Modification of gene activity in mouse embryos *in utero* by a tamoxifen-inducible form of Cre recombinase

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The ability to generate specific genetic modifications in mice provides a powerful approach to assess gene function. When genetic modifications have been generated in the germ line, however, the resulting phenotype often only reflects the first time a gene has an influence on – or is necessary for – a particular biological process. Therefore, systems allowing conditional genetic modification have been developed (for a review, see [1]); for example, inducible forms of the Cre recombinase from P1 phage have been generated that can catalyse intramolecular recombination between target recognition sequences (*loxP* sites) in response to ligand [2–5]. Here, we assessed whether a tamoxifen-inducible form of Cre recombinase (Cre–ER^{TM}) could be used to modify gene activity in the mouse embryo *in utero*. Using the enhancer of the *Wnt1* gene to restrict the expression of Cre–ER^{TM} to the embryonic neural tube, we found that a single injection of tamoxifen into pregnant mice induced Cre-mediated recombination within the embryonic central nervous system, thereby activating expression of a reporter gene. Induction was ligand dependent, rapid and efficient. The results demonstrate that tamoxifen-inducible recombination can be used to effectively modify gene function in the mouse embryo.

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Received: **29 June 1998**
Revised: **22 October 1998**
Accepted: **30 October 1998**

Published: **23 November 1998**

Current Biology 1998, 8:1323–1326
http://biomednet.com/elecref/0960982200801323

Results and discussion

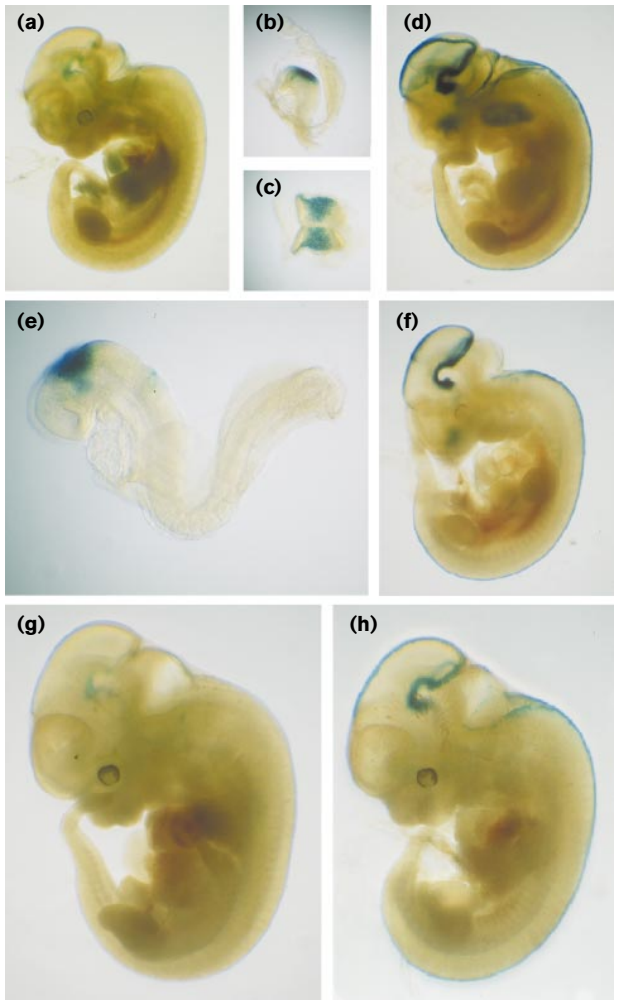
We have developed a transgenic reporter system to determine whether an inducible form of Cre recombinase can be used for recombination-mediated modulation of gene activity in the developing mouse embryo. A construct that could function as a reporter for Cre recombinase activity *in vivo* was derived from the *Wnt1*-enhancer-based expression construct pWEXPZ2, which is active from 8.0 days *post coitum* (dpc) in the neural plate of the mouse embryo [6,7]. In brief, a cassette containing the *neo* gene under the control of the promoter for phosphoglycerate kinase (PGKneobpA) flanked by *loxP* sites was inserted just 5′ to a *lacZ* reporter to prevent translation of *lacZ* mRNA until Cre-mediated excision of the *neo* cassette (for details, see Supplementary material published with this paper on the internet). Transfected R1 embryonic stem (ES) cells were used to generate mice. To determine whether the reporter could be activated appropriately, zygotes from each line were injected with a plasmid encoding Cre recombinase. Recombination was initially scored using a PCR-based assay and by staining for β-galactosidase activity. Two lines, R170 (two copies of reporter) and R44 (three copies of reporter) showed virtually no reporter activity prior to recombination (Figure 1a). Following Cre expression in the zygote, the *neo* cassette was excised and β-galactosidase activity was detected in the *Wnt1* expression pattern, that is, initially in the midbrain (Figure 1b,c) and, after neural tube closure, in the dorsal and ventral midlines of the midbrain and caudal diencephalon, the midbrain–hindbrain junction and in the dorsal spinal cord (Figure 1d). Although both reporter lines were tested in all subsequent studies with equivalent results, R170 showed stronger expression and we focused on this line.

To express Cre within the *Wnt1* expression domain, the cDNA for Cre was placed under the control of the *Wnt1* enhancer. Transgenic mouse strains that expressed this construct were mated with the R170 reporter line to determine whether Cre-mediated activation of the reporter could take place within the *Wnt1* expression domain (Cre function is restricted to the *Wnt1* expression domain in these lines; data not shown). In embryos carrying both transgenes, β-galactosidase activity was always detected within the midbrain by 8.5 dpc ($n > 4$; Figure 1e), and the complete pattern of expression by 11.5 dpc ($n > 10$; Figure 1f). Thus, the reporter can be activated within the *Wnt1* domain, and activation — which requires intragenic recombination, transcription and translation — occurred in less than 12 hours.

Next, we generated Cre fusion proteins using the hormone-binding domain of a mutant mouse estrogen receptor, ER^{TM}, which fails to bind the naturally

occurring ligand 17β-estradiol at normal concentrations but retains relatively high affinity for the synthetic ligand 4-hydroxytamoxifen (4-OHT) [8–10]. Estrogen receptor fusion proteins are inactive and are probably sequestered in the cytoplasm, bound to heat shock protein-90, but are released on binding of ligand (reviewed in [11]). A transient transfection assay using a cell line containing an integrated single-copy reporter was used to test Cre fusion proteins with or without a nuclear localisation signal, and different peptide linkers between the Cre and ER^{TM} components. Of these, a form lacking a nuclear localisation signal and containing a short peptide linker displayed a high level of inducibility with 4-OHT (data not shown). Similar Cre–ER fusion proteins have been reported by others [3,5], which can function in a ligand-dependent manner in adult mice [3,12,13].

Figure 1



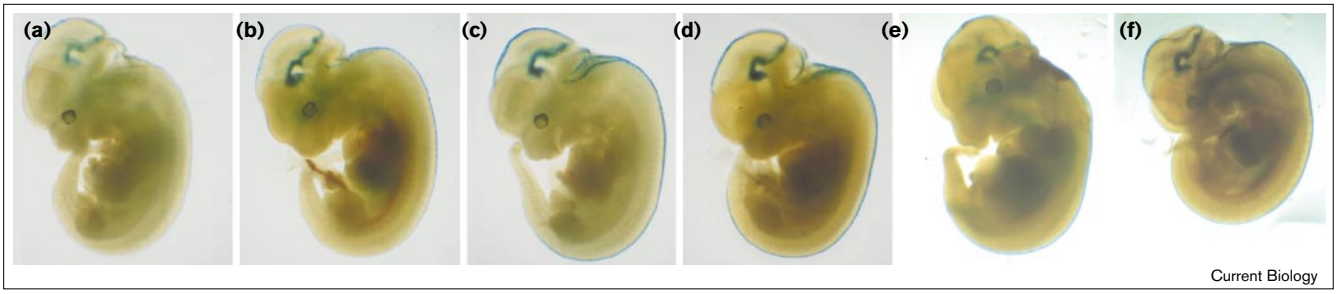
Inducible Cre-mediated recombination in the developing central nervous system *in utero*. **(a)** Practically no β-galactosidase activity was detected in 11.5 dpc embryos that were transgenic for the Cre reporter alone, except for weak ventral midbrain staining, which appeared after 48 h, in all lines tested. Consequently, staining for β-galactosidase activity was terminated after 24 h in all experiments. **(b–d)** Representative embryos at (b,c) 8.5 dpc and (d) 11.5 dpc in which recombination had occurred in the zygote, resulting in β-galactosidase activity in the *Wnt1* expression pattern; (b,d) are lateral views; (c) is a dorsal view of the embryo in (b) showing expression in the developing midbrain. **(e,f)** In embryos that were transgenic for the Cre reporter and the Cre-expressing transgene, β-galactosidase activity was detected in the *Wnt1* expression pattern at (e) 8.5 dpc and (f) 11.5 dpc, indicating that Cre-mediated recombination had occurred in the developing neural tube. **(g)** In 11.5 dpc embryos carrying the Cre reporter and the $Cre–ER^{TM}$-expressing transgene, no other sites of β-galactosidase activity (compared with the reporter alone) were detected when pregnant mice were injected with carrier (corn oil) alone. **(h)** Injection of tamoxifen, however, induced strong β-galactosidase staining in the *Wnt1* expression pattern showing that Cre recombinase activity is inducible. Representative embryos are shown.

The *Cre–ER^{TM}* cDNA was placed under the regulation of the *Wnt1* enhancer. Three expressing transgenic lines were tested and the two that expressed at higher levels gave the best, and essentially identical, results. For brevity, we only discuss line 10. To determine if recombination occurred in the absence of ligand, we crossed lines R170 and *Cre–ER^{TM}*-10 and stained for β-galactosidase activity at 11.5 dpc. All embryos determined by geno typing to be carrying both transgenes showed a staining pattern that was indistinguishable from embryos carrying only the reporter, whether pregnant mice were uninjected or injected with corn oil (carrier; $n > 14$; Figure 1g). When the pregnant mice from this intercross were injected intraperitoneally with 1.0 mg tamoxifen (TM) per day for four consecutive days (from 7.5 to 10.5 dpc) prior to collection at 11.5 dpc, a regimen described in [3], strong and reproducible β-galactosidase staining was obtained in the *Wnt1* pattern in all embryos carrying both transgenes ($n = 17$; Figure 1h). However, although most embryos were viable at 11.5 dpc, pregnancies failed shortly thereafter ($n > 4$ litters) presumably due to the anti-estrogenic properties of TM (reviewed in [14]).

To develop an effective dosing strategy over our period of interest, between 7.5 to 10.5 dpc, we injected different doses of TM and scored viable pups at term. A single 2.0 mg injection of TM at 8.5 dpc led to a rapid loss of embryos, whereas two consecutive injections of 1.0 mg at 7.75 and 8.5 dpc, or 8.5 dpc and 9.5 dpc, led to approximately 50% to 75% lethality at term. A single 1.0 mg injection was, however, not lethal ($n > 4$ litters, in each case).

As 1.0 mg TM appeared to be the highest dose tolerated without significant lethality, we tested the effectiveness of a single injection at 7.5, 8.5, 9.5 or 10.5 dpc to activate the reporter, scoring embryos at 11.5 dpc (Figure 2a–d). All embryos carrying both transgenes collected from mice injected at 9.5 dpc ($n = 5$) or 10.5 dpc ($n = 7$) showed extensive β-galactosidase activity throughout the *Wnt1* expression domain, comparable to that seen previously in embryos receiving four consecutive administrations of

Figure 2



Temporal analysis of inducible Cre-mediated recombination. One injection of tamoxifen (1.0 mg) was administered at **(a)** 7.5 dpc, **(b)** 8.5 dpc, **(c)** 9.5 dpc and **(d)** 10.5 dpc, and embryos analysed at 11.5 dpc. Representative embryos are shown. At earlier stages (a,b), some β-galactosidase staining was detected but only in areas where the transgene was expressed at early stages. Later injections (c,d) led to strong β-galactosidase staining in the entire *Wnt1* pattern. **(e)** Injection of 0.5 mg tamoxifen at 9.5 dpc. **(f)** Injection of 1.0 mg 4-OHT at 9.5 dpc was not more efficient than 1.0 mg tamoxifen in activating the reporter.

TM. Thus, a single dose of TM induced rapid (within 24 hours) recombination-mediated activation of the reporter. Injection of 1.0 mg TM at 7.5 dpc reproducibly gave very limited reporter activation that was restricted to the midbrain, consistent with the early midbrain-restricted expression of the *Wnt1* enhancer ($n = 5$). All numbers refer to embryos carrying both transgenes, all of which gave very similar results. Activation was also less efficient at 8.5 dpc ($n = 11$) and restricted, in the spinal cord, to more rostral regions. Less efficient recombination after injection at 7.5 and 8.5 dpc may reflect lower levels of Cre–ER^{TM} protein, but we note that *Wnt1–Cre* lines activate the reporter efficiently by 8.5 dpc. More likely, the establishment of a chorio-allantoic placental connection around 9.0 dpc increases the availability of ligand to the embryo. These results also indicate that induction of Cre activity is transient.

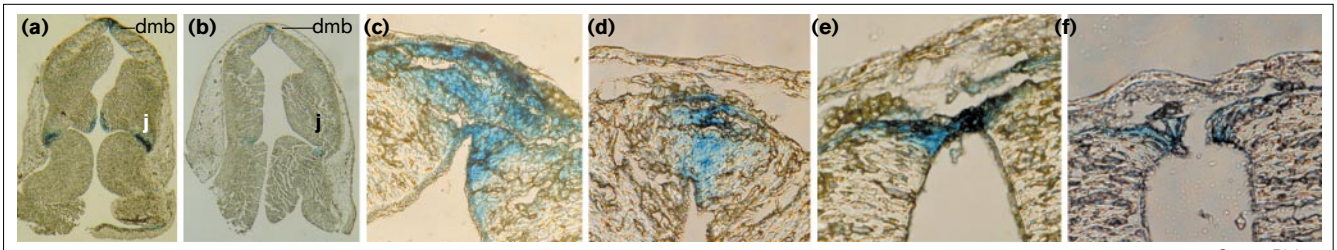
To assess whether lower concentrations of TM induce recombination, we examined reporter activation following injection of 0.5 mg and 0.1 mg TM at 9.5 dpc. No activation was observed with a 0.1 mg dose, indicating that this concentration of TM is below the threshold for activation of Cre–ER^{TM} ($n = 19$, data not shown). Injection of 0.5 mg TM led to a reproducibly reduced frequency of *lacZ*-expressing cells ($n = 12$; Figure 2e). Thus, by lowering the dose and changing the time of TM injection, it is possible to modulate the efficiency and spatial distribution of recombination in target cells. This could be a considerable advantage in some approaches, allowing the production of genetic mosaics in which gain or loss of function is restricted to small populations of cells in an otherwise wild-type background, a standard approach in genetic analyses of *Drosophila* development.

It is thought that TM is converted by the maternal liver into 4-OHT, which is bound more efficiently by the ER [14]. Thus, injection of 4-OHT may induce Cre activity more efficiently; this may, however, be offset by the longer serum half-life of TM (12 hours, compared with 6 hours for 4-OHT) [15]. Our results indicated that injection of a range of concentrations of 4-OHT from 0.1 mg to 2.0 mg at 9.5 dpc gave similar results to TM. No recombination was observed at 0.1 mg; 2.0 mg led to a rapid termination of pregnancy, and 1.0 mg was significantly better than 0.5 mg (data not shown). Interestingly, 1.0 mg 4-OHT was not more effective than 1.0 mg TM ($n = 3$; compare Figure 2c and 2f).

These experiments indicated that the most useful regimen was a single 1.0 mg injection of tamoxifen at 9.5 dpc or later. Analyses of tissue sections indicated that, following a single 1.0 mg injection of tamoxifen at 9.5 dpc, recombination had occurred in around 25% of the cells when compared with the zygotically recombined allele, which represents 100% recombination (for examples, see Figure 3). Note that the recombined transgene expressed *lacZ* from 8.0 dpc, compared with 9.5 dpc (at the earliest) for the transgene in which recombination had been induced by tamoxifen.

In summary, we have demonstrated that ligand-inducible recombination can be used to modulate gene activity in developing mouse embryos. Although the system we describe is simple and effective, there is clearly room for improvement. The biggest problem is the high concentration of ligand required, which is necessitated by the fact that the point mutation that allows preferential binding of 4-OHT rather than 17β-estradiol also decreases the binding affinity for 4-OHT [13]. Consequently, the dose of ligand required to activate Cre–ER^{TM} is quite close to that which interferes with the maintenance of pregnancy. Potential solutions include screening other ligands for their ability to induce Cre–ER^{TM} activity or screening for other ER variants that fail to bind 17β-estradiol efficiently but retain a high affinity for TM or 4-OHT.

Figure 3



Embryo sections showing *lacZ* expression from the Cre reporter following ligand-induced recombination. **(a)** Coronal section through the brain of a representative 11.5 dpc embryo – carrying the zygotically recombined Cre reporter – stained for β-galactosidase activity. **(b)** Similar section of a representative 11.5 dpc embryo in which recombination of the reporter was induced by injection of 1.0 mg tamoxifen at 9.5 dpc. **(c)** High-power view of the dorsal midbrain in (a). **(d)** High-power view of the dorsal midbrain in (b). **(e)** Section through the spinal cord at the level of the dorsal root ganglion and forelimb from a 11.5 dpc embryo carrying the zygotically recombined Cre reporter. Note that migrating neural crest cells stain for β-galactosidase activity. **(f)** Section at the same level as in (e) from an 11.5 dpc embryo in which recombination of the reporter had been induced by injection of 1.0 mg tamoxifen at 9.5 dpc. All sections are 6 μm; dmb, dorsal midbrain; j, midbrain–hindbrain junction.

Supplementary material

Supplementary materials and methods are published with this paper on the internet.

Acknowledgements

We thank Malcolm Parker for pBSK+MERG525 (ER^{TM}), Pierre Vassalli for pCAGGS-Cre, Klaus Rajewsky for pMC-CRE, Benoit St-Jacques for the floxed PGKneobpA construct, Retha Newbold for helpful advice on tamoxifen administration and our colleagues for their help and comments on the manuscript. This work was supported by a postdoctoral fellowship from the Human Frontiers Science Program to P.S.D., a Markey Exploratory Research Grant to P.S.D. and A.P.M. and grants HD30249 and NS33642 from the NIH to A.P.M.

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Supplementary material

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Current Biology 23 November 1998, **8**:1323–1326

Supplementary materials and methods

Construction of transgenes

A reporter construct for Cre recombinase activity *in vivo* was derived from a *Wnt1*-enhancer-based expression construct pWEXPZ2 [S1]. A PGKneobpA cassette [S2] which had been flanked with *loxP* sequences (Benoit St-Jacques and A.P.M., unpublished) was subcloned as a *Sal*I–*Bam*HI fragment into pSL1180 (Pharmacia) with a *Bgl*II–*Xba*I fragment containing the *Escherichia coli lacZ* cDNA. This placed the floxed PGKneobpA cassette upstream of the *lacZ* cDNA. A *Swa*I–*Bam*HI fragment containing this array was subcloned into pWEXPZ2 from which the *lacZ* tag had been removed. This placed the array between the *Wnt1* promoter and *Wnt1* enhancer in pWEXPZ2 (Figure S1).

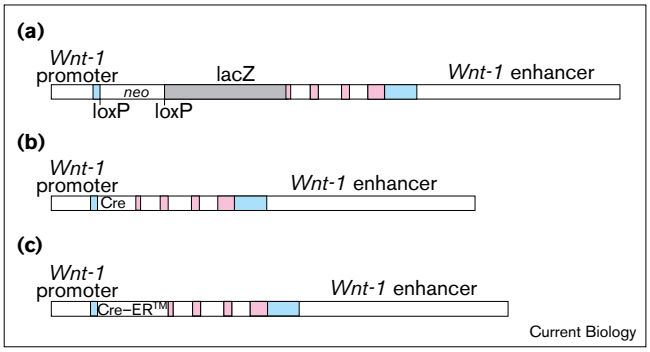
To generate *Cre–ER*^{TM}, a *Cla*I–*Eco*RI fragment from pCAGGS–Cre [S3], containing the cDNA encoding Cre recombinase, was subcloned into Bluescript (Stratagene) and modified using PCR with a KS primer and the primer 5′-TCGCGGATCCCCATCTTCCAGCAGGCGCACC-3′ to generate a *Bam*HI site at the last codon of the Cre cDNA. Following DNA sequence verification, this PCR product was subcloned back into pCAGGS–Cre together with a *Bam*HI–*Eco*RI fragment from pBSK+MERG525 containing the cDNA encoding the hormone-binding domain (amino acids 281–599) of the mutant mouse estrogen receptor, ER^{TM} [S4]. Subsequently, a *Cla*I–*Eco*RI fragment was removed from this plasmid in order to insert a pair of annealed oligonucleotides (5′-GATCAGGCTGGTGCCATGGGC-3′; 5′-GATCGCC-CATGGCACCAGCCT-3′) at the *Bam*HI site, introducing the amino acids QAGAMG between the two components of the fusion protein. This modified *Cla*I–*Eco*RI fragment was then subcloned into Bluescript (Stratagene) with a *Xho*I–*Cla*I fragment from pBS185 (GIBCO BRL) containing part of the Cre cDNA to regenerate the complete Cre–ER^{TM} cDNA. From this plasmid an *Apa*I–*Eco*RI fragment was subcloned into pWEXPZ2 from which the *lacZ* tag had been removed. This placed the cDNA encoding Cre–ER^{TM} between the *Wnt1* promoter and *Wnt1* enhancer in pWEXPZ2 (Figure S1). In initial tests of Cre–ER^{TM} fusion proteins, a Cre cDNA containing sequence encoding a nuclear localisation signal was derived from pMC–Cre. The Cre-expressing transgene will be described elsewhere.

Generation and genotyping of transgenic mice

For electroporation into ES cells, the Cre recombinase reporter construct was digested with *Sal*I and separated from the plasmid backbone by gel electrophoresis and recovered by electroelution as described [S1]. Electroporation of R1 ES cells [S5] with the Cre recombinase reporter construct and selection for cells in which integration had occurred with G418 (GIBCO BRL) was performed essentially as described [S6]. ES cell clones were screened for integration events by Southern analysis of genomic DNA digested with *Sph*I using a radiolabelled probe generated from a 400 bp *Xho*I–*Sph*I genomic fragment from *Wnt1* essentially as described [S7]. The endogenous *Wnt1* gene was detected as a 5 kb band whilst the transgene was detected as a 4.35 kb band. Of 134 clones analysed, 23 clones appeared to contain a single integrated copy of the transgene; 42 two copies; 33 multiple copies; 18 rearranged copies and 18 no copies. As *Wnt1* is not expressed in ES cells [S8], chimeric males were generated directly from six of the clones judged to contain a single copy of the transgene by injection into C57BL/6J recipient blastocysts to generate chimeras [S9]. Chimeric males were intercrossed to C57BL/6J females and agouti offspring were initially genotyped by Southern analysis of genomic DNA using the probe described above. Subsequently Cre recombinase reporter transgenic offspring were genotyped using a PCR-based assay using oligonucleotides identical to sequence at the 3′ end of the PGKneobpA cassette (5′-GCAGGCATGCTGGGGATGCGG-3′) and one identical to the 5′ end of the *lacZ* cDNA (5′-GCGATTAAGTTGGGTAACGC-3′). Amplification was achieved using 40 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C.

Removal of the PGKneobpA cassette by Cre-mediated recombination in zygotes was achieved essentially as described [S3]. Gel-purified supercoiled pCAGGS–Cre plasmid was injected at 0.5 ng/μl into the pronucleus of zygotes from Cre reporter × B6CBAF1 crosses and surviving two-cell embryos were transferred into pseudopregnant SWB recipients. These offspring were initially genotyped by Southern analysis of genomic DNA digested with *Bgl*II using the probe described above and subsequently by a PCR-based assay using oligonucleotides identical to sequence in the *Wnt1* promoter (5′-TAAGAGGCCTATAAGAGGCGG-3′) and that identical to the 5′ end of the *lacZ* cDNA described above using the same reaction conditions. No PCR reaction products were detected using these oligonucleotides and the unrecombined reporter allele. Loss of the PGKneobpA cassette was additionally assessed by PCR using the strategy described for the unrecombined reporter. Of the six lines generated three (9, 44 and 170) were judged to contain a functional Cre reporter transgene. The remaining three clones contained transgenes which failed in this assay and PCR analysis indicated that this was due to the fact that the *Wnt1* promoter had been lost from the transgene.

Figure S1



Wnt1-enhancer-based Cre recombinase reporter and Cre expression transgenes. **(a)** Schematic diagram of the Cre reporter transgene. The presence of the *loxP*-flanked PGKneobpA cassette (*neo*) prevents translation of the *lacZ* cDNA (grey box) until it is deleted by Cre-mediated recombination. The pink and blue boxes represent, respectively, translated and untranslated parts of *Wnt1* exons in the pWEXPZ2 transgene vector. **(b)** Schematic diagram of transgene expressing Cre; details as in (a). **(c)** Schematic diagram of transgene expressing the tamoxifen-inducible Cre–ER^{TM}; details as in (a).

Generation of transgenic mice by pronuclear injection of transgene DNA was performed essentially as described [S1,S10]. For the *Wnt1–Cre* construct of the fifteen founders generated, eight transmitted *Wnt1–Cre* through the germ line and six expressed the transgene in the *Wnt1* pattern. For the *Cre–ER*^{TM} construct, thirteen transgenic founder lines were generated and five out of six male founders transmitted an expressing transgene as judged by whole mount *in situ* hybridisation. Mice carrying the *Cre–ER*^{TM} transgene were initially identified by Southern analysis of genomic DNA digested with *Bam*HI using radiolabelled probes generated from a 600 bp *Bam*HI fragment of the *Cre* cDNA. Transgenic *Cre–ER*^{TM} offspring were subsequently genotyped using a PCR-based assay similar to that described above but with oligonucleotides identical to sequence in the 3′ of the *Cre* cDNA (5′-AACCTGGATAGTGAAACAGGGGC-3′) and sequence in the 5′ of the mouse estrogen receptor hormone-binding domain cDNA (5′-TTC-CATGGAGCGAACGACGAGACC-3′). All embryos were numbered by limb removal during dissection and yolk sacs collected for genotyping by PCR as previously described [S10].

Whole-mount β-galactosidase staining

Analysis of β-galactosidase activity in whole embryos and subsequent histological analysis was performed as described [S10].

Ligand administration

Tamoxifen (Sigma) or 4-hydroxytamoxifen (RBI) were dissolved in warm corn oil (Sigma) at the desired concentration and stored at 4°C. Intraperitnoeal injections of 0.1 ml were given as indicated. Corn oil was injected as a control.

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