

An inducible RNA interference system for the functional dissection of mouse embryogenesis

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ABSTRACT

Functional analysis of multiple genes is key to understanding gene regulatory networks controlling embryonic development. We have developed an integrated vector system for inducible gene silencing by shRNAmir-mediated RNA interference in mouse embryos, as a fast method for dissecting mammalian gene function. For validation of the vector system, we generated mutant phenotypes for *Brachyury*, *Foxa2* and *Noto*, transcription factors which play pivotal roles in embryonic development. Using a series of *Brachyury* shRNAmir vectors of various strengths we generated hypomorphic and loss of function phenotypes allowing the identification of *Brachyury* target genes involved in trunk development. We also demonstrate temporal control of gene silencing, thus bypassing early embryonic lethality. Importantly, off-target effects of shRNAmir expression were not detectable. Taken together, the system allows the dissection of gene function at unprecedented detail and speed, and provides tight control of the genetic background minimizing intrinsic variation.

INTRODUCTION

The mouse is the most widely used mammalian model in genetic research, due to the availability of inbred strains, a complete and annotated genome sequence and the ability to modify the genome by transgenic techniques in embryonic stem (ES) cells. Targeted inactivation of gene loci by homologous recombination or conditional mutagenesis using site-specific recombinases are widely applied (1–3). However, these techniques are time consuming and costly.

RNA interference is a natural mechanism of the cell to modulate gene function at the mRNA level. The main endogenous triggers of RNAi are 22-nt single-stranded RNAs (the so-called microRNAs or miRNAs), which are transcribed as long precursors from RNA polymerase II promoters (4). Experimental RNAi employs RNAs that mimic the structure of miRNA intermediates, such as short hairpin RNAs (shRNAs) or short hairpin RNAs in a miRNA context (shRNAmir). RNAi is routinely used for gene silencing in cellular systems, and is fast and cost efficient. However, methods for *in-vivo* RNAi in the mouse have not yet found wide application (5–9).

We present an integrated transgenic system for shRNAmir-mediated gene silencing in the mouse. The system consists of a series of vectors allowing graded downregulation of gene function and thus, the generation of mutant phenotypes of various strengths (hypomorphs to loss of function phenotypes). We demonstrate for the first time the generation of phenocopies of known genetic null mutants by application of *in vivo* RNAi in the mouse embryo. Since our system is inducible, it allows to bypass early embryonic lethality. In addition, this system controls for unrelated morphological phenotypes which frequently arise as a consequence of *in vitro* manipulation of ES cell clones. Finally, we show that gene silencing with our shRNAmir transgene system is achieved virtually without off-target effects. Our RNAi toolkit can therefore provide a flexible and economic alternative to gene ablation for the analysis of gene function in the mouse.

MATERIALS AND METHODS

Vectors

We inserted the recipient transgene into the XbaI site of pROSA26-1 and targeted the *Gt(ROSA)26Sor* locus (10). The recipient transgene was assembled in pBluescript SK

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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(Stratagene) and contained the following elements (5' to 3'): a SV40 splice acceptor fused to a murinized version of tTS or rtTA (Clontech) with a SV40 polyadenylation signal, the 1.2-kb chicken β -globin insulator (5'HS4), a PGK-hygromycin selection cassette flanked by opposing loxP and lox5171 sites (11,12), and a promoter-less neomycin resistance gene with bidirectional polyadenylation signal, followed by another chicken β -globin insulator. Exchange vectors were assembled in pBluescript SK and contained the following elements (5' to 3'): optimized tetS binding sites (TRE-tight, Clontech), CAGGS promoter (13), a transgene containing either one or two shRNAs-mir in a murine mir155 context (Invitrogen) followed by the thymidine kinase polyadenylation signal or the coding sequence of EGFP, one shRNAmir flanked by splice donor and acceptor sites and the rabbit β -globin polyadenylation signal, the chicken β -globin core insulator and a PGK promoter that complements the promoter-less neomycin resistance gene of the recipient locus upon integration. Exchange vectors were flanked by loxP and lox5171 sites. Maps and sequences of the recipient transgene and exchange vectors are available upon request.

Cell culture and *in vitro* RNAi

ES cells were cultured in knockout Dulbecco's Modified Eagle's Medium (Gibco) containing 15% of fetal calf serum and 1000 U ml⁻¹ LIF (Esgro) according to standard protocols. We used both C57BL/6 (Shimizu and Schrewe, unpublished) and G4 ES cells (14) for *Gt(ROSA)26Sor* targeting. Clones that had undergone homologous recombination were identified by Southern blot analysis of genomic DNA digested with BamHI, using a probe external of the 5' *Gt(ROSA)26Sor* homology arm, and the neomycin resistance gene as an internal 3' probe, and termed ROSA26S and ROSA26A. For RMCE, 3 × 10⁵ ROSA26S or ROSA26A ES cells were transfected with 5.0 μg of exchange vector and 0.1 μg of Cre recombinase expression vector using Lipofectamine 2000 (Invitrogen). Cells were split four hours after transfection and selected with 250 μg ml⁻¹ of Geneticin (Gibco) starting the following day. Positive recombinants were identified by Southern blot analysis of genomic DNA digested with HindIII or BamHI/HindIII, using the neomycin selection cassette as probe. For *in vitro* induction, cells were cultured in the presence of 2 ng ml⁻¹ doxycycline (Fluka). qRT-PCR was done using random-hexamer primed cDNA prepared according to manufacturer protocols (Invitrogen), the Step One Plus detection system (Applied Biosystems) and SYBR green (Applied Biosystems). Data were analyzed with Step One Software v2.1 (Applied Biosystems) using the C_T($\Delta\Delta C_T$) method. Per target gene, four individual shRNAmirs in pCDNA6.2GW/EmGFP miR (Invitrogen) were tested for interference efficacy by co-transfection with an expression vector carrying the murine target gene cDNA into human SW480 cells and subsequent qRT-PCR, as above. shRNAmirs for *in vivo* experiments were: T shRNAmir1 (KD1-T to KD4-T): TGC TGT TAG TTA GCT CCT TGA AGC GCG TTT TGG CCA

CTG ACT GAC GCG CTT CAG AGC TAA CTA A; T shRNAmir2 (KD1-T, KD4-T): TGC TGT AGA AGA TCC AGT TGA CAC CGG TTT TGG CCA CTG ACT GAC CGG TGT CAT GGA TCT TCT A; Foxa2 shRNAmir1 (KD1-Foxa2): TGC TGT AGG ATG ACA TGT TCA TGG AGG TTT TGG CCA CTG ACT GAC CTC CAT GAA TGT CAT CCT A; Foxa2 shRNAmir2 (KD1-Foxa2): TGC TGT GTA CGA GTA GGG AGG TTT GGG TTT TGG CCA CTG ACT GAC CCA AAC CTC TAC TCG TAC A; Noto (KD2-Noto): TGC TGT AAG GTA GCT CTG ATC CTG TGG TTT TGG CCA CTG ACT GAC CAC AGG ATG AGC TAC CTT A. Sequences of qRT-PCR primers are available upon request.

Mice

Fully ES-cell-derived embryos and mice were generated by tetraploid embryo complementation as previously described (15). For transgene induction *in vivo*, doxycycline was administered to mice at 4 mg ml⁻¹ in a 1% sucrose solution in drinking water. For timed induction intervals, the day of plug was assumed to be E0.5. Whole-mount *in situ* hybridization was performed using protocols and probes described in the MAMEP database (<http://mamep.molgen.mpg.de>). For northern blot analysis, whole embryo RNA was isolated in Trizol (Invitrogen), according to manufacturer's instructions. Thirty micrograms RNA/lane were separated on a 15% denaturing TBE gel and transferred to a GeneScreen Plus membrane (PerkinElmer). Hybridization was performed in OligoHyb buffer (Ambion) with a ³²P-end-labeled oligonucleotide complementary to the predicted siRNA.

Expression profiling

For gene expression profiling, four independent replicate profiles of each group were prepared. RNAs were isolated from tissue domains using MinElute columns (Qiagen), labeled using the MessageAMP Kit (Ambion) and probes were hybridized to Illumina Sentrix MouseRef-8v2 microarrays, according to manufacturer's instructions. Expression data was processed using Bead Studio GX software (Illumina), using the cubic spline normalization method, and background subtraction. Marginally expressed genes were removed for analysis by a detection value cutoff of one in at least four out of eight profiles from the respective tissue domain. Deregulated genes were selected by more than 2-fold change in expression with a *P*-value < 0.05 in an unpaired Welch *t*-test, without multiple testing correction. Comparison of randomized expression profiles was between 18 sets that contained equal numbers of profiles of induced and noninduced forebrains. For analysis of off-target regulation, Sylamer was used as suggested by the authors (see www.ebi.ac.uk/enright/sylamer/sylamer.html). In short, identifiers of expressed genes (detection cutoff as above) in caudal end or forebrain data sets were ordered by induced versus non-induced fold change expression. Next, Sylamer was used to determine the distribution of occurrence of 6-, 7- and 8-mer sequences complementary to the experimental seeds (shRNAmir 1: TAACTA, AACTAA, TAAC

TAA, CTA ACTA, GCTA ACTA, CTA ACTAA; shRNAmir2: CTTCTA, TCTTCT, TCTTCTA, ATCTTCT, GATCTTCT, ATCTTCTA) in these ordered lists, by analyzing a genome-wide set of murine 3' UTR sequences (16).

RESULTS

We decided to establish an integrated vector system with the following features: (i) single copy transgenes should be integrated into a defined recipient locus to avoid variability caused by the integration site; (ii) the RNAi construct should be inducible in order to allow temporal control of RNAi and to control for the integrity of the transgenic ES cell clones used in the experiments; and (iii) a series of RNAi vectors for the generation of graded phenotypes (hypomorphs to loss of function mutants) should be accommodated.

We modified ES cells by integrating the tetracycline-dependent transcriptional silencer (tTS) or the reverse tetracycline-dependent transcriptional activator (rtTA), followed by a cassette for recombinase-mediated cassette exchange (RMCE), into the first intron of *Gt(ROSA)26Sor* (termed ROSA26S or ROSA26A

recipients). Next, we constructed exchange vectors containing RNAi transgenes compatible with tTS or rtTA control and the PGK promoter providing G418 resistance upon successful RMCE (Figure 1A). For tTS-mediated control of transgene expression we constructed three exchange vectors containing either two shRNAmir (KD1), or a single shRNAmir (KD2) or one intronic shRNAmir and the coding sequence of EGFP (KD3), driven by the CAGGS promoter. In this setup, tTS is ubiquitously expressed under control of the ROSA26 promoter and represses the RNAi constructs until the block is released by exposure of the cells/embryos to doxycycline. In the rtTA vector system (KD4), the RNAi vector is silent in the absence of doxycycline and induced by exposure of the cells/embryos to the drug via activation of rtTA, which is constitutively expressed from the ROSA26 locus.

Two efficacious shRNAmir hairpins directed against *Brachyury* (*T*) were identified in *in vitro* assays (see 'Materials and Methods' section, and Supplementary Figure S1). *T* is a T-box transcription factor, which is essential for embryonic mesoderm formation and notochord development (17). Transient transfection of ROSA26S or ROSA26A ES cells with exchange vectors

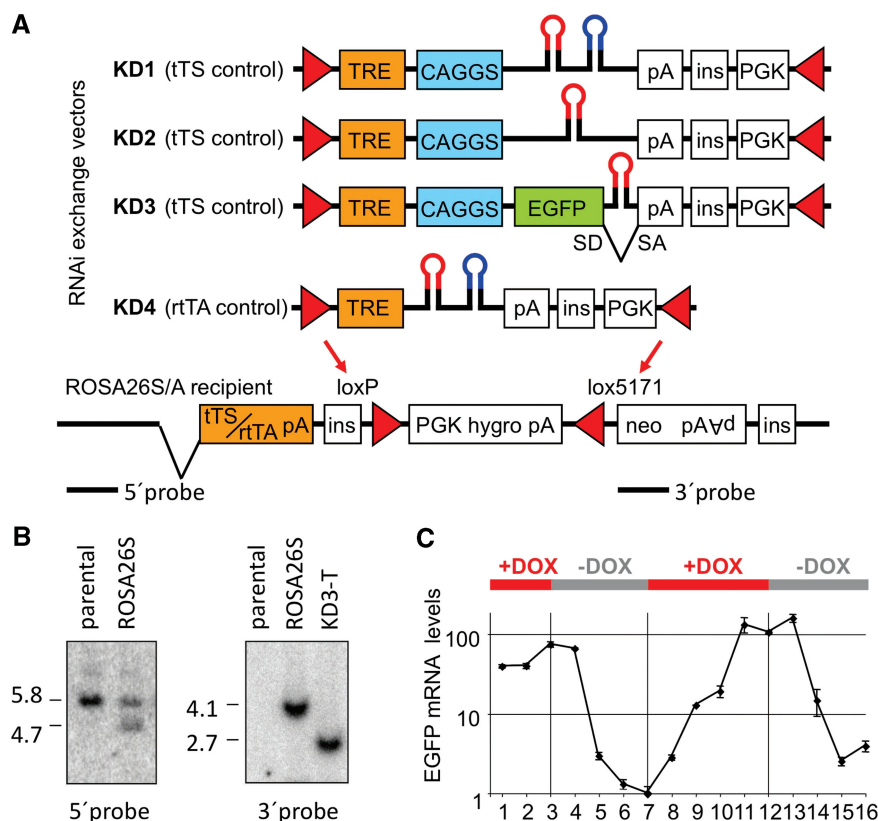


Figure 1. Vector system for inducible RNAi in mouse embryos. (A) Schematic representation of ROSA26A/ROSA26S recipient loci and KD1 to KD4 exchange vectors used for RNAi. The recipient locus contains either the tTS (for KD1-KD3) or the rtTA (for KD4) gene under control of the ROSA26 promoter; RNAi exchange vectors are integrated directionally by RMCE using Cre recombinase. Different shRNAmir hairpins are indicated in red and blue. TRE, tTS/rtTA responsive element; SD, SA, splice donor and acceptor sites; ins, chicken globin insulator. (B) Southern blot analysis of parental and recombined ES cell clones. Positions of probes are indicated in (A). (C) Reversible induction of transgene expression *in vitro*. ES cells carrying the KD3-T exchange vector were subjected to cycles of repression (-Dox) and re-induction (+Dox) for 16 days. Levels of EGFP transgene expression were measured by qRT-PCR. Error bars represent technical replicates.

and a Cre recombinase expression vector efficiently produced clones with a complete combined transgene system (Figure 1B). The addition of doxycycline to the medium of recombined ES cells led to transgene activation, which was reversible by doxycycline withdrawal (Figure 1C).

ES cells containing the vectors KD1-T to KD3-T were used to generate mouse embryos by tetraploid complementation (18). Embryos derived from untreated foster mothers were phenotypically normal (128/128 embryos; Figure 2A). Embryos derived from foster mothers exposed to doxycycline in the drinking water, however, showed various malformations. Embryos expressing KD1-T (98/98) were indistinguishable from *T/T* loss of

function mutants (data not shown, see ref. 17). At E9.5, this phenotype is characterized by absence of the notochord (marked by *Shh*) caudal to the midbrain, the absence of allantois derivatives and the presence of few irregular somites (marked by *Uncx*) combined with an excess of neural tissue at the caudal end and early arrest of posterior development, leading to lethality around E10. Embryos expressing KD2-T showed either the loss of function phenotype of KD1-T embryos (18/33) or a weaker phenotype (15/33): axial elongation had proceeded up to the tailbud, many though still irregular somites had formed and the notochord was detectable up to the forelimb bud. An even milder phenotype was observed in embryos expressing KD3-T (23/23). Though notochord

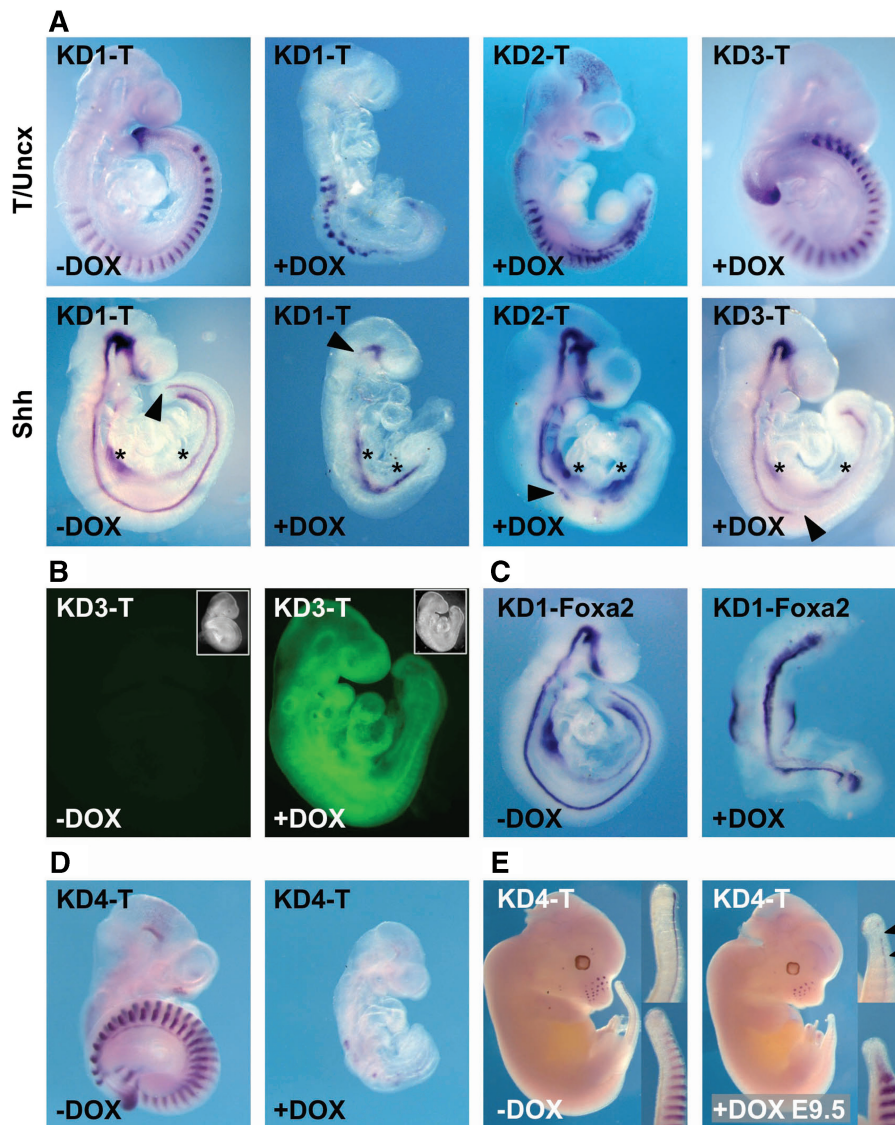


Figure 2. Analysis of embryonic phenotypes obtained by RNAi. RNAi constructs are indicated on the top, induction schemes on the bottom of each panel. (A, C and D) E9.5-10.0 transgenic control embryos (–Dox) or embryos induced for shRNAmir expression (+Dox) targeting *T* (KD1-T to KD4-T) or *Foxa2* (KD1-Foxa2) gene transcripts were hybridized *in situ* with *T* plus *Uncx* (A, D) or *Shh* (A, C) specific probes. Arrowheads mark the caudal end of the notochord, asterisks mark the endoderm, visualized by *Shh* transcripts. (B) Fluorescence images of E9.5 KD3-T control (–Dox) or induced (+Dox) embryos showing ubiquitous EGFP expression. Inserts show bright field images. (E) E12.5 control (left) and mutant embryo (right) induced for RNAi at E9.5 using the rtTA controlled KD4-T construct. Inlays show *Shh* (upper) or *Uncx* (lower) expression detected by *in situ* hybridization. Arrowheads mark discontinuous *Shh* expression.

formation was also arrested at the forelimb bud, somites appeared quite regular in size and number, allantois development occurred in some embryos and the caudal end displayed *T* transcripts, which were not or hardly detectable in KD1-T and KD2-T embryos. Tail outgrowth failed or was abnormal in all embryos of this series showing a *T* RNAi phenotype (Figure 2A). Thus, in addition to the loss of function phenotype, we were able to generate embryos with hypomorphic phenotypes, which are never observed in standard knockout alleles.

We detected enhanced green fluorescent protein (EGFP) fluorescence throughout the embryos induced for KD3-T expression, suggesting that the RNAi vector system can be applied to all tissues (Figure 2B), and found correct processing of the KD1-T to KD3-T shRNAmirs (Supplementary Figure S2).

In order to validate the system with additional genes, we introduced transgenes containing two shRNAmirs directed against *Foxa2* or one shRNAmir against *Noto* into ES cells and derived embryos as before. Again, control embryos for the *Foxa2* and *Noto* models raised in the absence of doxycycline were phenotypically normal (9/9 and 13/13, respectively). Induction of the KD1-*Foxa2* shRNAmir resulted in major malformations in a fraction of embryos (4/11), resembling those described for *Foxa2* loss of function mice (Figure 2C and data not shown; see also ref. 19). Induced embryos with a KD2-*Noto* shRNAmir showed defects in embryonic turning (2/14), in line with the *Noto* loss of function mice, which also show turning defects with incomplete penetrance (Supplementary Figure S3, see also ref. 20). We conclude that RNAi via the shRNAmir transgene system described here is well suited for generating phenocopies of loss of function as well as hypomorphic phenotypes in mouse embryos.

Since the induction of transgenes repressed by tTS during post-implantation development is difficult to control (unpublished data and ref. 21), for temporally controlled gene silencing we switched to rtTA, which provides strong target gene induction with very low background activity in the noninduced state. ES cells containing the KD4-T construct comprising two shRNAmir hairpins directed against *T* were introduced into embryos using tetraploid complementation. Noninduced KD4-T embryos dissected at E9.5 or E12.5 were phenotypically normal (17/17 embryos). Embryos induced to express KD4-T throughout development displayed a phenotype, which was indistinguishable from E9.5 *T* loss of function or KD1-T embryos (16/16 embryos; Figure 2D). However, when KD4-T was induced at E9.5 of development, early lethality was avoided and the phenotype was confined to the tail. Embryos examined at E12.5 showed an arrest of mesoderm formation and notochord development in the mid-tail, indicating that loss of *T* function induced by KD4-T RNAi commenced one day after the doxycycline treatment was initiated (12/12 embryos; Figure 2E). These results demonstrate the suitability of the rtTA KD4 vector system for temporally controlled RNAi in the embryo.

Next, we asked if the specificity of the KD1-T RNAi phenotype was also reflected by deregulation of genes

involved in the processes controlled by *T*. To test this, we analyzed RNA derived from the caudal ends of KD1-T expressing E8.5 embryos (6–8 somites) using microarrays. This early stage was chosen since compositional imbalance between mesoderm and neural tissue is still low. We compared caudal ends from induced versus noninduced embryos (Figure 3A). Statistical analysis identified 37 significantly deregulated genes (>2-fold and $P < 0.05$; 9 up-, 28 down-regulated). Among the downregulated genes, we found the experimental RNAi target *T*, *Tbx6* and several markers of the presomitic mesoderm, as well as the notochord marker *Shh* (Figure 3B). These data confirm the specificity of the RNAi phenotype generated by KD1-T expression. We conclude that analysis of RNAi models is suitable for the functional analysis of genes in embryonic development.

A major concern when using experimental RNAi is the occurrence of off-target effects, i.e. unwanted modulation of the function of additional mRNAs. To determine if our experimental embryos show evidence of unspecific RNA interference, we analyzed gene expression in the forebrain, which does not express *T*, and consequently should not be affected by KD1-T shRNAmir expression. When we compared induced versus noninduced forebrains, we found only three deregulated genes (>2-fold and $P < 0.05$; 1 up-, 2 downregulated; Figure 3C and D), less than in comparisons between randomized groups of forebrain expression profiles (data not shown). We also determined whether transcripts that contain RNA motifs complementary to the seed sequence of our experimental shRNAmirs are underrepresented in the expression profiles, which could be an indicator of off-target regulation. Using the Sylamer tool (16), we could not detect such a bias in the genome-wide forebrain or caudal end data sets (Supplementary Figure S4). Taken together, off-target effects were not apparent at the transcriptome level in embryos induced to express the KD1-T shRNAmir transgene.

DISCUSSION

In summary, we present a novel single-copy, single-locus integrated transgenic system for shRNAmir mediated RNAi in the mouse. By use of this system, we demonstrate for the first time conditional RNAi in the mouse embryo that phenocopies the genetic null mutant. We present a panel of vectors allowing the generation of various hypomorphs of different strengths, which have not been possible to obtain with loss of function alleles. This set of tools is suitable for investigating the function of genes playing multiple roles in development in more detail than previously possible with knockout alleles. Previously, constitutive high-dosage expression of shRNA transgenes under control of the H1 RNA polymerase III promoter has been used for functional RNAi in the mouse embryo (5,6). This approach did, however, not allow direct comparison of induced versus noninduced control embryos or temporal control over RNAi, and in some experiments showed more severe phenotypes than

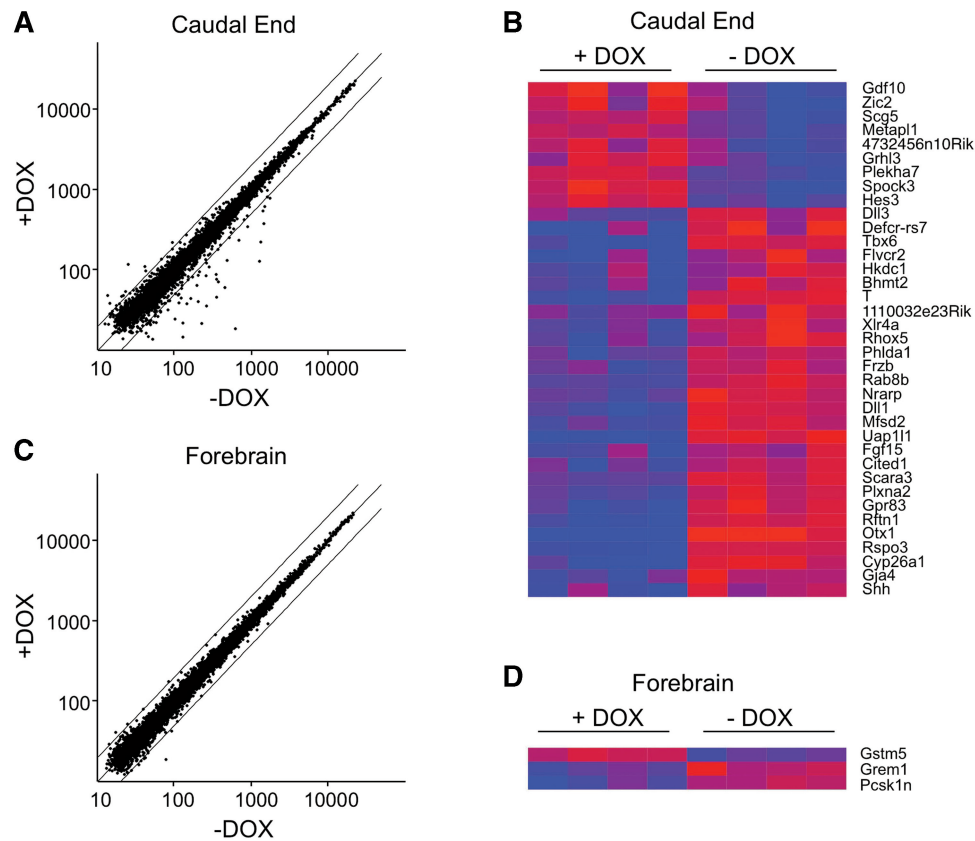


Figure 3. Expression analysis of the KD1-T RNAi model. (A and C) Scatter plot analysis of gene expression in the caudal ends or forebrains of KD1-T transgenic E8.5 control (–Dox) or induced (+Dox) embryos, as determined by microarray hybridization. Diagonal lines represent 2-fold deregulation. (B and D) Heat maps of genes found to be deregulated more than 2-fold ($P < 0.05$) in caudal ends or forebrains of four embryos each (red, upregulated; blue, downregulated; gene symbols on the right).

the respective null mutant. Other vector-based RNAi systems have been used in adult mice (7–9); however, embryonic phenotypes were not reported. An inducible system in the ROSA26 locus based on high-level expression of shRNAs under control of RNA polymerase III promoters has been used to generate a mouse diabetes model (8).

We propose that the system presented here has distinct advantages in generating null and hypomorphic mutants with minimal off-target regulation. In particular, the possibility to derive genetically identical experimental and control embryos ensures compatibility with high-throughput applications, such as expression profiling or massively parallel sequencing.

Modified exchange vectors may allow tissue-specific or multiple gene interference in the future. We provide tTS and rtTA variants of the system to allow adaptability to a wide range of applications. In addition, the system can be applied to various defined genetic backgrounds.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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