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Generation and Characterization of a Tamoxifen-Inducible *Eomes*^{CreER} Mouse Line

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Summary

Transgenic mouse lines expressing inducible forms of Cre-recombinase in a tissue-specific manner are powerful genetic tools for studying aspects of development and various processes in the adult. The T-box transcription factor *eomesodermin* (*Eomes*) plays critical roles for maintenance and differentiation of different pools of stem and progenitor cells from early embryonic stages to adulthood. These include trophoblast stem cells, epiblast cells during the generation of the primary germ layers, neurogenic intermediate progenitor cells in embryonic and adult cortical neurogenesis, and maturing natural killer and T cells. Here, we report on the generation and analysis of an *Eomes*^{CreER}-targeted allele by placing the tamoxifen-activatable *Cre-recombinase* (*CreER*) under the control of the *Eomes* genomic locus. We demonstrate that *CreER* expression recapitulates endogenous *Eomes* transcription within different progenitor cell populations. Tamoxifen administration specifically labels *Eomes*-expressing cells and their progeny as demonstrated by crossing *Eomes*^{CreER} animals to different Cre-inducible reporter strains. In summary, this novel *Eomes*^{CreER} allele can be used as elegant genetic tool that allows to follow the fate of *Eomes*-positive cells and to genetically manipulate them in a temporal specific manner.

Keywords

mouse; Eomesodermin; Tbx2; tamoxifen-activatable CreER; lineage tracing

T-box proteins (Tbx factors) comprise a family of 17 transcription factors defined by their highly conserved, DNA-binding domain, the so-called T-box. Tbx factors frequently show

highly restricted spatiotemporal expression patterns and loss-of-function mutants exhibit profound developmental phenotypes (Naiche *et al.*, 2005). The T-box gene *Eomesodermin* (*Eomes*, also called *Tbr2*) becomes first expressed in cells of the trophoblast lineage from early postimplantation stages onward marking the population of undifferentiated, multipotent trophoblast stem cells (TSCs) (Niwa *et al.*, 2005; Russ *et al.*, 2000; Strumpf *et al.*, 2005). *Eomes*-positive TSCs are found in the extraembryonic ectoderm (ExE) at early pregastrulation stages and become later restricted to the chorion until extraembryonic expression ceases around embryonic day 9.5 (E9.5). *Eomes* is required to maintain TSCs in an undifferentiated state and to allow for the expansion of TSCs, which constitute the cellular source for the embryonic part of the placenta (Russ *et al.*, 2000; Strumpf *et al.*, 2005). During onset of gastrulation starting from E6.25, a second *Eomes* expression domain arises in the epiblast at the site of primitive streak initiation where it is maintained for a short time period until E7.5 (Ciruna and Rossant, 1999; Russ *et al.*, 2000). The conditional deletion of *Eomes* in the epiblast has uncovered crucial functions for specification of two of the germ layers, namely, for the formation of definitive endoderm (Arnold *et al.*, 2008a; Teo *et al.*, 2011) and the cranio-cardiac mesoderm (Costello *et al.*, 2011). Accordingly, the deficiency of *Eomes* in the epiblast leads to developmental arrest at the primitive streak stage shortly after gastrulation onset due to combined defects of mesoderm and endoderm formation (Arnold *et al.*, 2008a; Costello *et al.*, 2011).

During cerebral corticogenesis, *Eomes* expression is first found in Cajal-Retzius cells of the developing fore-brain vesicles (Bulfone *et al.*, 1999; Russ *et al.*, 2000). From E12.5 *Eomes* marks the pool of neurogenic intermediate progenitor cells (IPCs) located in the embryonic subventricular zone (SVZ) that gives rise to cortical projection neurons (Englund *et al.*, 2005; Hevner *et al.*, 2006; Hodge *et al.*, 2008). Deletion of *Eomes* function in the developing cortex results in deficits of early and late neurogenesis due to a gross reduction of IPC proliferation and major disturbances of neuronal differentiation and cortical patterning (Arnold *et al.*, 2008b; Elsen *et al.*, 2013; Sessa *et al.*, 2008). Similarly, human cases of a chromosomal translocation 2215 kb upstream to the *Eomes* locus were identified that leads to severe microcephaly in homozygously affected individuals. Most likely, this translocation leads to the loss of regulatory elements for *Eomes* expression during cortical neurogenesis (Baala *et al.*, 2007). During adult murine neurogenesis, *Eomes* is expressed in intermediate progenitors of the mature brain and is essential for glutamatergic neurogenesis (Brill *et al.*, 2009; Hodge *et al.*, 2008; Hodge *et al.*, 2012).

Additional sites of adult *Eomes* expression include central memory CD8⁺ T cells (Intlekofer *et al.*, 2005; Pearce *et al.*, 2003) and natural killer (NK) cells (Gordon *et al.*, 2012; Tayade *et al.*, 2005) of the adaptive and innate immune system, respectively. Maintenance of both cell types depends on *Eomes* function, explaining deficiencies in the immune responses of *Eomes* conditional mutants (Banerjee *et al.*, 2010; Gordon *et al.*, 2011).

In conclusion, numerous studies using constitutive or conditional gene deletion approaches have established central functions of the transcription factor *Eomes* during lineage specification, differentiation, and maintenance of various stem and progenitor cell populations. To characterize *Eomes*-expressing cells and their progeny, mouse lines harboring reporter genes in the endogenous *Eomes* locus were previously established, such

as insertions of eGFP (Arnold *et al.*, 2009) or the Cre-recombinase (Costello *et al.*, 2011). These reporter alleles allow for studies of cells during the time of *Eomes* expression (*Eomes*^{GFP}) or for lineage tracing of progeny of *Eomes*-expressing cells by combining the *Eomes*^{Cre} allele with Cre-inducible reporter alleles.

To study roles of *Eomes*-expressing cells and their progeny at defined developmental stages, we generated a novel *Eomes* allele by integrating the tamoxifen (Tx)-activatable version of *Cre* (*CreER*^{T2}, hereafter simply referred to as *CreER*) (Feil *et al.*, 1997) into the *Eomes* locus via homologous recombination in embryonic stem (ES) cells. We inserted the cDNA of *Cre recombinase* that is fused to a mutated ligand-binding domain of the human estrogen receptor (*ER*) into the translational start site of the endogenous *Eomes* locus, thereby deleting ~500 bp of the exon 1 coding region (Fig. 1a). For positive and negative ES cell selection, a loxP-flanked neomycin cassette and a thymidine kinase cassette (pMCI.TK) were integrated into the targeting vector (Fig. 1a). ES cell clones were screened by Southern blot (Fig. 1b) and correctly targeted clones were used for the generation of chimeras by morula aggregation. Following germline transmission, F1 progeny and subsequent generations were genotyped by polymerase chain reaction (PCR) resulting in a 327-bp band for the wild-type and a 202-bp band for the targeted allele (Fig. 1c). Heterozygous *Eomes*^{CreER} mice are viable, fertile, and show no obvious abnormalities.

To evaluate the novel *Eomes*^{CreER} allele, we first tested if *CreER* expression resembles the patterns of endogenous *Eomes* mRNA. In situ hybridizations were performed using probes for *Eomes* and *CreER* on E7.25 *Eomes*^{CreER/+} whole-mount embryos and on E14.5 brain sections. Comparative expression analysis shows that *CreER* expression recapitulates the spatiotemporal distribution of endogenous *Eomes* transcription (Fig. 2). Thus, *Eomes* and *CreER* expression is found in the primitive streak and ExE of E7.25 embryos (Fig. 2a,b) and in cells of the SVZ of the developing cerebral cortex at E14.5 (Fig. 2c,d). We conclude that *CreER* expression faithfully recapitulates the patterns of *Eomes* transcription, suggesting that the insertion of cDNA for *CreER* and a polyA-signal into the transcriptional start site of exon 1 does not disturb transcriptional regulation from the locus. Similar to previously established *Eomes* reporter alleles (Arnold *et al.*, 2009; Costello *et al.*, 2011) the *Eomes*^{CreER} allele acts as a null allele leading to a heterozygous genetic background for *Eomes*. Accordingly, no homozygous *Eomes*^{CreER/CreER} embryos could be recovered from *Eomes*^{CreER/+} intercrosses at E7.5 (*n*=43, data not shown) owing to requirements of *Eomes* function in the trophectoderm lineage (Russ *et al.*, 2000; Strumpf *et al.*, 2005). No obvious phenotypic alterations indicative for haploinsufficiency could be observed in heterozygous *Eomes*^{CreER/+} embryos and adult animals.

To test for recombination activity of the Tx-activated *CreER* during gastrulation stages, *Eomes*^{CreER/+} mice were first crossed with animals carrying a Creactivatable mGFP-reporter allele (Muzumdar *et al.*, 2007). Pregnant females were intraperitoneally (i.p.) injected with either a single dose of Tx at E5.5 or injected on two consecutive days at E5.5 and E6.5. Treatment before gastrulation onset induces reporter expression in a few cells in the ExE and rarely in cells of the primitive streak when embryos are analyzed at E6.5 (Fig. 3a). Dual injection at E5.5 and E6.5 results in mGFP expression in the ExE and in cells of the primitive streak (Fig. 3b). In a second approach, *Eomes*^{CreER/+} males were crossed with

females carrying the Rosa26 LacZ-reporter allele (R26^R) (Soriano, 1999) and pregnant females injected with Tx at E6.5 or twice at E6.5 and E7.5. At E8.5 resulting embryos show LacZ-positive cells in the developing heart, the aorta, the primitive gut tube, and in the visceral yolk sac after single injections (Fig. 3c – f). Dual application of Tx at E6.5 and E7.5 significantly increases the number of labeled cells, indicating the dependency on concentration and timing of Tx administration for activation of the CreER recombinase (Fig. 3g – j). Thus, the *Eomes*^{CreER} allele can be used to follow *Eomes*-expressing cells during gastrulation at the level of single cells in a close-to-clonal analysis. Alternatively, higher numbers of *Eomes*-expressing cells can be marked using repeated, high-dose injections of Tx.

During development of the brain, *Eomes* is expressed dynamically in neurogenic regions, such as the embryonic cerebral cortex and cerebellum (Arnold *et al.*, 2008b; Englund *et al.*, 2005; Hodge *et al.*, 2008). Additionally, *Eomes* is also found in the neurogenic niches of the adult brain including the olfactory bulb and the adult dentate gyrus (Hevner *et al.*, 2006; Hodge *et al.*, 2008). To test for recombination in neurogenic brain regions during development we first crossed *Eomes*^{CreER/+} animals to R26^R LacZ-reporter mice and injected Tx on days E14–E16. Dorsal views of resulting whole-mount brains at E17 show activation of the LacZ-reporter gene in the hemispheres of *Eomes*^{CreER}-positive brains, but not in control brains (Fig. 4a,c). Coronal sections reveal LacZ-positive cells that range from the SVZ toward the cortical plate in the hemisphere of *Eomes*^{CreER/+} embryos, but not in sections of control brains (Fig. 4b,b',d,d').

In a second set of experiments, *Eomes*^{CreER/+} mice were bred with *Ai14*-reporter mice, in which the red-fluorescent *tdTomato* reporter is expressed after Cre-mediated recombination (Madisen *et al.*, 2012). Pregnant females were injected at E14 and offspring analyzed at postnatal day 0.5 (P0.5). Red-fluorescent *tdTomato* can be detected in middle to upper layer neurons of the cerebral cortex and in migrating unipolar brush cells of the cerebellum (Fig. 4e,e',f). When adult animals were treated with Tx, neurogenic progenitor cells and resulting neurons are correctly labeled in the neurogenic niche of the dentate gyrus, in the cerebellum, and in the olfactory bulb (Fig. 4g,g',h,i). Double immunofluorescence staining to detect *tdTomato* and *Eomes* protein shows that *tdTomato* is expressed in many but not all *Eomes*-positive cells, e.g., in the olfactory bulb (Fig. 4i'). Thus, administration of a single dose of Tx induces mosaic recombination by *Eomes*^{CreER} in a subset of *Eomes*-positive cells but does not lead to ectopic recombination. We estimate that recombination efficiencies in the developing cortex reach 60–80% of *Eomes*-expressing cells, whereas recombination rates are considerably lower during earlier stages of development, possibly reflecting reduced bioavailability of injected Tx during early pregnancies.

Next, we wanted to test whether the CreER allele also functions in *Eomes*-expressing cells of the innate immune system. Splenic NK cells are innate lymphocytes that can be identified based on the presence of the cell surface receptors NKp46 and CD49b. The liver harbors an additional NK cell population that expresses the death-ligand TRAIL but not CD49b. Previous studies suggested that *Eomes* is only expressed in CD49b⁺ NK cells but not in TRAIL⁺ NK cells (Gordon *et al.*, 2012). To test if the *Eomes*^{CreER} allele is able to specifically label CD49b⁺ NK cells we fed female mice carrying the *Eomes*^{CreER} allele and

a Cre-inducible GFP-reporter allele (Muzumdar *et al.*, 2007) with Tx-containing food and analyzed splenic and hepatic NK cells by fluorescence activated cell sorting (FACS). In agreement with previous findings, only CD49b⁺ NK cells in spleen and liver show *GFP* expression after *Eomes*^{CreER} activation, whereas TRAIL⁺ NK cells of the liver do not exhibit GFP-reporter expression (Fig. 5). Thus, *Eomes*^{CreER} marks and distinguishes different NK cell lineages after administration of Tx in adult mice.

In summary, we present a novel *Eomes*^{CreER} allele that allows for Tx-inducible activation of the Cre-recombinase in *Eomes*-expressing stem and progenitor cells during development and in adulthood. This genetic tool will be instrumental to resolve some of the eminent questions regarding the cellular fate and mechanisms of lineage decisions of *Eomes*-expressing progenitors. For example, limited-number cell labeling in combination with time-lapse imaging of early gastrulation-stage embryos will enable to delineate the origin and emergence of cardiac mesoderm and definitive endoderm. Additionally, this allele will allow to further resolve neurogenesis from embryonic and adult IPCs, e.g., with respect to temporal differences in neuronal subtype specification and contribution to the different layers of the embryonic cerebral cortex. In combination with Cre-inducible alleles that impact on cellular signaling or behavior, this allele will further enable studies toward mechanisms of lineage specification and lineage-specific behavior in descendants of *Eomes*-expressing progenitors. In conclusion, this *Eomes*^{CreER} allele will be a valuable tool for future genetic studies in different areas of progenitor cell research.

MATERIALS AND METHODS

Generation of the *Eomes*^{CreER}-Targeted Allele

The targeting vector for the generation of an *Eomes*^{CreER}-targeted allele comprises an 8.25-kb HpaI-HpaI fragment of the *Eomes* locus. A CreERT².pA cassette and a LoxP-flanked neomycin resistance cassette were integrated between the SphI site at the translational start site and an EagI site, thereby deleting ~500 bp of the coding region of exon 1. The 3' homology region of the targeting construct was flanked with a pMC1.TK negative selection cassette. Linearized targeting vector was electroporated into CCE ES cells, and neomycin and FIAU-resistant ES cell colonies were screened by Southern blot using a 3' external probe on EcoRV-digested DNA (wt allele: 15 kb and targeted allele: 7 kb). Generation of chimeras was performed by morula aggregation. F1 progeny and subsequent generations were genotyped by multiplex PCR at 60° C annealing temperature to detect wild-type allele (wt, 327 bp) and CreER allele (202 bp) using the following primers: forward primer 5'-GAGGGAGGAAGGGGACATTA-3'; reverse primer1 5'-CAGGTTCTTGCGAACCTCAT-3'; reverse primer2 5'-CAGGTTCTTGCGAACCTCAT-3'. Animals carrying the *Eomes*^{CreER} allele are available to the research community and can be requested from the corresponding author.

Animals

R26^R-reporter (Soriano, 1999), *Ai14*-reporter (Madisen *et al.*, 2012), and *mGFP*-reporter mice (Muzumdar *et al.*, 2007) were described previously. Tamoxifen (Tx) (Sigma-Aldrich, Taufkirchen, Germany, T5648; 20 mg/ml) and progesterone (Sigma-Aldrich P0130; 10

mg/ ml) were diluted in corn oil (Sigma-Aldrich C8267). For CreER induction at E5.5, E6.5, and E7.5 pregnant females were injected i.p. with 4 mg of Tx and 2 mg of progesterone per injection. For analysis of embryonic brains, pregnant females received repeated i.p. injections of 1 mg of Tx/0.5 mg of progesterone at E14, E15, and E16. Adult mice (age 8 weeks) received a single dose of Tx (180 mg/kg, i.p.) followed by analysis after 2 days or 5 months. For labeling of NK cells, adult female mice were fed with Tx-enriched food (TAM400, Harlan Laboratories, Horst, Netherlands) for 21 days and analyzed thereafter.

Histological Analysis

In situ hybridization on whole-mount embryos and paraffin sections was performed according to standard protocols (Nagy *et al.*, 2003) using *Eomes*- and *CreER*-specific probes. LacZ staining was carried out as described (Nagy *et al.*, 2003). For histological analysis of LacZ-stained embryos and brains, specimens were postfixed in 4% paraformaldehyd/phosphate-buffered saline, dehydrated through ethanol series, and embedded in paraffin before sectioning at 8 μ m. Cryostat sections of embryos and Eosin counterstaining was performed according to standard protocols (Nagy *et al.*, 2003).

For detection of red fluorescent protein (RFP)-reporter expression, animals were perfused and studied by detection of native RFP fluorescence or immunofluorescence to detect RFP (Clontech 632496, Mountain View, California, 1:1,000) in combination with DCX or *Eomes/Tbr2* as described (Hodge *et al.*, 2008). Images were collected on a Zeiss (Jena, Germany) Axiovision or Zeiss LSM710 microscope and processed for brightness and contrast using Photoshop.

Flow Cytometry

Spleen and liver lymphocytes were isolated as previously described (Gordon *et al.*, 2012), followed by antibody staining as described (Klose *et al.*, 2013). In brief, single-cell suspensions of purified cells were kept on ice and Fc receptors were blocked using CD16/CD32 antibody. NK cells in spleen and liver were identified as lineage-negative (CD4, CD8a, CD19, and Gr-1) CD45⁺ NKp46⁺ cells, which were further divided based on the expression of CD49b and TRAIL. All antibodies were purchased from eBioscience (Frankfurt, Germany). FACS data were acquired on a LSR Fortessa (BD Biosciences, Heidelberg, Germany) and analyzed using FlowJo software (TreeStar, Ashland, Oregon).

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FIG. 1.

Generation of the *Eomes*^{CreER} allele. (a) Strategy to introduce *CreER* into exon 1 of the *Eomes* locus by homologous recombination in ES cells. The *CreER* cassette was placed into the transcriptional start site of the endogenous *Eomes* locus, followed by a removable, LoxP-flanked *neomycin* selection cassette (PGK.*neo*). (b) Drug-resistant ES cell clones were screened by Southern blot of EcoRV-digested genomic DNA using a 3' external probe [indicated as red bar in (a); wild-type allele (wt, 15 kb), targeted allele (T, 7 kb)]. (c) PCR genotyping distinguishes wild-type (wt, 327 bp) and *CreER* reporter allele (*CreER*, 202 bp). Genotyping primers are indicated as purple arrowheads in (a). E, EagI; H, HindIII; neo, neomycin-positive selection cassette; S, SphI; RV, EcoRV, TK, thymidine kinase negative selection marker.

FIG. 2.

CreER mRNA expression recapitulates the endogenous *Eomes* transcription pattern. (**a** and **b**) Whole-mount in situ hybridization of E7.25 *Eomes*^{CreER/+} embryos using probes for (a) *Eomes* and (b) *CreER*. Expression of *Eomes* and *CreER* is similarly found at the primitive streak (asterisk) and in the ExE (arrow). (**c** and **d**) In situ hybridization of coronal sections from the cerebral cortex of an E14.5 *Eomes*^{CreER/+} embryo. Note the almost identical distribution of *CreER* and endogenous *Eomes* mRNAs in (a and b) the E7.25 embryo and in (c and d) cells of the embryonic ventricular (VZ) and subventricular zone (SVZ). Scale bar: 200 μ m.

FIG. 3.

Reporter activation by $Eomes^{CreER}$ at gastrulation stages. (a and b) Females carrying the Cre-inducible mGFP reporter allele were mated to $Eomes^{CreER/+}$ males and pregnant females received Tx at (a) E5.5 and (b) E5.5 plus E6.5. Resulting embryos were analyzed at E6.5 and E7.5 by cryosections for mGFP expression and DAPI counterstaining. mGFP labeling is found in cells of the ExE (red arrowhead) and in the primitive streak (asterisk). Dual application at E5.5 and E6.5 resulted in increased number of labeled cells. (c-j) $Eomes^{CreER/+}$ males were crossed with females carrying the R26^R LacZ-reporter allele and pregnant females received Tx at (c-f) E6.5 and (g-j) E6.5 plus E7.5. Embryos were recovered at E8.5, stained for LacZ expression, and used for histological analysis at the indicated plane of section. (c-f) Single-dose injections of Tx at E6.5 leads to reporter activation in a limited number of cells in the visceral endoderm (c, blue arrow), in the

primary gut tube (e, white arrowhead), the aorta (e, asterisk), and in the developing myocardium (f, black arrowhead). (g–j) Dual injections at E6.5 and E7.5 result in significantly increased recombination activity in a larger number of cells. LacZ-reporter activity is present in the visceral yolk sac (h, arrow), in the myocardium (j, black arrowhead), in head mesenchyme (j, grey arrowhead), and gut endoderm (j, white arrowhead). a, anterior; p, posterior; r, right side; l, left side. Scale bar: 200 μ m.

FIG. 4.

Detection of $Eomes^{CreER}$ -mediated cell labeling in the developing and adult brain. (a and c) $Eomes^{CreER/+}$; R26^R, and control embryos were induced by repeated Tx injections of the pregnant mother from E14 to E16 and analyzed for reporter activation in the brain by LacZ staining at E17. Dorsal views of control (a) and $Eomes^{CreER/+}$ (c) brains and corresponding coronal sections (b, b', d, and d') demonstrate $Eomes^{CreER}$ -induced reporter-activation accurately labeling cells in the embryonic subventricular zone (SVZ) and their progeny in the cortical plate (CP). (e–i') Analysis of induction of the *Ail4* reporter that produces the red

fluorescent protein, tdTomato, following *Eomes*^{CreER}-mediated recombination in developing and adult brains. (e) Sagittal section of a neonatal (P0.5) mouse brain treated with Tx on E14 showing *tdTomato* expression in the cerebral cortex (Ctx) and associated axon pathways, and in cerebellum (Cb). (e') Within the cortex, tdTomato-positive cells are seen in layer 3 (L3) but not L2 or marginal zone (MZ), consistent with differentiation of *Eomes*-expressing intermediate progenitor cells into cortical projection neurons. (f) In the cerebellar vermis, tdTomato is detected in cells migrating through the cerebellar white matter, consistent with unipolar brush cells (UBCs). (g and g') In the adult hippocampus *tdTomato*-expressing cells can be observed in the subgranular layer (SGL) of the dentate gyrus (DG) 2 days after Tx treatment. Double labeling with tdTomato and DCX (green), a marker of intermediate progenitors and new neurons, shows partially overlapping expression. (h, i, and i') Cell labeling in the adult cerebellum (h) and olfactory bulb (i and i') 5 months after Tx treatment. *TdTomato*-expressing cells are abundant in folia IX and X of the cerebellum, matching the characteristic distribution of unipolar brush cells. In the olfactory bulb, reporter-expressing cells are seen in the peri-glomerular layer. (i') Double labeling using an *Eomes*-specific antibody and *tdTomato* expression demonstrates that in the olfactory bulb all reporter-positive cells (red) show *Eomes* protein (green), but only a subset of *Eomes*-expressing cells are marked with tdTomato. *Eomes*-, tdTomato-double positive cells are indicated with arrowheads in (i').

FIG. 5.

Labeling of NK cell subpopulations by the *Eomes*^{CreER} allele. *Eomes*^{CreER/+} female mice carrying a Cre-inducible GFP reporter were fed with Tx-enriched food for 21 days and different NKp46⁺ NK cell populations from spleen and liver were analyzed by flow cytometry for GFP-reporter expression. One representative result demonstrates that reporter activation by *Eomes*^{CreER} is only detected in *Eomes*⁺ CD49b⁺ NK cells, but not in *Eomes*⁻ TRAIL⁺ NK cells.