Generation of a Conditional Null Allele of NADPH Oxidase Activator 1 (*Noxa1*)

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Abstract

NADPH oxidase complexes are multiprotein assemblies that generate reactive oxygen species in a variety of mammalian tissues. The canonical phagocytic oxidase consists of a heterodimeric, enzymatic core comprised of the transmembrane proteins, CYBB and CYBA and is regulated, in part, by an "organizing" function of NCF1 and an "activating" activity of NCF2. In contexts outside of the phagocyte, these regulatory functions may be encoded not only by *NCF1* and *NCF2*, but alternatively by their respective paralogues, *NOXO1* and *NOXA1*. To allow tissue-specific dissection of *Noxa1* function in mouse, we have generated an allele of *Noxa1* suitable for conditional inactivation. Moreover, by crossing *Noxa1* conditional allele carriers to B6.129S4-*Meox2^{tm1(Cre)Sor}*/J mice, we have generated first, *Noxa1*-null heterozygotes, and ultimately, *Noxa1*-null homozygotes. Through the thoughtful use of tissue-specific, *Cre*-expressing mouse strains, the *Noxa1* conditional allele will offer insight into the roles of NOXA1 in the variety of tissues in which it is expressed.

Keywords

Cre-loxP; gene targeting; conditional inactivation; FLP-FRT; NADPH oxidase; *Noxa1*

NADPH oxidase (NOX) complexes are multiprotein assemblies with the enzymatic function of transferring electrons across biological membranes to oxygen (O_2) thereby creating superoxide ($O_2^{\bullet-}$) and a cascade of subsequent reactive oxygen species (ROS). Due to their reactivity, ROS interact with a variety of small inorganic molecules and biological macromolecules and can cause irreversible oxidative damage to these targets specifically, as well as to cells and organisms more generally. Nonetheless, systems have evolved to harness this reactivity into beneficial biological processes including host defense and a myriad of other reversible regulatory processes modulating such events as protein processing, cellular signaling, gene expression and cell differentiation. As a result of these opposing phenomena, NOX family NADPH oxidases are involved in a variety of physiological and pathophysiological processes (Bedard and Krause, 2007).

NADPH oxidase was initially discovered in phagocytic cells (phagocytic oxidase or phox) where it plays a vital role in host defense against bacterial and fungal pathogens. In fact, functional deficits within various components of the phagocytic oxidase complex underlie a genetically heterogeneous family of primary immune deficiencies known as the chronic

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granulomatous diseases (CGDs, OMIM #s 233690, 233700, 233710 and 306400) (Holland, 2010; Matute *et al.*, 2009; Segal *et al.*, 2009).

The phagocytic oxidase (Figure 1a) has been extensively studied and is now known to consist of a heterodimeric flavocytochrome at its enzymatic core. In turn, the heterodimer is comprised of CYBB, an NADPH-, FAD- and iron- (heme-) binding, six-span transmembrane protein; and, CYBA, a two-span transmembrane protein (for other naming conventions see Methods). Activation of the heterodimer, that is normally quiescent, requires the recruitment of at least two cytosolic factors including an "organizing" function of NCF1; and an "activating" activity of NCF2.

Expansion of the NOX family of NADPH oxidases occurred with the identification of a number of *CYBB* paralogues in the mammalian genome that are expressed in a wide variety of cells and tissues (Figure 1b). In humans, the NOX family includes six additional members — *NOX1*, *NOX3*, *NOX4*, *NOX5* and the dual oxidase (NADPH oxidase and peroxidase) members, *DUOX1* and *DUOX2*. *Nox5* is absent in rodents (Nauseef, 2008). With regard to the organizing cytosolic factor NCF1, here too a paralogue has been identified, known as NOXO1 (Banfi *et al.*, 2003).

Likewise, an activator subunit paralogous to NCF2 has been identified and is known as NOXA1 (Banfi *et al.*, 2003). NOXA1 is expressed in a variety of cell lines including guinea pig gastric mucosal cells, basilar artery epithelial cells, airway-like normal human bronchial epithelial cells and vascular smooth muscle cells (Ago *et al.*, 2005; Ambasta *et al.*, 2006; Bedard and Krause, 2007; Kawahara *et al.*, 2005; Lavigne and Eppihimer, 2005). It is expressed in a number of organs including the spleen, stomach, colon, small intestine, lung, and uterus as well as the prostate, thyroid, and salivary glands (Banfi *et al.*, 2004; Bedard and Krause, 2007; Geiszt *et al.*, 2003). Interestingly, NOXA1 is also expressed in the inner ear (Banfi *et al.*, 2004) where it may play a role in an emerging complex consisting of NOX3, CYBA and NOXO1 (Banfi *et al.*, 2004; Bergstrom *et al.*, 2004; Kiss *et al.*, 2006; Nakano *et al.*, 2008; Paffenholz *et al.*, 2004). In human, the *NOXA1* transcription unit spans 11,010 base pairs (bp) from nucleotides 140,317,847 to 140,328,857 on human Chromosome 9 (9q34.3, February 2009, GRCh37/hg19 genome assembly) in a region of conserved linkage synteny with the proximal end of mouse Chromosome 2. The domain structures of human and mouse NCF2 and NOXA1 proteins are similar (Figure 2) with the N-terminal halves of each protein containing multiple tetratricopeptide (TPR) repeats, the central region containing conserved activation domains (AD) and the C-terminal end of each protein containing both phox and Bem1 (PB1) and Src homology 3 (SH3) domains. In NCF2 additional SH3 domains are present centrally, adjacent to the AD.

Although a number of studies have been conducted *in vitro* to assess the interaction of NOXA1 with other factors, its wide expression profile, ability to interact with multiple NOX partners and functional similarity to NCF2 make it difficult to assess the physiological and phenotypic significance of specific interactions in whole animals.

Accordingly, in order to allow tissue-specific dissection of the phenotypes caused by *Noxa1* deficiency, we used the *Cre-loxP* based conditional mutagenesis approach (Furuta and Behringer, 2005) to generate a *loxP*-flanked (floxed) allele of *Noxa1* suitable for conditional inactivation (Figure 3). The mouse *Noxa1* transcription unit spans 9,478 bp from nucleotides 24,941,190 to 24,950,668 on mouse Chromosome 2 and consists of 12 exons (NCBI 37 Mouse Genome Assembly, Figure 3a). To generate the *Noxa1^{neo-flox}* targeting vector, we used a recombineering approach (Figure 3b) (Copeland *et al.*, 2001; Liu *et al.*, 2003; Warming *et al.*, 2005). Initially, a retrieval vector was prepared using 300–500 bp arms generated by PCR from the 5' and 3' regions of the *Noxa1* gene. This retrieval vector was

then used to capture, from a C57BL/6J-derived BAC, a 10.6 kb fragment of the *Noxa1* gene extending from within exon 1 to beyond exon 12. A mini-targeting vector, constructed from two small homology arms in intron 2 and the floxed PGK/EM7 neomycin/kanamycin selection cassette from PL452, was introduced into the retrieved *Noxa1* segment by recombination. Following transfer of the construct into SW105 and the induction of Cre recombinase with arabinose, the selection cassette was excised leaving only a single *loxP* site remaining in intron 2. Likewise, a second mini-targeting vector, constructed from two small homology arms in intron 6 and the FRT-flanked PGK/EM7 neomycin/kanamycin selection cassette and *loxP* site of PL451, was also introduced into the retrieved *Noxa1* segment by recombination. The design of the completed targeting construct (Figure 3c) permits removal of the PGK/EM7 neomycin/kanamycin selection cassette from the *Noxa1^{neo-flox}* allele (Figure 3d) by mating to a FLPe deleter mouse line (generating the *Noxa1^{flox}* allele, Figure 3e) or removal of both *Noxa1* genomic sequence (including exons 3 through 6) and the selection cassette by mating to a *Cre* deleter line (generating the *Noxa1^{del1}* allele, Figure 3f). This excision event results in the deletion of amino acids 88 to 220 encoding multiple TPR domains and the AD, and an ensuing frameshift in amino acids 221 to 444 causing functional loss of the PB1 and Src homology-3 domains of the NOXA1 protein.

The *Noxa1^{neo-flox}* targeting vector was electroporated into B6(Cg)-*Tyr^{c-2J}*/J-PRX-B6-albino #1 ES cells (The Jackson Laboratory Item 000058C02) and 384 colonies were picked for analysis. Initial screening was performed by Southern analysis using a 5' probe on *Eco*RI-digested DNA (Figure 4a). For the wild type-allele of *Noxa1*, this probe identifies a 14.3 kb fragment (Figure 3a, green line). In targeted clones in which recombination occurred as desired within the outer half of the 5' arm, the retention of the intron two *loxP* site and the presence of an *Eco*RI site within this site result in the generation a novel 4.4 kb band (Figure 3d, green line). Among the 307 scored clones, four such clones were identified. In targeted clones in which recombination occurred inopportunely within the inner half of the 5' arm, the loss of the distal *loxP* site in intron 2 (Figure 3d, red circle) and the retention of the novel *Eco*RI site within the selection cassette result in the generation a novel 8.3 kb band (Figure 3d, red line). Among 307 scored clones, twenty such clones were identified. Of the four properly prepared clones, one was confirmed to be properly targeted using a 3' probe (Figure 3d, blue line, data not shown) and injected into C57BL/6J host blastocysts. Resulting albino/black male chimeras were mated to B6(Cg)-*Tyr^{c-2J}*/J females. ES-cell derived pups were identified by their albino coat color. Germline transmission of the *Noxa1^{neo-flox}* allele was confirmed by PCR with primers flanking the distal (intron 2) *loxP* site (See Methods). All subsequent crosses were *inter se* or to C57BL/6J mice not harboring the *Tyr^{c-2J}* (albino) allele of the original ES cells. Male and female *Noxa1^{neo-flox}*/+ heterozygotes (Figure 4b, lane 2) were bred to each other and *Noxa1^{neo-flox}*/*Noxa1^{neo-flox}* homozygotes were identified by PCR amplification across the region of the distal *loxP* site, scoring for the absence of the wild type-band (data not shown).

Mice both heterozygous and homozygous for the *Noxa1^{neo-flox}* allele were viable, fertile and did not exhibit any obvious, overt phenotypic abnormalities; indicating that neither the *loxP* site in intron 2, nor the FRT-PGK/EM7 *Neo*-FRT-*loxP* site in intron 6 has any obvious, adverse effects on any critical *Noxa1* function.

The *Noxa1^{flox}* allele was generated by mating *Noxa1^{neo-flox}* mice to the B6.Cg-Tg(ACTFLPe)9205Dym/J deleter strain, which expresses FLPe recombinase in the male and female germlines (Rodriguez *et al.*, 2000). Excision of the PGK/EM7 *Neo* cassette was demonstrated by PCR spanning the FRT-*loxP* site, which results in an 85 bp increase in the size of the *Noxa1^{flox}* product (Figure 4b, lane 3) when compared to the wild type-allele

(Figure 4b, lane 4) and a 101 bp decrease when compared to the *Noxa1*$^{neo-flox}$ allele (Figure 4b, lane 2).

Mice heterozygous for the *Noxa1*^{del1} allele were produced by mating *Noxa1*$^{neo-flox}$ mice to B6.129S4-*Meox2*$^{tm1(Cre)Sor}$/J mice, which express Cre recombinase widely in the early embryo (Tallquist and Soriano, 2000). Excision was shown by PCR spanning the entire deleted region of the allele, which results in a novel 259 bp product (Figure 4b, lane 7). *Noxa1*^{del1}/+ heterozygotes show no obvious phenotypic abnormalities.

Mice homozygous for the *Noxa1*^{del1} allele were created by intercrossing *Noxa1*^{del1} heterozygotes. Homozygosity of the *Noxa1*^{del1} allele was demonstrated by PCR, scoring for the absence of the *Noxa1*$^{neo-flox}$ allele (Figure 4b, lane 6). RT-PCR analysis of temporal bone/inner ear RNA confirms an absence of *Noxa1*-derived sequences from within the *Cre*-mediated deletion; and moreover, when compared to +/+ samples, weak amplification from exons 1 and 2 upstream of the deletion suggests that the deleted, frameshifted *Noxa1*^{del1} transcript is subject to nonsense-mediated decay (Figure 4c). *Noxa1*^{del1}/*Noxa1*^{del1} homozygotes were viable, showed no overt phenotype and were produced in the expected (1:2:1) Mendelian ratio. Possible more subtle phenotypes of *Noxa1*^{del1} homozygotes or tissue-specific *Noxa1* ablation studies are beyond the scope of this manuscript. Mice bearing the *Noxa1*$^{neo-flox}$ allele are available for distribution by contacting the corresponding author or from The Jackson Laboratory's Genetic Resource Science Repository (http://research.jax.org/repository/index.html).

Through the thoughtful use of specific recombinase-expressing mouse strains, the conditional allele of *Noxa1* will provide greater insight into the roles of NOXA1 in the variety of cells and organs where it is expressed.

METHODS

Mice

All mice were obtained from The Jackson Laboratory including strains C57BL/6J (Stock 000664), B6(Cg)-*Tyr*$^{c-2J}$/J (Stock 000058), B6.Cg-Tg(ACTFLPe)9205Dym/J (Stock 005703), B6.129S4-*Meox2*$^{tm1(Cre)Sor}$/J (Stock 003755) and CByB6F1/J (Stock 100009). All mice were housed on a bedding of white pine shavings and fed NIH-31 5K54 (4% fat) or NIH-31 5K52 (6% fat) diet and acidified water, *ad libitum*. All experiments were performed with the approval of The Jackson Laboratory Institutional Animal Care and Use Committee (IACUC) and in compliance with all applicable laws and guidelines.

Nomenclature

All mouse strains are referred to by official nomenclature. All mouse gene symbols (first letter upper case, italicized) and human gene symbols (all letters upper case, italicized) and proteins (all letters upper case) are referred to by official nomenclature throughout the body of the text. Relationships to other naming conventions and full names are shown here: Cytochrome b-245, beta polypeptide=CYBB=NOX2=gp91^{phox}; Cytochrome b-245, alpha polypeptide=CYBA=p22^{phox}; Neutrophil cytosolic factor 1=NCF1=p47^{phox}; and Neutrophil cytosolic factor 2=NCF2=p67^{phox}.

The unaltered *Noxa1* conditional allele containing the FRT-flanked neomycin/kanamycin resistance cassette and *loxP*-flanked *Noxa1* exons is named *Noxa1*^{tm1Brg} by official nomenclature, but is referred to throughout this publication as *Noxa1*$^{neo-flox}$. The FLP-altered allele having lost the neomycin/kanamycin resistance cassette is named *Noxa1*$^{tm1.1Brg}$ by official nomenclature, but is referred to throughout this publication as *Noxa1*^{flox}. The *Cre*-altered allele having lost the neomycin/kanamycin resistance cassette

and *Noxa1* exons 3 through 6 is named *Noxa1*$^{tm1.2Brg}$ by official nomenclature, but is referred to throughout this publication as *Noxa1*^{del1}.

Generation of the *Noxa1* conditional gene-targeting vector

The *Noxa1* conditional gene-targeting vector was constructed from BAC clone RP23-261J20 (Strain: C57BL/6J) using a recombineering approach (Copeland *et al.*, 2001; Liu *et al.*, 2003; Warming *et al.*, 2005). Genomic DNA from the 5' and 3' ends of the *Noxa1* gene was amplified from the BAC with oligonucleotide pairs A & B and Y & Z, digested with *Not*I and *Hin*DIII (A & B) or *Hin*DIII and *Spe*I (Y & Z) and cloned together into the *Not*I and *Spe*I digested targeting vector, PL253.

This plasmid was linearized with *Hin*DIII and used to retrieve a portion of the *Noxa1* gene (from mouse Chromosome 2, position 24,939,966 to 24,950,622; UCSC Genome Browser, July 2007 assembly) contained on the same BAC.

To insert a single *loxP* site into intron 2, a mini-targeting vector was constructed using arms that were amplified from the BAC using oligonucleotide pairs C & D and E & F, digested with *Not*I and *Eco*RI (C & D) or *Bam*HI and *Sal*I (E & F) and cloned along with the *Eco*RI/*Bam*HI fragment of PL452 into pBluescript II KS(+). After introducing the mini-targeting vector into the retrieval product by recombination, the neomycin/kanamycin selection cassette and one of the *loxP* sites were removed (leaving a single *loxP* site remaining) by electrotransforming the construct into the recombineering strain, SW105, and inducing Cre recombinase expression with arabinose (Warming *et al.*, 2005).

An FRT-flanked neomycin/kanamycin selection cassette and an additional *loxP* site were then introduced into intron 6 by recombination using a similar approach. In this instance a second mini-targeting vector was constructed using arms that were amplified from the BAC using oligonucleotide pairs G & H and I & J, digested with *Not*I and *Eco*RI (G & H) or *Bam*HI and *Sal*I (I & J) and cloned along with the *Eco*RI/*Bam*HI fragment of PL451 into pBluescript II KS(+). This second mini-targeting vector was then introduced into the modified retrieval product by recombination to complete the conditional *Noxa1* gene-targeting vector.

As constructed, the final vector is designed to delete the selection cassette upon interaction with FLP recombinase and exons 3 through 6 upon interaction with Cre recombinase. The *Cre*-induced deletion causes the ablation of amino acids 88 to 220 from any hypothetical, altered NOXA1 protein and, due to a frame shift, would direct the incorporation of 19 novel amino acids prior to a premature termination codon.

Generation of mice carrying the *Noxa1* conditional allele

The *Noxa1* conditional gene-targeting vector was linearized with *Sal*I, electroporated into B6(Cg)-*Tyr*$^{c-2J}$/J-PRX-B6-albino #1 ES cells (The Jackson Laboratory Item 000058C02) and cultured under G418 and FIAU selection.

Clones were assayed for proper targeting by Southern analysis. Oligonucleotides 5'F & 5'R and 3'F & 3'R were used to amplify unique probe sequences from mouse genomic DNA. Next, the genomic DNAs of 384 ES clones were digested with *Eco*RI, blotted and analyzed with the 5' probe. Of 307 scored clones, four clones contained a hybridizing 4.4 kb band expected for properly targeted clones that had retained the 5' *loxP* site. An additional 20 clones contained a hybridizing 8.3 kb band expected for targeted clones that had lost the 5' *loxP* site. All clones contained a hybridizing 14.3 kb band arising from the wild type-allele.

All four clones that retained the 5' *loxP* site were injected into C57BL/6J blastocysts and transferred into the oviducts (on day 0.5 post coitus) or the uterine horns (on day 2.5 *post coitum*) of CByB6F1/J pseudopregnant females. The resulting male black and albino chimeras were mated to B6(Cg)-*Tyr^{c-2J}* females. Germline transmission was observed for one clone and the resulting offspring were identified by their albino coat color. Transmission of the targeted allele, and retention of the *loxP* in intron 2, was confirmed by *Noxa1* conditional allele-specific PCR with oligonucleotides, *Noxa1-lox*PF and *Noxa1-lox*PR, which flank the *loxP* site in intron 2.

Genotyping

The presence or absence of the *Meox2-Cre* knockin, *Meox2^{tm1(Cre)Sor}* (Tallquist and Soriano, 2000), was ascertained using the three primers oIMR1542, oIMR1871, and oIMR3671 as recommended by the distributor. Using this assay, the knockin allele gives rise to an approximately 300 bp product. The wild type-allele gives rise to an approximately 410 bp product. All products were resolved on 2% SeaKem LE agarose gels (Lonza).

The presence or absence of the actin-FLPe transgene, TgN(ACTFLPe)9205Dym (Rodriguez *et al.*, 2000), was ascertained using the four primers oIMR7338, oIMR7339, oIMR1348, and oIMR1349 as recommended by the distributor. Using this assay, an internal control amplicon derived from the interleukin 2 (*Il2*) gene gives rise to a 324 bp product. The transgene gives rise to a 725 bp product. All products were resolved on 1.5% SeaKem LE agarose gels (Lonza).

After exposure to FLP recombinase, mice carrying the *Noxa1^{neo-flox}*, *Noxa1^{flox}* or *Noxa1^+* (wild type-) alleles were distinguished with a genotyping assay containing the three primers *Noxa1*-465F, *Neo*-788R, and *Noxa1*-2481R (Figure 4b, lanes 1–4; Figure 5). Using this assay the *Noxa1^{neo-flox}* allele gives rise to a 344 bp product, the *Noxa1^{flox}* allele gives rise to a 243 bp product and the *Noxa1^+* allele gives rise to a 158 bp fragment. All products were resolved on 2% SeaKem LE agarose gels (Lonza).

After exposure to Cre recombinase, mice carrying the *Noxa1^{neo-flox}*, *Noxa1^{del1}* or *Noxa1^+* (wild type-) alleles were distinguished with a genotyping assay containing the three primers *Noxa1*-3769F, *Noxa1*-2481R, and *Noxa1*-3970R (Figure 4b, lanes 5–8; Figure 5). Using this assay the *Noxa1^{neo-flox}* allele gives rise to a 222 bp product, the *Noxa1^{del1}* allele gives rise to a 259 bp product and the *Noxa1^+* allele gives rise to a 132 bp fragment. All products were resolved on 2% SeaKem LE agarose gels (Lonza).

All PCR reactions were performed using the following standard cycling conditions: Preheating at 95°C for 3 min; 40 cycles of amplification at 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; and a final extension of 72°C for 9 min 30 sec.

RT-PCR

Total RNA was isolated from individual *Noxa1^{del1}*/*Noxa1^{del1}* and +/+ temporal bone explants (containing the inner ear) using an RNeasy Mini Kit (Qiagen) according to the manufacturer's directions. First strand synthesis reactions (8 µL RNA, 200–300 ng) were run in accordance with the SuperScript III (Invitrogen) random hexamer protocol. PCR reactions (30 µL) were performed on each cDNA and on no-reverse transcriptase controls to amplify two segments of the *Noxa1* transcript (*Noxa1* primers ex-1F and ex-2R, upstream; and primers ex-4F and ex-6R, within; the *Cre*-mediated deletion) and the *Ap3d1* transcript (cDNA quality control). Each reaction used 2 µL cDNA, 5 Prime Master Mix (5 Prime, final concentration 1X) and the appropriate primers (200 nM) (Table 1). Amplification was performed on a MJ Research PTC-200 Thermocycler (Bio-Rad) and involved a 2:30 minute denaturing step at 95°C followed by 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds,

and a 72°C extension step for 90 seconds. Products were resolved on a 2% SeaKem LE Agarose gel (Lonza).

Acknowledgments

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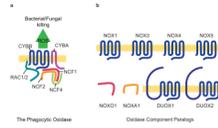
Figure 1. The phagocytic NADPH oxidase complex and its paralogues
a. Schematic representation of the canonical NADPH oxidase of phagocytes. **b.** Paralogues of phagocytic oxidase components include the CYBB paralogues NOX1, NOX3, NOX4, NOX5, and the dual oxidase proteins DUOX1 and DUOX2; the NCF1 paralogue, NOXO1; and the NCF2 paralogue, NOXA1.
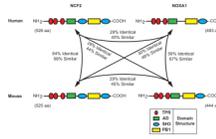
Figure 2. Domain structure and sequence conservation among human and mouse NCF2 and NOXA1 proteins
Schematic representations of the conserved domain structures of the human and mouse NCF2 and NOXA1 proteins are shown, as are the percentage of amino acid identity and similarity among the four proteins. Tetratricopeptide repeat (TPR) domains, red ellipses; activation domains (AD), green rectangles; Src homology 3 (SH3) domains, blue ellipses; phox and Bem1 (PB1) domains, yellow rectangles.
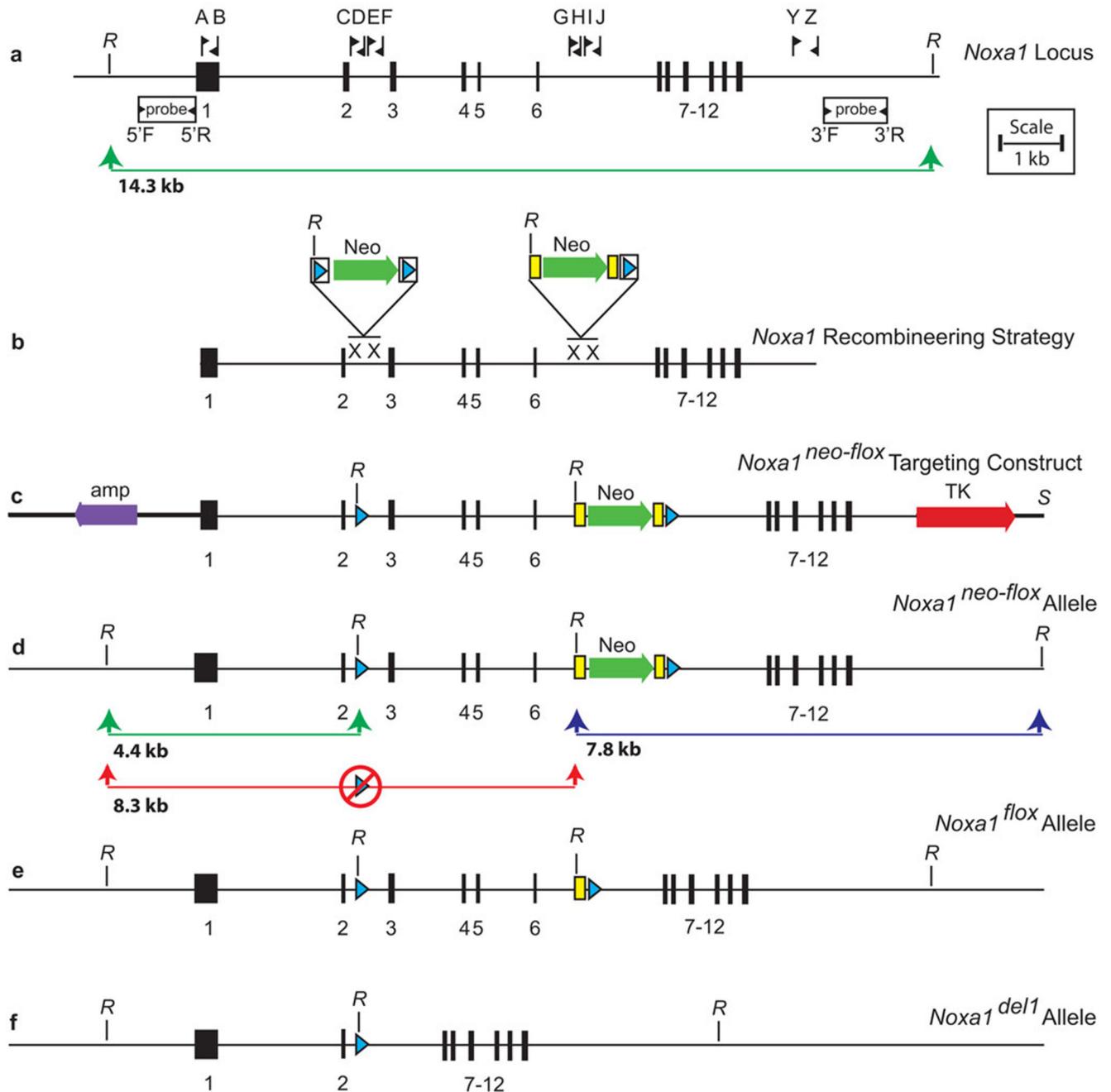
Figure 3. Construction of a conditional allele of *Noxa1* using a recombineering approach
Schematic representations of the endogenous *Noxa1* locus, the *Noxa1* conditional allele
construction strategy and construct, and the *Noxa1* conditional (and derivative) alleles are
shown. **a.** The endogenous *Noxa1* locus of mouse. **b.** Recombineering strategy used to
generate the *Noxa1* targeting construct. **c.** The *Noxa1* targeting construct. **d.** The properly
targeted *Noxa1*$^{neo\text{-}flox}$ allele detected by Southern analysis as hybridizing 4.4 kb (5' probe,
green line) and 7.8 kb (3' probe, blue line) fragments. Improperly targeted clones lacking the
intron 2 *loxP* site (red circle) were detected by a hybridizing 8.3 kb band (5' probe, red line)
e. The *Noxa1*^{flox} allele following FLPe-mediated removal of the neomycin resistance
cassette. **f.** The *Noxa1*^{del1} allele following *Cre*-mediated removal of exons 3 through 6. Fine

horizontal lines, genomic DNA; bold horizontal lines, vector DNA; numbered black rectangles, exons; lettered flags, oligonucleotide primers; white rectangles, 5' and 3' probes; green arrows, neomycin resistance cassettes (*Neo*); bold red arrow, negatively selectable thymidine kinase (TK) cassette; purple arrow, vector ampicillin resistance gene; blue triangles, *loxP* sites; yellow rectangles, FRT sites; X, sites of recombination; *R*, *Eco*RI.
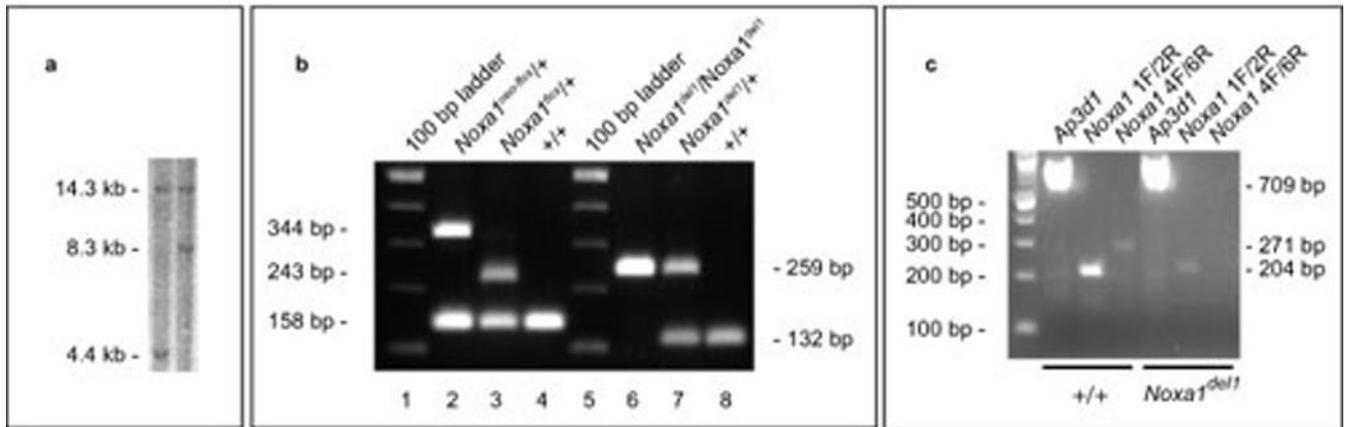
Figure 4. Southern, genotyping and RT-PCR assays to characterize the *Noxa1* conditional allele
a. Southern analysis of targeted ES cell colonies identifies clones showing retention (left lane) or loss (right lane) of the *loxP* site in intron 2. **b.** Genotyping assays. Lanes 1–4 and lanes 5–8 showing the amplification products of two genotyping assays used to distinguish among the *Noxa1*$^+$, *Noxa1*$^{neo\text{-}flox}$, *Noxa1*^{flox} and *Noxa1*^{del1} alleles (for details see Methods). **c.** RT-PCR analysis of *Noxa1* transcripts from +/+ and *Noxa1*^{del1}/*Noxa1*^{del1} temporal bone/ inner ear RNA. Amplification of cDNA sequences upstream of the *Cre*-mediated deletion (primers 1F and 2R) is reduced in *Noxa1*^{del1} homozygotes. Amplification of cDNA sequences within the *Cre*-mediated deletion (primers 4F and 6R) is absent in *Noxa1*^{del1} homozygotes. Amplification of cDNA sequences from *Ap3d1* serves as a control for the quality of the RNA.
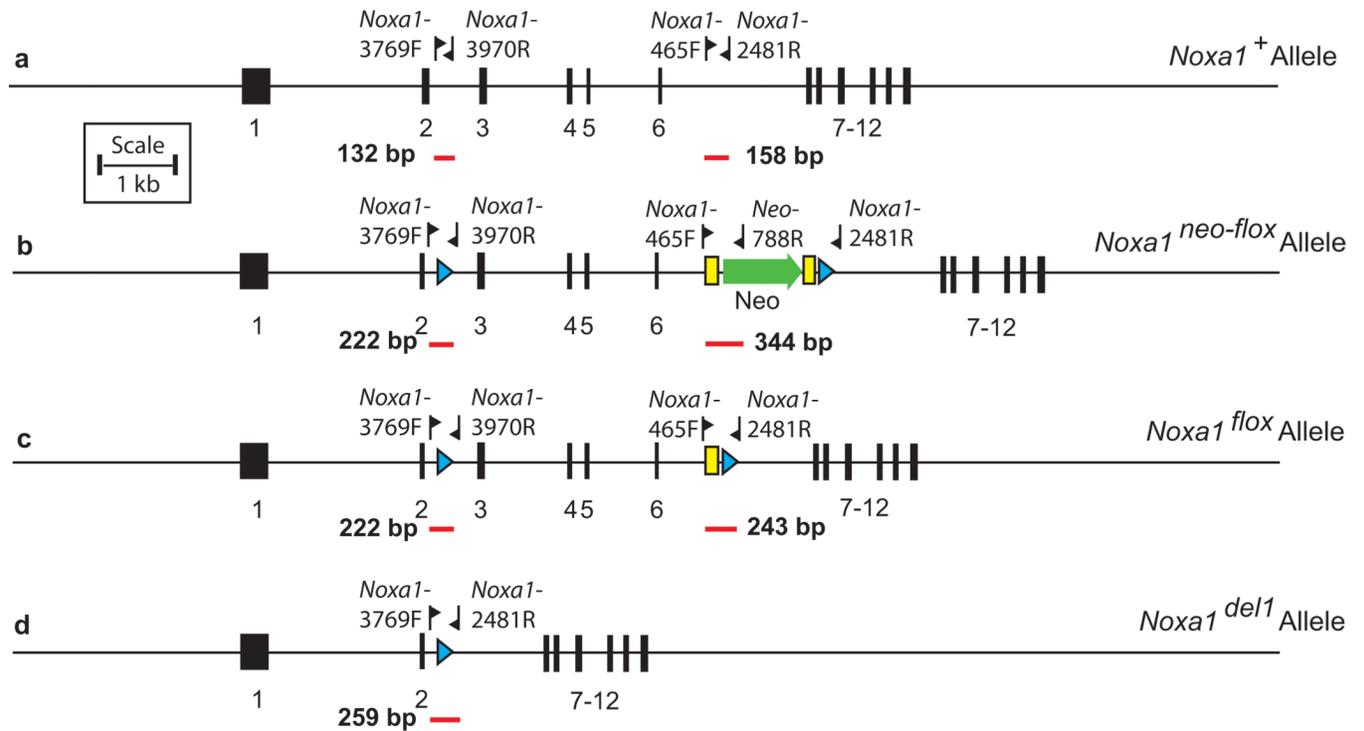
Figure 5. Genotyping strategies to distinguish among the *Noxa1$^+$*, *Noxa1$^{neo-flox}$*, *Noxa1^{flox}* and *Noxa1^{del1}* alleles

a. The *Noxa1$^+$* allele. **b.** The *Noxa1$^{neo-flox}$* allele. **c.** The *Noxa1^{flox}* allele. **d.** The *Noxa1^{del1}* allele. Fine horizontal lines, genomic DNA; numbered black rectangles, exons; labeled flags, oligonucleotide primers; green arrow, neomycin resistance cassette (*Neo*); blue triangles, *loxP* sites; yellow rectangles, FRT sites; labeled red lines, amplification products.

Table 1

Oligonucleotides

Oligonucleotides (Integrated DNA Technologies) used for construction of the *Noxa1* conditional gene targeting vector, amplification of Southern analysis probes, genotyping and RT-PCR are shown. Each oligonucleotide used in the vector construction consists of an arbitrary CGCAT sequence at the 5' end (italicized), any of various restriction enzyme recognition sites internally (bold) and sequences derived from within the *Noxa1* gene at the 3' end (plain text). The remaining oligonucleotides do not contain the CGCAT sequence or restriction enzyme recognition sites.

Used for construction of the *Noxa1* conditional gene-targeting vector

A, *CGCAT***GCGGCCGC**TCAAGGACCGACTGTCACAA

B, *CGCAT***AAGCTT**ATGCACACAGCCCCTGTTA

C, *CGCAT***GCGGCCGC**GTCTCTGCAAACCCATGCTT

D, *CGCAT***GAATTC**CTCCTCCCTTCCTCCTCTTG

E, *CGCAT***GGATCC**AGGGTTACAGGCCCCAGTG

F, *CGCAT***GTCGAC**AGGGGACCATGCCTATATCC

G, *CGCAT***GCGGCCGC**CAAGCCCTTGTGAAGAGGAG

H, *CGCAT***GAATTC**GCTGGCAGATGCAGGTTTA

I, *CGCAT***GGATCCC**TTCATAAGGGTCACACAGTGAA

J, *CGCAT***GTCGAC**CCTGCATCAGTGGAGAGTCA

Y, *CGCAT***AAGCTT**TCTTAGGGCAGTCACCTGCT

Z, *CGCAT***ACTAGT**GTGGGGTTTTCTTGGACAGA

Used for the generation of Southern probes

5' probe-F, TTAGCTGTCCTGCGTCACAC

5' probe-R, TTCGTCAGGCCTATCTGTCC

3' probe-F, ACGTGAGGTTCAGGAAATGG

3' probe-R, GCTTTCCTCTGCTCATGGTC

Used for genotyping

To confirm germline transmission of the *Noxa1* conditional allele, *Noxa1[neo-flox]*, and presence of the *loxP* site in intron 2

*Noxa1-lox*PF, ACCAGGGCTTATCCCAAGAT

*Noxa1-lox*PR, TCCCCAAACCCCTTCTAACT

To test for the presence of *Meox2[tm1(Cre)Sor]* knockin

oIMR1542, GGGACCACCTTCTTTTGGCTTC

oIMR1871, AAGATGTGGAGAGTTCGGGGTAG

oIMR3671, CCAGATCCTCCTCAGAAATCAGC

To test for the presence of the Tg(ACTFLPe)9205Dym transgene

oIMR7338, CTAGGCCACAGAATTGAAAGATCT

oIMR7339, GTAGGTGGAAATTCTAGCATCATCC

oIMR1348, CACTGATATTGTAAGTAGTTTGC

oIMR1349, CTAGTGCGAAGTAGTGATCAGG

To distinguish among the *Noxa1[neo-flox]*, *Noxa1[del1]* and *Noxa1^{+}* alleles after exposure to Cre recombinase

Noxa1-3769F, ACCAGGGCTTATCCCAAGAT

Noxa1-2481R, CCTCTACCCTCTGTGGGACA

Noxa1-3970R, ACTTTCCCCAGTCACCCTTC

To distinguish among the *Noxa1^neo-flox*, *Noxa1*^flox or *Noxa1^+* alleles after exposure to FLP recombinase

Noxa1-465F,CCCTAGGACTTAAACCTGCATC

Noxa1-2481R,CCTCTACCCTCTGTGGGACA

Neo-788R,GGGGAACTTCCTGACTAGGG

Used for RT-PCR

Ap3d1-ex1F, TCCGCAACCACAAGGAGGACGA

Ap3d1-ex8R, TCGATCAGCTTCTTGCCCAGCC

Noxa1 cDNA ex-1F, AGGGGATCAGATACGGGACT

Noxa1 cDNA ex-2R, GCCATGCAGGTGTCCTTAGT

Noxa1 cDNA ex-4F, TACAACATGGCATCAGCACA

Noxa1 cDNA ex-6R, CGTTGTGGTCATCAGGAATG