a construct similar to the one described did not succeed. The lack of success may have been the result of one of the following. First, a strong CAGGS promoter was used (not an endogenous promoter as, for example, the *Rosa26* promoter). Second, a single poly-A region was included for transcription termination of the drug-resistance gene within the LSL portion of the construct, which may not have been sufficient to prevent leaky

(undesirable) transcription and expression of the downstream A3B mini-gene. Third, in the previous attempt, animals were generated by transfection of a transgene into ES cells, a technique that typically introduces multiple copies of the transgene (unlike a single copy transgene that is generated by knock-in to an endogenous locus). Together, these differences caused leaky expression of the *A3B* mini-gene, resulting in inactivation of the transgene by the third generation of animals. PCR and sequencing analyses indicated that the catalytic domain of the gene had been lost completely and the adjacent fluorescence reporting *RFP* gene had accumulated 14 different base substitution mutations. These observations are consistent with leaky levels of A3B ultimately causing cytotoxicity and directly mutating and inactivating its own transgene.

In one aspect, this disclosure describes a transgenic mouse that can conditionally express human A3B. In another aspect, this disclosure describes a vector that includes a human *A3B* mini-gene and can express the human A3B protein in a mouse. A transgenic mouse, as described herein, may include one or more of the vectors including A3B described herein.

In some embodiments, the *A3B* gene may include at least a portion of a beta-globin gene. In some embodiments, the *A3B* gene includes SEQ ID NO:1. In some embodiments, the *A3B* gene includes a sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 95%, homology to SEQ ID NO:1. In some embodiments, the *A3B* gene includes a naturally occurring minor allele in the human population. In some embodiments, the *A3B* gene includes a naturally occurring major allele in the human population. In some embodiments, the *A3B* gene encodes a protein that includes Thr at position 146. In some embodiments, the *A3B* gene encodes a protein that includes Lys at position 146.

In some embodiments, the *A3B* gene can include the *A3B* coding sequence shown in CAPITAL letters in SEQ ID NO:1. In some embodiments, the *A3B* gene includes a coding sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 95%, homology to the *A3B* coding sequence shown in CAPITAL letters in SEQ ID NO:1.

In some embodiments, the vector containing *A3B* includes a first region having homology to a mouse gene. In some embodiments, the mouse gene is, for example, *Rosa26*, *hypoxanthine guanine phosphoribosyl transferase* (*HPRT*), *Cd6*, etc. In some embodiments, the first region having homology to a mouse gene includes a constitutive low-mid-copy promoter. In some embodiments, the promoter is a non-essential murine gene promoter. In some embodiments, *A3B* is expressed from a promoter included in the first region having homology to a mouse gene. In some embodiments, the promoter is a *Rosa26* promoter.

In some embodiments, the vector containing *A3B* includes a second region having homology to a mouse gene. In some embodiments, the mouse gene is, for example, *Rosa26*, *hypoxanthine guanine phosphoribosyl transferase* (*HPRT*), *Cd6*, etc. In some embodiments, the second region having homology to a mouse gene is homologous to the same mouse gene as the first region. In some embodiments, both the first region and the second region are homologous to at least a portion of to the *Rosa26* gene.

In some embodiments, the mouse and/or vector includes a site-specific recombination cassette. In some embodiments, the site-specific recombination cassette includes *loxP* sites, which are acted upon by the Cre recombinase. In some embodiments, the site-specific recombination cassette includes *FRT* sites (Dymecki et al., 1996, *Proc Natl Acad Sci USA.* 93(12):6191-6196). While described in the context of an exemplary embodiment in which a site-specific recombination cassette is a *lox*-STOP-*lox* (LSL) cassette, the vectors and methods described herein can provide other site-specific recombination cassettes including, for example, a *FRT* recombination cassette.

In some embodiments, the mouse and/or vector includes a *lox*-STOP-*lox* (LSL) cassette. In some embodiments, the LSL cassette includes a constitutive and/or heterologous promoter. In some embodiments, the LSL cassette includes a first polyadenylation (poly-A) region. In some embodiments, the LSL cassette includes polyadenylation (poly-A) regions from more than one gene. The first poly-A region can include at least one *PGK* poly-A region and/or at least one SV40 poly-A regions. In some embodiments, the LSL cassette may be operably linked to the *Rosa26* promoter. In some embodiments, it may be operably linked to other endogenous, non-essential promoters.

In some embodiments, the LSL cassette further includes a drug resistance gene. The drug resistance gene can provide resistance to, for example, G418; gentamycin; neomycin; puromycin; blasticidin, including blasticidin S, blasticidin S HCl; histidiol, including L-histidinol dihydrochloride; hygromycin, zeocin, etc. In some embodiments, the drug resistance gene encodes a protein that provides resistance to a drug that includes, for example, G418, gentamycin, neomycin, puromycin, blasticidin, histidiol, hygromycin, etc. In some embodiments, the drug resistance cassette may include a neomycin/G418 resistance gene. A drug resistance gene may be used, for example, to select cells that include the vector.

In some embodiments, the LSL cassette may include a *PGK* promoter, a drug resistance gene, a *PGK* poly-A region and 2x SV40 poly-A regions. In some embodiments, the first region having homology to a mouse gene and the second region having homology to a mouse gene flank

the LSL cassette and the *A3B* gene. For example, a first region having homology to the *Rosa26* promoter and a second region having homology to a portion of the *Rosa*26 gene can flank the LSL cassette and the *A3B* gene.

In some embodiments, a vector that includes A3B can conditionally express A3B. For example, in one embodiment, a vector contains a *Rosa26* targeting vector that includes a *Rosa26* promoter, a *lox*-STOP-*lox* (LSL) cassette, and an A3B gene. The LSL cassette may be positioned between the *Rosa26* promoter and the *A3B* gene such that transcription termination/poly-A regions in the LSL cassette prevent the *A3B* gene from being expressed, as shown, for example, in FIG. 2. In the presence of Cre, at least a portion of the LSL cassette will be deleted resulting in the *A3B* gene being operably linked to the *Rosa26* promoter, as shown, for example, in FIG. 2 and FIG. 3.

In some embodiments, a mouse that includes A3B can conditionally express A3B in any mouse tissue. In other embodiments, a mouse that includes A3B can conditionally express A3B in specific mouse tissues. In some embodiments, for example, when the mouse and/or vector includes a LSL cassette, the conditional expression of A3B may be Cre-inducible; when the mouse and/or vector includes a *FRT* recombination cassette, the conditional expression of A3B may be *flippase* (*Flp*)-inducible. For example, depending on the locations of Cre-expression in a mouse, A3B may be expressed in all mouse tissues or only in specific mouse tissues. In some embodiments, the conditional expression of A3B may be inducible by, for example, tetracycline and/or doxycycline.

In some embodiments, the mouse and/or vector includes at least one poly-A region that is operably linked to an A3B gene. In some embodiments, the poly-A region may be further operably linked to the *Rosa26* targeting vector. In some embodiments, the *A3B* gene is flanked on the 3' side by the poly-A region. The poly-A region may be operably linked to the *A3B* gene such that at least one poly-A region may be included on an mRNA that encodes an A3B protein and that is expressed in the mouse and/or by the vector. The poly-A region may include, for example, a bovine growth hormone polyadenylation signal (BGHpA), a *PGK* poly-A region, an SV40 poly-A region, etc.

In one embodiment, the transgenic mouse or "Mutamouse" has a *Rosa26::LSL-A3Bi* cassette that includes a *Rosa26* targeting vector that includes a *Rosa26* promoter, an LSL cassette, a human intron-containing *A3B*, and a bovine growth hormone polyadenylation signal (BGHpA). In at least one embodiment, the LSL cassette includes a *PGK* promoter, a drug resistance gene, a PGK poly-A region and/or 2x SV40 poly-A regions. In some embodiments, the *Rosa26* promoter and the LSL cassette are operably linked. In some embodiments, for example, after being bred with a mouse expressing Cre, the transgenic mouse has *Rosa26::L-A3Bi* cassette, that is a collapsed LSL cassette

that includes a single loxP site, wherein the cassette includes an operably linked *Rosa26* targeting vector and human intron-containing *A3B*, and bovine growth hormone polyadenylation signal (BGHpA).

In a further aspect, this disclosure describes methods of making a transgenic mouse or a cell or tissue isolated from a mouse that can express human A3B. The transgenic mouse may be transformed with one or more of the vectors of this disclosure which are able to express human A3B.

In some embodiments, fertilized eggs may be transfected with one or more of the vectors of this disclosure which are able to express human A3B.

In further embodiments, one or more of the vectors of this disclosure that are able to express human A3B are introduced into an embryonic stem cell. In some embodiments, the embryonic stem cell is a mouse embryonic stem cell. In one embodiment, the embryonic stem cell may be a C57BL/6 embryonic stem cell. In some embodiments, the vector may be introduced into an embryonic stem cell by transfection. In some embodiments, the embryonic stem cell may be transfected by electroporation with a linearized vector. In some embodiments, a single copy transgene may be generated by knock-in to an endogenous locus. In some embodiments, the embryonic stem cells may be selected based on a selection system contained in the vector. For example, if the vector contains a neomycin/G418 resistance protein, the embryonic stem cells may be selected for resistance to treatment with neomycin and/or G418. In some embodiments, the embryonic stem cells may be expanded. Analysis including, for example, PCR analysis, Southern blot analysis, etc., may be performed to identify homologous recombinants.

Following transfection and, optionally, selection and screening, the embryonic stem cell can be injected into a blastocyst. In one embodiment, transfected and selected C57BL/6 embryonic stem cells are injected into BALB/c blastocysts. The blastocysts are allowed to grown into a mouse, and resulting chimeric mice with a high percentage of black coat color may be further selected and bred to obtain germline transmission.

In some embodiments, the transgenic mice may be bred with a Cre-expressing mouse. The Cre-expressing mouse may express Cre in all tissues or only in specific tissues. For example, a *Rosa26::LSL-A3Bi* mouse may be crossed with a CMV-Cre mouse to obtain doubly heterozygous progeny expressing Cre in all tissues. Cre recombinase catalyzes the excision of the LSL cassette and reduces it to a single loxP “L” site (as shown schematically in FIG. 2). Cre recombination may be confirmed by, for example, PCR analysis, immunoblotting, etc.

In one embodiment, embryonic stem cells may be transfected by electroporation with a *Rosa26::LSL-A3Bi* targeting linearized by SacII digestion. G418 may be used to select drug resistant embryonic stem cell clones. The clones may be expanded, and homologous recombinants may be identified with PCR and/or Southern blot analysis. Embryonic stem cells including *Rosa26::LSL-A3Bi* may be microinjected into into BALB/c blastocysts. Resulting chimeras with a high percentage of black coat color may be further selected and bred to obtain germline transmission. Mice expressing *Rosa26::LSL-A3Bi* may be maintained through crosses or breeding with wild-type C57BL/6N mice or by intercrossing *Rosa26::LSL-A3Bi* heterozygous or homozygous knock-in animals. Mice expressing *Rosa26::LSL-A3Bi* may be identified by PCR of the *Rosa26* region. Animals expressing *Rosa26::LSL-A3Bi* may be crossed with CMV-Cre animals to obtain doubly heterozygous progeny expressing Cre in all tissues. Expression of A3B may be tested by, for example, PCR, immunoblotting, etc.

The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Generation of Targeting Construct

First, a previously reported intron-containing APOBEC3B (A3Bi) cDNA (Lackey et al., 2013, *Cell Cycle* 12(5):762-772; Lackey et al., 2012, *J Mol. Biol.* 419(5):301-314) was amplified by high fidelity PCR using 5'-ACGCGTACCATGAATCCACAGA (SEQ ID NO:5) and 3'-ACGCGTtcaGTTTCCCTGATTC (SEQ ID NO:6) in order to flank the cDNA with MluI cleavage sites (underlined). This cDNA encodes a human A3B protein that matches GenBank NM_004900.4, with the exception of a single Thr-to-Lys amino acid substitution at position 146 (ACG-to-AAG at the cDNA level) (available online at useast.ensembl.org/Homo_sapiens/Variation/Mappings?db=core;g=ENSG00000179750;r=22:38982347-38992804;v=rs5995649;vdb=variation;vf=4123122). This Lys146 variant is a naturally occurring minor allele in the human population. The 2 kbp amplified band was excised, gel extracted, cloned into pJET1.2, and confirmed by Sanger sequencing (FIG. 1). This MluI-flanked A3Bi cDNA was subcloned into similarly digested *Rosa26* targeting vector (Talabot-Ayer et al., 2015, *J. Immunol.* 194(2):750-760) provided by inGenious

Targeting Laboratory (Ronkonkoma, NY) to generate the Cre-inducible A3Bi gene targeting and expression vector (FIG. 2).

Validation of Targeting and Inducible A3Bi Expression Construct

The entire LSL-A3Bi cassette was subcloned into pcDNA3.1 for validation experiments by double digesting the *Rosa26* targeting vector and pcDNA3.1 with NheI and ClaI. The appropriate bands (*Rosa26*=6621bp, pRH302=5388bp) were excised, gel extracted and ligated. Resulting clones were confirmed by sequencing. Resulting expression vector was co-transfected with pEGFP (Clontech Laboratories, Inc., Mountain View, CA) +/- Cre recombinase into 293T cells with TransitLT-1 (Mirus Bio LLC, Madison, WI) per manufactured protocols. 48 hours post transfection cells were harvested for immunoblot, RNA isolation, flow cytometry, and DNA deaminase activity assays (FIG. 3).

Immunoblots for A3B Protein Detection

Lysates ran on a 12.5% acrylamide gel and probed with rabbit anti-A3B 5210-87-13 1:1000 (described in U.S. Provisional Patent Application No. 62/186,109, filed June 29, 2015) and mouse anti-tubulin 1:40,000 (Novus Biologicals LLC, Littleton, CO). Secondary antibodies (LI-COR Biosciences, Inc., Lincoln, NE) were used at 1:20,000 and scanned using ODYSSEY imaging system (LI-COR Biosciences, Inc., Lincoln, NE). A representative immunoblot is shown in FIG. 3.

RTqPCR for A3B Expression Quantification

Total RNA was isolated with RNAeasy (Qiagen N.V., Hilden, Germany), with QIAshredder (Qiagen N.V., Hilden, Germany) and on column DNase treatment per manufacturer protocol. cDNA was prepared and A3B mRNA expression was quantified by RT-qPCR as described (Refsland et al., 2010, *Nucleic Acids Res.* 38(13):4274-4284). A3B was amplified using 5'-GACCCTTTGGTCCTTCGAC-3' (SEQ ID NO:7) and 5'-GCACAGCCCCAGGAGAAG-3' (SEQ ID NO:8). Human TATA-binding protein (TBP) was amplified using primers 5'-CCCATGACTCCCATGACC-3' (SEQ ID NO:9) and 5'-TTTACAACCAAGATTCACTGTGG-3' (SEQ ID NO:10).

Flow Cytometry

Cells were assayed for GFP fluorescence by flow cytometry to confirm equal transfection between reactions.

DNA Deaminase Activity Assays

Cell extracts were used for DNA deaminase activity assays as described (Olson et al., 2013, *ChemMedChem* 8(1):112-117). Cells were suspended in HED buffer (25 mM HEPES, 5 mM EDTA, 10% glycerol, 1 mM DTT, 1 tablet proteasome inhibitor (Roche, Basel, Switzerland) per 50 ml buffer, freeze/thawed once, and vortexed to promote lysis. Cell debris removed by centrifugation and cleared lysates were incubated two hours with a fluorescently labeled ssDNA substrate (RSH5195=5'-ATTATTATTATTCGAATGGATTTATTTATTTATTTATTTATTT-fluorescein -3') (SEQ ID NO:11). These conditions support A3B dependent ssDNA cytosine deamination and excision of the resulting uracil by endogenous uracil DNA glycosylase (UNG2). Cleavage of deaminated substrates was promoted by adding NaOH to a final concentration of 0.1 M and incubating samples at 98°C for 5 minutes. The resulting samples were fractionated on a 15% acrylamide gel, imaged (TYPHOON, GE Healthcare Life Sciences, Pittsburgh, PA), and quantified by densitometry (IMAGEQUANT, GE Healthcare Life Sciences, Pittsburgh, PA). A representative DNA deaminase activity assay is shown in FIG. 3.

Generation of Knock-in Rosa26::LSL-A3Bi Animal or "Mutamouse"

The following procedures were performed under contract by inGenious Targeting Laboratory (Ronkonkoma, NY). Ten micrograms of functionally confirmed targeting vector were linearized by *Sac*II digestion and transfected by electroporation into C57BL/6 embryonic stem cells. G418 was used to select drug resistant clones, which were expanded for PCR analysis to identify homologous recombinants. Positive clones were confirmed with PCR and Southern blot analysis. Correctly targeted ES cells were microinjected into BALB/c blastocysts. Resulting chimeras with a high percentage of black coat color were mated to wild-type (WT) C57BL/6N mice to obtain germline transmission.

Animal Husbandry and Mouse Genotyping

All animals were maintained in AAALAC accredited animal facilities at the University of Minnesota, Twin Cities, under approved IACUC protocol 1302A30327. The *Rosa26::LSL-A3Bi* line was maintained through standard breeding crosses with WT C57BL/6N mice or by intercrossing heterozygous or homozygous knock-in animals. Genotypes were determined by PCR analysis of tail biopsy genomic DNA from 21-day-old mice. Genomic DNA was isolated using the PUREGENE protocol (Qiagen, N.V., Hilden, Germany). Primers RSH8980 5'-AGCACTTGCTCTCCCAAAGTC-3' (SEQ ID NO:3) (common WT forward), RSH8985 5'-TGCGAGGCCAGAGGCCACTTGTGTAGC-3' (SEQ ID NO:12) (targeted reverse), and RSH10347 5'-CACCTGTTCAATTCCCCTGC-3' (SEQ ID NO:13) (WT reverse) were used to amplify both WT and targeted *Rosa26* alleles. The WT *Rosa26* amplicon is 300 bp, and the knock-in *Rosa26::LSL-A3Bi* amplicon is 500 bp (FIG. 4).

A3B Expression In Vivo

To test for A3B function *in vivo*, *Rosa26::LSL-A3Bi* males were crossed with CMV-Cre females (The Jackson Laboratory, Bar Harbor, ME) to obtain doubly heterozygous progeny expressing Cre in all tissues. Cre recombinase catalyzes the excision of the transcription stop "LSL" cassette and reduces it to a single loxP "L" site (schematic in FIG. 2). Cre recombination was confirmed by PCR analysis of genomic DNA using RSH8980 5'-AGCACTTGCTCTCCCAAAGTC-3' (SEQ ID NO:3) (common WT forward) and RSH8984 5'-GCACATTTCTGCGTGGTACTGAGG-3' (SEQ ID NO:4), which yields a 590 bp amplicon (FIG. 5).

Testing A3B Activity In Vivo

A sibling pair (*Rosa26::L-A3Bi* CMV-Cre (note collapse of LSL cassette to single loxP (L) site) versus *Rosa26* CMV-Cre) was sacrificed and assorted tissues were harvested. Soluble proteins were extracted by physical separation and sonication, followed by centrifugation to remove insoluble components. A3B expression was analyzed by immunoblotting and A3B activity was determined using a fluorescently labeled single-stranded DNA substrate. mRNA levels were obtained as described above, A3B primers remain unchanged, human TBP primers were replaced with mouse-specific TBP primers 5'-

GGGGAGCTGTGATGTGAAGT-3'(SEQ ID NO:14) and 5'-CCAGGAAATAATTCTGGCTCA-3' (SEQ ID NO:15) (as above; representative data in FIG. 6).

The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

Sequence Listing Free Text

SEQ ID NO:2

MNPQIRNPME RMYRDTFYDN FENEPILYGR SYTWLCYEVK IKRGRSNLLW
DTGVFRGQVY FKPQYHAEMC FLSWFCGNQL PAYKCFQITW FVSWTPCPDC
VAKLAEFLSE HPNVTLTISA ARLYYYWERD YRRALCRLSQ AGARVKIMDY
EEFAYCWENF VYNEGQQFMP WYKFDENYAF LHRTLKEILR YLMDPDTFTF
NFNNDPLVLR RRQTYLCYEV ERLDNGTWVL MDQHMGFLCN EAKNLLCGFY
GRHAELRFLD LVPSLQLDPA QIYRVTWFIS WSPCFSWGCA GEVRAFLQEN
THVRLRIFAA RIYDYDPLYK EALQMLRDAG AQVSIMTYDE FEYCWDTFVY
RQGCPFQPWD GLEEHSQALS GRLRAILQNQ GN

What is claimed is:

1. A vector for expressing human APOBEC3B comprising:

a first region having homology to a mouse gene;

a *lox*-STOP-*lox* (LSL) cassette, wherein the LSL cassette comprises two *loxP* sites, a first promoter, a drug resistance gene, and a first poly-A region; and

a human *APOBEC3B* gene.

2. The vector of claim 1, wherein the first region having homology to a mouse gene comprises a constitutive low-mid-copy promoter.

3. The vector of either of claims 1 or 2, wherein the mouse gene comprises at least a portion of *Rosa26*.

4. The vector of any of claims 1-3, wherein the first region having homology a mouse gene comprises a *Rosa26* promoter.

5. The vector of any of claims 1-4, further comprising a second region having homology to the mouse gene.

6. The vector of claim 5, wherein the first region having homology to a mouse gene and the second region having homology to a mouse gene flank the LSL cassette and the human *APOBEC3B* gene.

7. The vector of claim 6, wherein the first region having homology to a mouse gene comprises a portion of *Rosa26* and the second region having homology to a mouse gene comprises a portion of *Rosa26*.

8. The vector of any claims 1-7, wherein the first poly-A region comprises a *PGK* poly-A region or an SV40 poly-A region.

9. The vector of claim 8, wherein the first poly-A region comprises a *PGK* poly-A region and an SV40 poly-A region.

10. The vector of any of claims 1-9, wherein the first poly-A region comprises a poly-A region from more than one gene.

11. The vector of any of claims 1-10, wherein the drug resistance gene encodes a protein that provides resistance to a drug comprising G418, gentamycin, neomycin, puromycin, blasticidin, histidiol, hygromycin, or zeocin.

12. The vector of any of claims 1-11, wherein the first promoter comprises a *PGK* promoter.

13. The vector of any of claims 1-12, wherein the *APOBEC3B* gene comprises an intron.

14. The vector of any of claims 1-13, wherein the *APOBEC3B* gene comprises a sequence having at least 90% homology to SEQ ID NO:1.

15. The vector of claim 14, wherein the *APOBEC3B* gene comprises SEQ ID NO:1.

16. The vector of any of claim 14, wherein the *APOBEC3B* gene comprises a naturally occurring minor allele in the human population.

17. The vector of any of claim 14, wherein the *APOBEC3B* gene comprises a naturally occurring major allele in the human population.

18. The vector of any of claims 1-17, wherein the *APOBEC3B* gene is flanked on the 3' side by a second poly-A region.

19. The vector of claim 18, wherein the second poly-A region comprises a bovine growth hormone polyadenylation signal (BGHpA).

20. A vector for expressing human APOBEC3B comprising:

a *Rosa26* promoter,
a human *APOBEC3B* gene, and
a polyadenylation region.

21. The vector of claim 20, wherein the polyadenylation region comprises a bovine growth hormone polyadenylation signal (BGHpA).

22. The vector of either of claims 20 or 21, wherein the *APOBEC3B* gene is an intron-containing *APOBEC3B* gene.

23. The vector of any of claims 20-22, wherein the *APOBEC3B* gene comprises a sequence having at least 90% homology to SEQ ID NO:1.

24. The vector of claim 23, wherein the *APOBEC3B* gene comprises SEQ ID NO:1.

25. The vector of any of claim 23, wherein the *APOBEC3B* gene comprises a naturally occurring minor allele in the human population.

26. The vector of any of claim 23, wherein the *APOBEC3B* gene comprises a naturally occurring major allele in the human population.

27. The vector of any of claims 20-26, wherein the *Rosa26* promoter, the *APOBEC3B* gene, and the polyadenylation region are operably linked to each other.

28. The vector of any of claims 20-27, further comprising a site-directed recombination cassette.

29. The vector of claim 28, wherein the site-directed recombination cassette is a *lox*-STOP-*lox* (LSL) cassette.

30. The vector of claim 28, wherein the site-directed recombination cassette is a *FRT*-STOP-*FRT* cassette

31. The vector of any of claims 28-30, wherein the site-directed recombination cassette comprises a *PGK* promoter and a poly-A region.

32. The vector of any of claims 28-31, wherein the site-directed recombination cassette further comprises a drug resistance gene.

33. The vector of claim 32, wherein the drug resistance gene encodes a protein that provides resistance to a drug comprising G418, gentamycin, neomycin, puromycin, blasticidin, histidiol, hygromycin, or zeocin.

34. A transgenic mouse, or a cell or tissue isolated therefrom, which is transformed with the vector of any of claims 1-33.

35. A method for generating a transgenic mouse comprising the steps of:
transfecting an embryonic stem cell with the vector of any of claims 1-34;
injecting the embryonic stem cell into a blastocyst; and
allowing the blastocyst to grow into a transgenic mouse.

36. The method of claim 35, further comprising breeding the transgenic mouse with a mouse comprising one or more tissues expressing Cre or Flp.

37. A transgenic mouse comprising a *Rosa26* promoter and an intron-containing human *APOBEC3B* gene that are operably linked to each other.

38. A transgenic mouse comprising a *Rosa26* promoter, an intron-containing human *APOBEC3B* gene, and a *lox*-STOP-*lox* cassette, wherein the *Rosa26* promoter and the *lox*-STOP-*lox* cassette are operably linked to each other.

FIG. 1

acgcgtaccATGAATCCACAGATCAGAAATCCGATGGAGCGGATGTATCGA
GACACATTCTACGACAACTTTGAAAACGAACCCATCCTCTATGGTCGG
AGCTACACTTGGCTGTGCTATGAAGTGAAAATAAAGAGGGGCCGCTCA
AATCTCCTTTGGGACACAGGGGTCTTTCGAGGCCAGGTGTATTTCAAG
CCTCAGTACCACGCAGAAATGTGCTTCCTCTCTTGGTTCTGTGGCAACC
AGCTGCCTGCTTACAAGTGTTTCCAGATCACCTGGTTTGTATCCTGGAC
CCCCTGCCCGGACTGTGTGGCGAAGCTGGCCGAATTCCTGTCTGAGCA
CCCCAATGTCACCCTGACCATCTCTGCCGCCCGCCTCTACTACTACTGG
GAAAGAGATTACCGAAGGGCGCTCTGCAGGCTGAGTCAGGCAGGAGC
CCGCGTGACGATCATGGACTATGAAGAATTTGCATACTGCTGGGAAAA
CTTTGTGTACAATGAAGGTCAGCAATTCATGCCTTGGTACAAATTCGA
TGAAAATTATGCATTCCTGCACCGCACGCTAAAGGAGATTCTCAGATA
CCTGATGGATCCAGACACATTCACTTTCAACTTTAATAATGACCCTTTG
GTCCTTCGACGGCGCCAGACCTACTTGTGCTATGAGGTGGAGCGCCTG
GACAATGGCACCTGGGTCCTGATGGACCAGCACATGGGCTTTCTATGC
AACGAGgtgagtccaggagatgtttcagcactgttgcctttagtctcgaggcaacttagacaactgagtattgatc
tgagcacagcagggtgtgagctgtttgaagatactggggttgggggtgaagaaactgcagaggactaactgggctg
agacccagtggcaatgttttagggcctaaggaatgcctctgaaaatctagatggacaactttgactttgagaaaagaga
ggtggaaatgaggaaaatgacttttctttattagatttcggtagaaagaactttcatctttcccctatttttgttattcgttttaa
aacatctatctggaggcaggacaagtatggtcattaaaaagatgcaggcagaaggcatatattggctcagtcaaagtg
gggaactttggtggccaaacatacattgctaaggctattcctatatcagctggacacatataaaatgctgctaatgcttca
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agagcgtgtgtttcttttaacgttttcagcctacagcatacagggttcatggtggcaagaagataacaagatttaaattat
ggccagtgactagtgctgcaagaagaacaactacctgcatttaatgggaaagcaaaatctcaggctttgagggaagtt
aacataggcttgattctgggtggaagctgggtgtgtagttatctggaggccaggctggagctctcagctcactatgggt
tcatctttattgtctcctttcatctcaacagGCTAAGAATCTTCTCTGTGGCTTTTACGGCCG
CCATGCGGAGCTGCGCTTCTTGGACCTGGTTCCTTCTTTGCAGTTGGAC
CCGGCCCAGATCTACAGGGTCACTTGGTTCATCTCCTGGAGCCCCTGCT
TCTCCTGGGGCTGTGCCGGGGAAGTGCGTGCGTTCCTTCAGGAGAACA
CACACGTGAGACTGCGCATCTTCGCTGCCCGCATCTATGATTACGACC
CCCTATATAAGGAGGCGCTGCAAATGCTGCGGGATGCTGGGGCCCAA
GTCTCCATCATGACCTACGATGAGTTTGAGTACTGCTGGGACACCTTTG
TGTACCGCCAGGGATGTCCCTTCCAGCCCTGGGATGGACTAGAGGAGC
ACAGCCAAGCCCTGAGTGGGAGGCTGCGGGCCATTCTCCAGAATCAG
GGAAACTGA**acgcgt**

FIG. 2

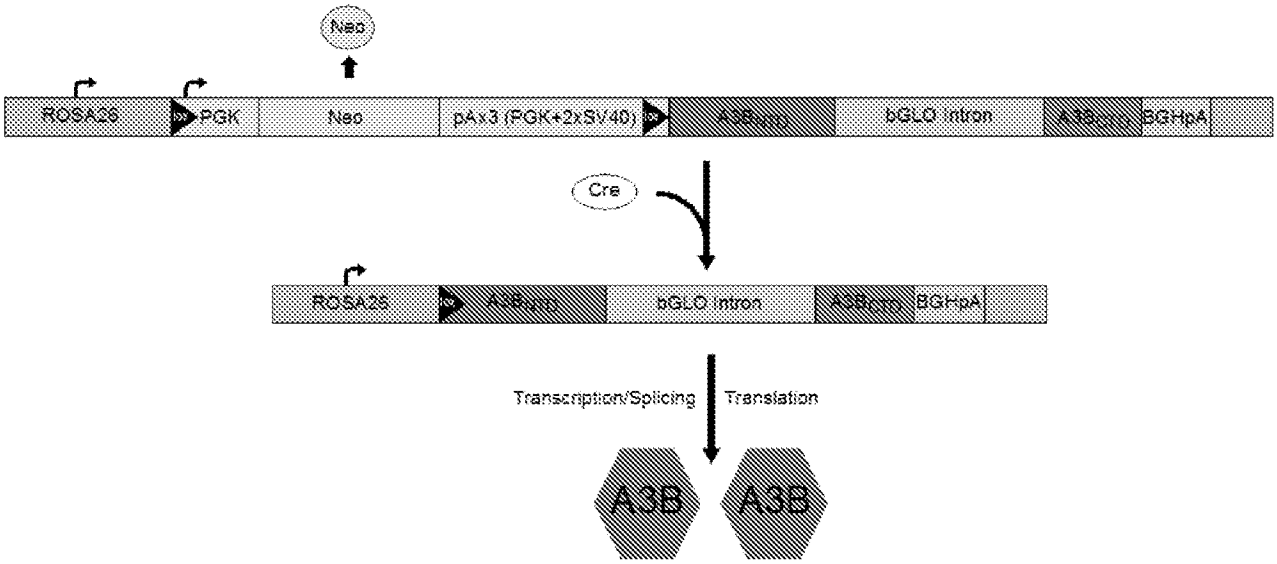

Neo
ROSA26
PGK
Neo
pAx3 (PGK+2xSV40)
bGLO Intron
BGHpA
Cre
ROSA26
bGLO Intron
BGHpA
Transcription/Splicing
Translation
A3B
A3B

FIG. 3

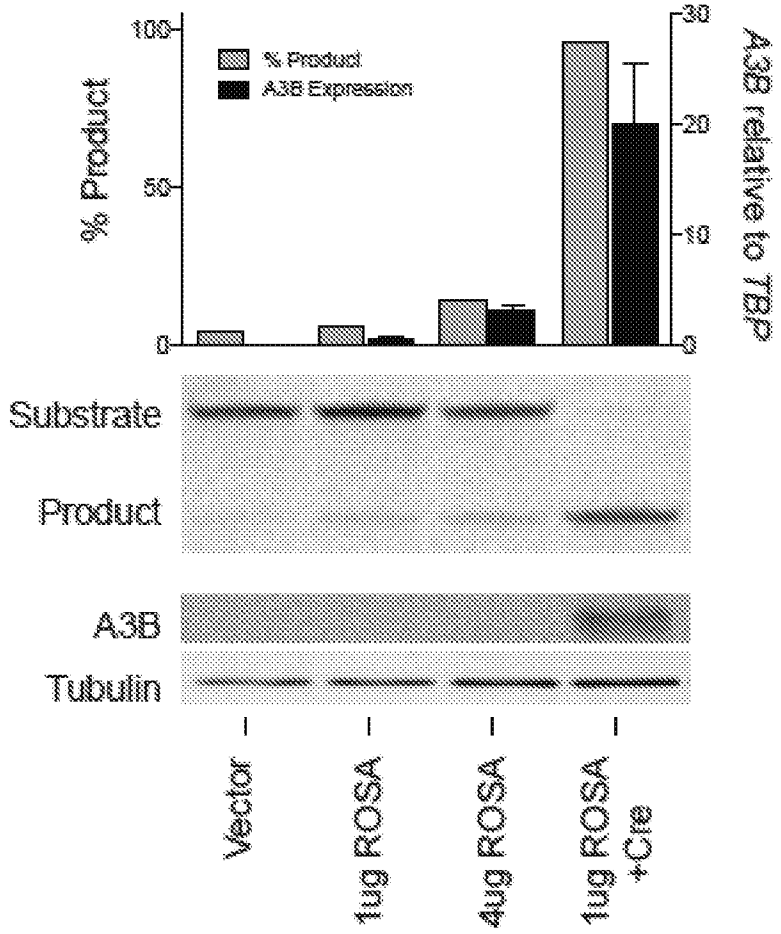

% Product
A3B Expression
% Product
A3B relative to TBP
100
50
0
30
20
10
0
Substrate
Product
A3B
Tubulin
Vector
1ug ROSA
4ug ROSA
1ug ROSA +Cre

FIG. 4

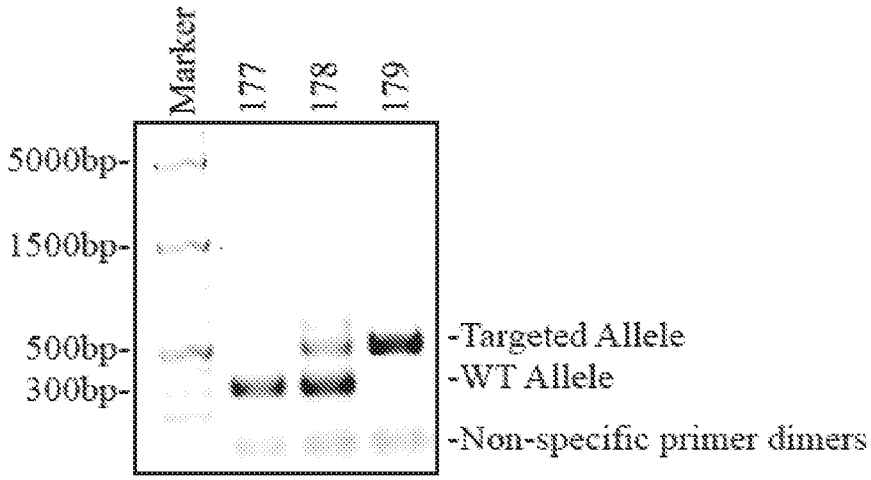

Marker
177
178
179
5000bp-
1500bp-
500bp-
300bp-
-Targeted Allele
-WT Allele
-Non-specific primer dimers

FIG. 5

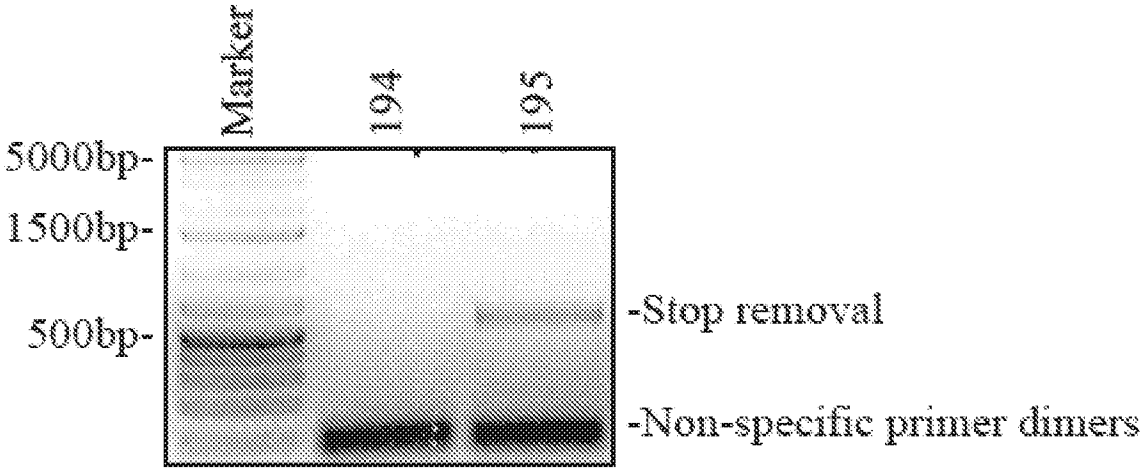
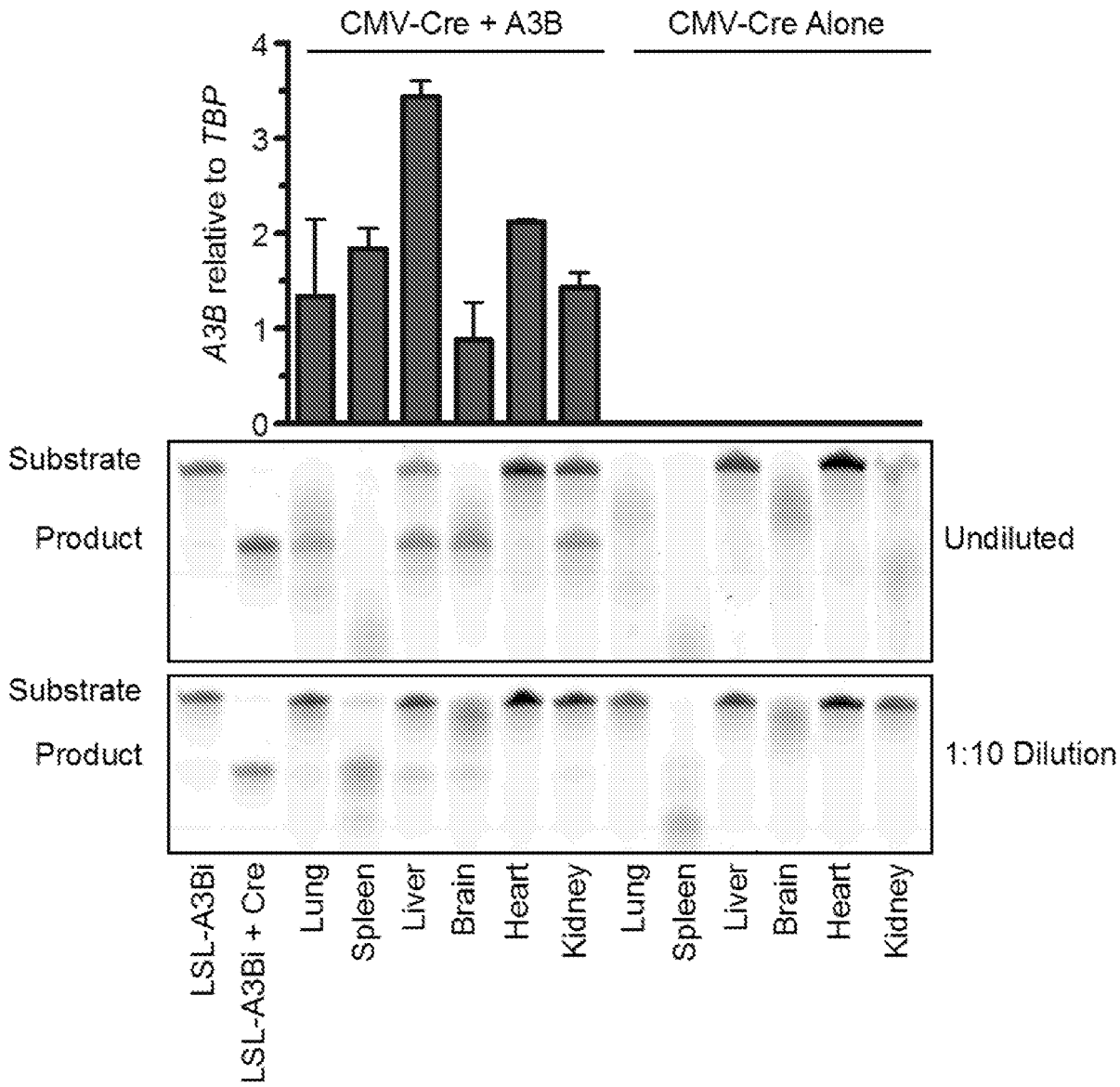

Marker
194
195
5000bp-
1500bp-
500bp-
-Stop removal
-Non-specific primer dimers

FIG. 6


CMV-Cre + A3B
CMV-Cre Alone
A3B relative to TBP
0
1
2
3
4
Substrate
Product
Undiluted
Substrate
Product
1:10 Dilution
LSL-A3Bi
LSL-A3Bi + Cre
Lung
Spleen
Liver
Brain
Heart
Kidney
Lung
Spleen
Liver
Brain
Heart
Kidney

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/40077

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/10, 15/85, 7/01, 15/00, 15/63, 15/79 (2016.01)
CPC - C12N 15/85, 15/63
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) Classifications: C12N 5/10, 15/85, 7/01, 15/00, 15/63, 15/79 (2016.01)
CPC Classifications: C12N 15/85, 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data), Google/Google Scholar, EBSCO, PubMed: APOBEC3B, A3B, ARP4, ARCD3, PHRBNL, APOBEC1, bK150C2.2, DJ742C19.2, express, vector, plasmid, virus, phage, contain, cassette, human, rosa, poly-A, lox-stop-lox, flox, lox, cre, lsl

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	(TALABOT-AYER, D et al.) Severe Neutrophil-Dominated Inflammation and Enhanced Myelopoiesis in IL-33-Overexpressing CMV/IL33 Mice. Journal of Immunology. 10 December 2014, Vol. 194, No. 2; pages 750-760; page 750, column 2, paragraph 3; page 751, column 1, paragraph 3; DOI: 10.4049/jimmunol.1402057	1-2, 3/1-2, 20-21, 22/20-21, 37-38
Y	(LACKEY, L et al.) APOBEC3B and AID Have Similar Nuclear Import Mechanisms. Journal of Molecular Biology. 22 June 2012, Vol. 419, No. 5; pages 301-314; page 6, paragraph 3, lines 18-21; DOI: 10.1016/j.jmb.2012.03.011	1-2, 3/1-2, 20-21, 22/20-21, 37-38
A	WO 2014/130364 A1 (THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK, et al.) 28 August 2014; entire document	1-2, 3/1-2, 20-21, 22/20-21, 37-38
A	WO 2015/089465 A1 (THE BROAD INSTITUTE INC., et al.) 18 June 2015; entire document	1-2, 3/1-2, 20-21, 22/20-21, 37-38
A	(LACKEY, L et al.) Subcellular Localization of the APOBEC3 Proteins During Mitosis and Implications for Genomic DNA Deamination. Cell Cycle. 1 March 2013, Vol. 12, No. 5; pages 762-772; DOI: 10.4161/cc.23713	1-2, 3/1-2, 20-21, 22/20-21, 37-38
P, Y	(CAVAL, V et al.) Molecular Basis of the Attenuated Phenotype of Human APOBEC3B DNA Mutator Enzyme. Nucleic Acid Research. 30 October 2015, Vol. 43, No. 19; pages 9340-9349; DOI: 10.1093/nar/gkv935	1-2, 3/1-2, 20-21, 22/20-21, 37-38

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2016 (08.09.2016)	11 OCT 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/40077

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-19, 23-36
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.