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Cre-mediated recombination can induce apoptosis in vivo by activating the p53 DNA damage-induced pathway

Jianjian Zhu^{*}, Minh-Thanh Nguyen^{*,1}, Eiichiro Nakamura^{*,2}, Junming Yang, and Susan Mackem^{3,†}

Cancer and Developmental Biology Laboratory, Center for Cancer Research, NCI-Frederick, Frederick, MD

Abstract

Cre-mediated apoptosis has been observed in many contexts in mice expressing Cre-recombinase, and can confound the analysis of genetically engineered conditional mutant or transgenic alleles. Several mechanisms have been proposed to explain this phenomenon. We find that the degree of apoptosis induced correlates roughly with the copy number of *loxP* sites present in the genome and that some level of increased apoptosis accompanies the presence of even only a few *loxP* sites, as occurs in conditional *floxed* alleles. Cre-induced apoptosis in this context is completely p53-dependent, suggesting that the apoptosis is stimulated by p53 activation in response to DNA damage incurred during the process of Cre-mediated recombination.

Keywords

transgenic mice; *loxP* sites; Cre recombinase; apoptosis

Introduction

Cre recombinase, encoded by the coliphage P1, recognizes a specific 34-bp sequence (*loxP* site) to catalyze site-specific concerted recombination, resulting in either excision of DNA sequences between two directly repeated *loxP* sites, or inversion of DNA sequences between two inverted sites (Hoess and Abremski, 1985; Abremski *et al*, 1986; rev. by Lewandoski, 2001 and Schmidt-Supprian and Rajewsky, 2007). Since Cre-*lox* technology was first developed for use in engineered mouse mutants, there have been several reports of cell death ensuing from Cre-mediated recombination in mouse embryos (Lewandoski and Martin, 1997; Schmidt *et al*, 2000; Loonstra *et al*, 2001; Naiche and Papaioannou, 2007; Gregoire and Kmita, 2008; Huh *et al*, 2010; rev. by Schmidt-Supprian and Rajewsky, 2007). The mechanisms that have been proposed to explain this phenomenon include deleterious gene deletions and genomic rearrangements resulting from recombination at cryptic, endogenous *loxP*-like sites, or genomic rearrangements with chromosome loss resulting from recombination of inverted *loxP* sequences in engineered transgenic mice. Recombination between directly repeated, perfect *loxP* sequences has been presumed to be without cytotoxic consequences, being normally a concerted reaction.

[†]author for correspondence: smack@helix.nih.gov Susan Mackem, Center for Cancer Research, NCI-Frederick, Cancer & Developmental Biology Laboratory, 1050 Boyles St., Bldg 539, Rm 121A, Frederick, MD 21702.

^{*}These authors contributed equally to this paper.

¹current addresses: Dept. Ophthalmology, Univ. Cincinnati, OH

²Dept. of Orthopedic Surgery, Univ. of Occupational and Environmental Health, Kitakyushu Fukuoka, 807-8555 Japan

³Cancer and Developmental Biology Laboratory, CCR, NCI

Recombination at endogenous genomic *loxP*-like sequences provides an explanation for apoptosis observed in several Cre lines independent of introduced engineered *loxP* consensus sequences, which may be particularly problematic at very early embryonic stages with high levels of proliferation (Schmidt *et al*, 2000; Loonstra *et al*, 2001; Naiche and Papaioannou, 2007). However, we have surveyed a number of Cre lines (AP2Cre, Hoxb6Cre, Prx1Cre, Col2CreER, Hoxb6CreER) that do not display appreciable apoptosis at sites of Cre expression in the absence of introduced *loxP* sites (unpubl. results), suggesting that recombination at cryptic endogenous sites is an inefficient and low probability event, perhaps most evident in the context of very high level, early embryonic, and/or sustained Cre expression. Inverted *loxP* sites can clearly cause cell death via chromosomal rearrangements and loss (Lewandoski and Martin, 1997), and this feature has in fact been exploited as a strategy to target the ablation of specific cell types in mice (Gregoire and Kmita, 2008). However, the accidental creation of inverted *loxP* sites in tandemly repeated transgenes due to the inclusion of palindromically inverted transgene copies is expected to be an infrequent event. Tandem arrays are usually integrated into the genome as direct repeats; palindromic inverted repeated sequences (ie. long hairpins) are inherently less stable during DNA replication, and occur infrequently on a random basis both in prokaryotes and eukaryotes including mammals (Gordenin *et al*, 1993; Waldman *et al*, 1999; Voineagu *et al*, 2008 and references therein). We recently noticed cell death associated with the presence of single copy, *floxed* allele in the absence of inverted *loxP* sites (Zhu *et al*, 2008), raising the question of how recombination of low copy number, directly repeated, perfect *loxP* consensus sites can lead to apoptosis.

In the present study, we surveyed several independently generated conditional transgenic lines containing tandemly repeated *loxP* sites and found that some degree of recombination-induced apoptosis occurs with high frequency in *loxP* site-containing transgenic lines, and the amount of apoptosis correlates roughly with *loxP* site frequency. Furthermore, whereas Cre-induced apoptosis in the context of *loxP*-containing lines not expected to contain inverted *loxP* sites occurred over a limited time interval following the onset of Cre expression, presumably because of loss of *loxP* substrate, sustained apoptosis was observed in the case of a transgenic line known to contain inverted *loxP* sites and capable of undergoing multiple rounds of recombination. A low, but detectable level of apoptosis was also observed in certain cases with engineered *loxP* sites known not to have an inverted orientation, suggesting that failed recombination can indeed occur even at perfect *loxP* consensus sequences, albeit with low frequency. Low level, incomplete recombination could result in apoptosis in a small percentage of cells due to DNA damage-induced signals following the rare occurrence of nicks or double strand breaks introduced into the DNA by Cre recombinase (Hoess and Abremski, 1985; Abremski *et al*, 1986). Consistent with this explanation, we found that removal of p53, the major regulator and effector of the apoptotic cascade induced in response to DNA damage (Vogelstein *et al*, 2000; Vousden and Lu, 2002), can rescue cell survival in the context of Cre-driven recombination.

Results and Discussion

Cre-induced apoptosis correlates directly with *loxP* copy number

To evaluate the frequency and severity of Cre-induced apoptosis, we focused on the embryonic limb bud as a model, since apoptosis and subsequent phenotypic consequences can be readily observed without affecting embryo viability in utero per se, and the early limb bud is also a highly proliferative tissue with a short cell cycle time (~12 hrs). A number of Cre drivers that target recombination to the early limb bud have been developed and characterized (Lowe *et al*, 2000; Sun *et al*, 2000; Ahn *et al*, 2001; Logan *et al*, 2002; Nelson and Williams, 2004; Nakamura *et al*, 2006; Grieshammer *et al*, 2008; Nguyen *et al*, 2009;). We surveyed several of these Cre lines with strong constitutive or conditional (tamoxifen-

dependent) Cre expression (AP2Cre, Hoxb6Cre, Prx1Cre, Hoxb6CreER, Col2CreER), but did not observe significant Cre-induced apoptosis or abnormal phenotypes in any of these lines without the introduction of genetically engineered *loxP* sites (data not shown; see also eg. in Fig 3A–B). Consequently, the occurrence of Cre-associated apoptosis cannot always be attributed to activity at cryptic *loxP*-like sites in the presence of high level and/or early timing of Cre expression, since the lines we evaluated include some that have both very robust and very early onset of Cre expression (eg. E8 for Hoxb6 promoter), yet have no appreciable apoptosis. To assess how frequently Cre-induced apoptosis occurs in transgenic lines that contain engineered *loxP* sites, we surveyed a number of different independently generated transgenic lines using a single Cre line, AP2Cre (MGI name Tg(TFAP2A-cre)1Will; Nelson and Williams, 2004), which expresses Cre recombinase uniformly in limb mesenchyme beginning at about the time limb buds first appear as swellings in the lateral plate mesoderm. This AP2Cre line was crossed with α -ACrys-*loxP*-TAg (Lakso *et al*, 1992), Col2-*loxP*-Hoxd12 (E. Nakamura and S. Mackem, unpubl.), and CMV-*loxP*-NT2 transgenic lines (K. Torigoe and Y. Yamada, unpubl.) containing different copy numbers of tandemly repeated *loxP* sites (probably ranging from several to ~250, see below). At E10.5, about 24 hours after onset of AP2Cre expression in the limb bud, varying levels of apoptosis were observed in the limb buds of different transgenic lines (Fig.1), but some apoptosis above background levels was observed frequently in transgenic lines. Although the amount of apoptosis was highly variable between the different lines, the level observed within a given line was very consistent. Notably, some of the *loxP*-containing transgenes surveyed are driven by promoters that are not appreciably expressed in early stage limb buds (Col2 promoter, see eg. Nakamura *et al*, 2006), or that are not expressed at all in the limb (α Acrystallin promoter, see Lakso *et al*, 1992), making it unlikely that the apoptosis is related to transgene expression *per se*. No obvious apoptosis was seen in transgenic embryos negative for the AP2Cre allele (data not shown), consequently the observed apoptosis was also unrelated to integration site effects of the transgene.

To determine whether the severity of apoptosis correlated directly with transgene copy number (*loxP* site frequency), QPCR was used to estimate the transgene copy number for α Acrys-*loxP*-TAg and Col2-*loxP*-Hoxd12 lines relative to a control single copy allele containing the same amplicon used (see Materials and Methods). Both the Col2-*loxP*-Hoxd12 and α Acrys-*loxP*-TAg lines had a very significant amount of apoptosis above endogenous levels (Fig 2), and above that observed in single copy *floxed* alleles (see Fig. 3), with a somewhat higher level of apoptosis apparent in the Col2-*loxP*-Hoxd12 (Fig 2D) than the α Acrys-*loxP*-TAg line (Fig 2C). We estimated the α Acrys-*loxP*-TAg transgenic line contains 32 transgene copies (64 *loxP* sites) in the hemizygote (comparable to a previous estimate of ~50; Lakso *et al*, 1992), and the Col2-*loxP*-Hoxd12 transgenic line contains 128 transgene copies (256 *loxP* sites) in the hemizygote. Hence apoptosis was most prominent in transgenic lines with high copy number *loxP* sites, and correlated roughly with *loxP* copy number. Some apoptosis, albeit at a much lower level, was also observed above endogenous apoptosis even when only 2 *loxP* sites are present, for at least certain *floxed* alleles. Increased apoptosis was not obvious for the widely used Rosa26LacZ reporter alone (Soriano, 1999), when either heterozygous (data not shown) or homozygous (Fig. 3E). However, we have consistently observed that recombination of a single *Shh*-*floxed* allele using either a conditional tamoxifen-dependent Cre driver (see Zhu *et al*, 2008), or the non-conditional AP2Cre (Fig. 3D,F), results in apoptosis above that present in control or in Cre +; *Shh*+/- embryos (Fig. 3A–C), which can only be attributed to recombination at the engineered *loxP* sites. Interestingly, compound mutant embryos positive for both the *Shh*-*floxed* allele and homozygous for the Rosa26LacZ reporter (Fig. 3F) have a somewhat higher level of apoptosis in the presence of Cre than do either those positive for the *Shh*-*floxed* allele or Rosa26LacZ alone, again suggesting that the level of apoptosis observed depends on the total *loxP* substrate dosage. These results indicate that Cre-induced apoptosis

is not solely a consequence of recombination at inverted *loxP* sites, or cryptic endogenous *loxP* sites. Although the possibility of an inverted *loxP* sequence embedded in the tandemly repeated transgene arrays we analyzed cannot be categorically excluded, we consider this a very unlikely general explanation for the observed apoptosis, which was present in most (5/6) of the transgenic lines that we checked, and which was also observed in previously well-characterized *floxed* knock-in alleles, at a reduced level. If apoptosis is directly related to the *loxP* dosage and the number of recombination events, then one would also predict that alleles containing inverted *loxP* sites, capable of undergoing multiple rounds of recombination efficiently (since no product is excised), should exhibit sustained apoptosis in the presence of Cre. We tested this using a modified Brainbow-type fluorescent reporter containing inverted *loxP* sites (Livet et al., 2007) under the control of a ubiquitous promoter (see Fig. 4A). In fact, this allele showed sustained apoptosis over several days (E10.5-E13.5, Fig. 4), in contrast to the other multi-copy *loxP* containing transgenic lines tested, in which apoptosis subsides within about 24 hrs of the onset of Cre expression (eg. CMV-*loxP*-NT2, Col2-*loxP*-Hoxd12, α Acrys-*loxP*-TAg, data not shown). By E13.5, apoptosis in the presence of AP2Cre has lessened (Fig. 4K-M), possibly owing to the decline in AP2 promoter expression by this stage (Zhang and Williams, 2003). Notably, the level of sustained apoptosis is high enough to begin producing digit phenotypes by E13.5 (Fig. 4M).

If Cre-induced apoptosis is solely a consequence of cryptic, endogenous, poor consensus *loxP* sequences, as has been proposed (Schmidt *et al*, 2000; Loonstra *et al*, 2001; Naiche and Papaioannou, 2007), then the apoptosis should depend only on the presence of Cre activity and not exogenously introduced transgenic *loxP* sequences, as observed here. In addition, the severity of apoptosis would correlate only with the expression level of Cre in a particular line, rather than the number of bona fide *loxP* sites introduced transgenically. The high frequency with which apoptosis is detected in *loxP* containing transgenic lines, and the variable level of apoptosis seen between different transgenic lines is also not readily explained by Cre-induced apoptosis owing to the inadvertent introduction of an inverted *loxP* site in tandemly repeated transgenes, which appears to result in a uniformly high level of apoptosis when inverted repeats are present, based both on our results, and other published work (Gregoire and Kmita, 2008).

Cre-induced apoptosis associated with high copy number *loxP* sites leads to phenotypes secondary to extensive cell death

We noticed that Cre-mediated recombination at the early blastocyst stage (using EIIa-Cre expressed ubiquitously in the inner cell mass (Lakso *et al*, 1996) was associated with a high level of prenatal mortality in the case of transgenic lines (such as α Acrys-*loxP*-TAg) containing multiple *loxP* sites (about 35% survival of Cre+; transgene+ offspring relative to the expected frequency, $p < 0.005$, $n = 80$, data not shown). This reduced viability suggested phenotypic consequences related to apoptosis in very early embryos, as did the apparent digit phenotypes following AP2Cre exposure of a Brainbow reporter allele with known inverted *loxP* sequences (Fig. 4M).

To further assess the phenotypic consequences of apoptosis induced by Cre expression at a later organogenesis stage, we turned again to the developing limb and analyzed the effect of AP2Cre-induced apoptosis on limb skeletal development. α Acrys-*loxP*-TAg;AP2cre embryos and Col2-*loxP*-Hoxd12;AP2cre embryos were harvested at E16.5-E17.5 to evaluate the limb skeleton. α Acrys-*loxP*-TAg;AP2cre embryos invariably had mild to moderately shorter limbs, affecting all proximodistal components, although the stylopod was often most severely affected (Fig. 5A). Since the α Acrystallin promoter driving the T-Antigen transgene is not expressed at all in the limb, the observed shortening of skeletal elements can be attributed entirely to the apoptosis induced by Cre-mediated recombination. Col2-*loxP*-Hoxd12;AP2cre embryos harvested at a late skeletal stage had a more severe phenotype

including complete loss of long bone elements as well as severe shortening, and variable loss of anterior digits (Fig. 5B). Since the Col2 transgenic promoter drives expression in chondrocytes, part of this phenotype could be related to enforced transgene expression. However, we have also analyzed the phenotypes of multiple primary founder embryos using the same transgene, but without conditional expression requiring Cre (i.e. no *loxP*-stop sequence present), and found that *Hoxd12* misexpression in cartilage resulted in a consistent, mild phenotype of shortened cartilage elements without any loss of digit or long bone elements (A. Bao, M-T. Nguyen, E. Nakamura and S. Mackem unpublished results; see also Fig. 6). The severe shortening and selective loss of anterior skeletal elements were clearly related to Cre-induced apoptosis. The limb reduction phenotypes observed following Cre-induced apoptosis are overall similar to those observed in mutants in which cell survival is drastically affected in the limb mesoderm (e.g. *Shh*, *Gremlin*, or *Fgf4,8* limb knockouts; Chiang *et al*, 2001; Michos *et al*, 2004; Sun *et al*, 2000).

Apoptosis triggered by Cre-mediated recombination is p53 dependent

Since Cre-related apoptosis is observed consistently in association with the presence of bona fide *loxP* consensus sites, and in this context appears to be dependent on the total *loxP* dosage, we speculated whether this might be a consequence of incomplete recombination by Cre recombinase (i.e. leaving single strand nicks or double strand breaks in the target DNA). Cre recombination is normally a highly concerted reaction, but in the context of poor *loxP* consensus matches, incompletely recombined intermediates with nicks or breaks can be detected readily (Abremski *et al*, 1986). A similar incomplete reaction has been observed even between recombination of two perfect consensus *loxP* sites ((Hoess and Abremski, 1985), albeit at a much lower frequency. p53 is situated at the crossroads of a network of signaling pathways that are essential for cell growth regulation and apoptosis induced by genotoxic and non-genotoxic stresses (Vogelstein *et al*, 2000; Vousden and Lu, 2002). DNA damage such as nicks and breaks normally stimulate a pathway leading to apoptosis mediated by p53 activity, and in fact p53 expression has been observed to increase in Cre-expressing cells (Huh *et al*, 2010). If a response to DNA damage is indeed the mechanism leading to apoptosis, then inactivation of p53 should rescue cell survival in the context of Cre-induced apoptosis.

To test this possibility, we generated α Acrys-*loxP*-TAg;AP2cre and Col2-*loxP*-*Hoxd12*;AP2cre embryos in a p53 null background, and found that apoptosis induced by Cre-mediated recombination in early limb buds in both of these lines is completely blocked when p53 is absent (Fig. 2C–F), suggesting that Cre-induced apoptosis results from p53 pathway activation. To determine whether the rescue of cell survival by p53 deletion was sufficient to restore normal organogenesis (in this case, normal skeletal development), we also examined the limb skeleton of Col2-*loxP*-*Hoxd12*;AP2cre embryos (which had the highest level of apoptosis at early stages). p53 null mutants do have some modest alterations in osteogenesis, which affects primarily late differentiation of osteoblasts, and does not significantly alter the formation of the cartilage model of the limb skeleton (Lengner *et al*, 2006; Wang *et al*, 2006). In the Col2-*loxP*-*Hoxd12*;AP2cre;p53^{-/-} embryos (18/18 embryos from 10 litters), both the formation of all long bone elements and normal digit numbers were restored (Fig. 6). Mild long bone shortening, compared to non-transgenic controls, was still present. This residual phenotypic abnormality in cartilage size was consistent with effects of *Hoxd12* misexpression in cartilage observed in non-conditional, transgenic Col2-*Hoxd12* founder embryos, as discussed above. Hence, the phenotypes attributable entirely to Cre-induced apoptosis (eg. loss of skeletal elements) were rescued by removal of p53.

Based on the ability of p53 removal to rescue both cell survival and normal development, we propose that the rare creation of nicks and double-stranded breaks in genomic DNA generated during Cre-mediated recombination (even between perfect *loxP* consensus sites)

are recognized through activation of the p53 pathway, in turn leading to apoptosis. If the frequency of failed ligation is relatively constant, the frequency of apoptotic cells should correlate with the total number of recombination events/cell (i.e. the more *loxP* sites present, the higher the frequency of apoptosis). The observed dependency of the *loxP*-related apoptosis on the number of *loxP* sequences suggests a mechanism related to the recombination of bona fide *loxP* recognition sites by Cre. Although Cre-mediated recombination is normally a highly concerted process, creation of nicks or double-stranded breaks have been demonstrated as a rare occurrence between such bona fide *loxP* sites (Hoess and Abremski, 1985), which can then act as signals for the repair mechanisms that also trigger apoptosis. Alternatively, the presence of a perfect *loxP* site may stimulate recombination with an endogenous, cryptic-poor consensus partner, resulting in a higher frequency of abortive recombination and production of DNA nicks and breaks (Hoess and Abremski, 1986). Depending on the proximity of cryptic *loxP* sites, this might explain the context dependence observed for the induction of apoptosis by recombination of certain *floxed* alleles, but not others (eg. *Shh*-*floxed* vs. *Rosa26LacZ* alone, see Fig. 3).

This study adds to our understanding of the mechanisms of apoptosis frequently associated with use of the Cre-*loxP* system. Since conditional targeting is such a widespread and important tool to control gene expression spatially and temporally, as previously cautioned (Loonstra *et al*, 2001; Naiche and Papaioannou, 2007; rev. by Schmidt-Supprian and Rajewsky, 2007), it is important to be aware that Cre-mediated apoptosis might contribute to phenotypes. Consequently, appropriate controls are essential to carefully assess the contribution of Cre-related ‘background’ apoptosis to cell death and phenotypes in conditional knockout lines. p53 deletion may in some instances also be a useful strategy for inhibiting the DNA-damage related apoptosis and assist in revealing underlying phenotypes using conditional knockout alleles. The potential problems associated with Cre-induced apoptosis in engineered and conditionally expressed transgenes can be most easily circumvented by the inclusion of a single *FRT* site for Flp-mediated recombination (rev. by Lewandoski, 2001), which will reduce a tandemly repeated array to a single copy transgene integrant. Alternatively, ES cell transfection or lentiviral vector strategies also produce single or low copy transgene integrants (rev. by Park, 2007).

Materials and Methods

Mouse strains

The α Acrys-*loxP*-Tag (Lakso *et al*, 1992), EIIACre (Lakso *et al*, 1996), AP2Cre (MGI name Tg(TFAP2A-cre)1Will; Nelson and Williams, 2004), Hoxb6CreER (Nguyen *et al*, 2009), p53 null mutant (Donehower *et al*, 1992), and *Shh-Floxed* (Lewis *et al*, 2001) mouse lines used have all been previously described. A Col2-*loxP*-Hoxd12 transgene was generated by introducing the Hoxd12 coding sequence (Knezevic *et al*, 1997) into a Col2-promoter/enhancer vector (see Nakamura *et al*, 2006), and inserting a 1.2kbp ‘stop sequence’ flanked by *loxP* sites (Lakso *et al*, 1992) between the Col2 promoter and the Hoxd12 coding sequence to prevent expression prior to recombination. A modified CAGG-Brainbow-2.1 reporter construct was generated by replacing the CMV promoter in Brainbow-2.1 (Livet *et al*, 2007) with a ubiquitous CAGG promoter (Niwa *et al*, 1991). The Col2-*loxP*-Hoxd12 and CAGG-Brainbow-2.1 transgenic lines were generated by pronuclear microinjection of DNA into FVB/n zygotes as described by Hogan *et al*. (1994). CMV-*loxP*-NT2;AP2Cre embryos were kindly provided by Y. Yamada and K. Torigoe. All alleles were maintained on a predominantly FVB/n background. Noon on the date of the vaginal plug was defined as E0.5.

Detection of cell death

Apoptosis was detected using either Nile Blue Sulfate or Lysotracker Red staining (see Zucker et al, 1999). Embryos were dissected in phosphate buffered saline (PBS) and immediately transferred into either 0.1% Nile Blue sulfate (Sigma) solution PBS for staining and bright-field photography, or into 5 μ M Lysotracker Red (Invitrogen L-7528) in PBS. Lysotracker Red stained embryos were dehydrated in methanol and cleared in Murray's clear (BABB) (Sigma), prior to fluorescence photography.

Skeletal staining

Embryos (E16.5-E17.5) were stained in alcian blue/alizarin red, cleared in glycerol/KOH and photographed as previously described (Zhu *et al*, 2008).

Determination of transgene (*loxP*) copy number

The transgene copy number in the α Acrys-*loxP*-TAg and Col2-*loxP*-Hoxd12 lines were determined by qPCR of an amplicon within the SV40 polyA additional signal that was present in all of the lines used, and compared to the RosaLacZ reporter line, which is known to contain 4 SV40 polyA addition sites in the single copy heterozygous knock-in allele (Soriano, 1999). To control for differences in DNA quantity and quality, an endogenous wild type Gli3 gene amplicon was used as an internal control. qPCR was performed with a Roche Lightcycler 480. Reactions were performed in duplicate on genomic DNA from 2 different mice for each of the lines. From the qPCR results, normalized against the Gli3 amplicon, the α Acrys-*loxP*-TAg transgenic line contains 32 transgene copies and the Col2-*loxP*-Hoxd12 transgenic line contains 128 transgene copies per hemizygous allele. The sequences of primers used are given below.

SV40 Late orientation polyA signal (includes 5' linker sequences, italicized):

Forward: 5'-GGGAAGCTTCATAATCAGCCATACCAC-3'

Reverse: 5'-GGGGTCGACTGATAAGATACATTGATG-3'

Gli3:

Forward: 5'-GGCCCAAACATCTACCAACACATAG-3'

Reverse: 5'-GTTGGCTGCTGCATGAAGACTGAC-3'

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Figure 1.

Varying levels of apoptosis in the limb buds of several conditional transgenic lines crossed to the AP2Cre driver line. Embryos were harvested at E10.5 and stained with Nile Blue Sulfate. Forelimb buds are shown in all the panels. The transgenic lines used are indicated at the top of each panel.

Figure 2.

Apoptosis in conditional transgenic lines crossed to AP2Cre in a $p53^{+/-}$ or $p53^{-/-}$ background. Lysotracker Red staining for apoptosis (Zucker et al, 1999) in E10.5 forelimb buds. Extensive apoptosis in both α ACrys-loxP-TAg;AP2Cre; $p53^{+/-}$ (C) and Col2-loxP-Hoxd12;AP2cre; $p53^{+/-}$ embryos (D) was blocked in a p53 null background (E,F), whereas the endogenous apoptosis normally seen in the limb bud at this stage (Fernandez-Teran et al, 2006) was not affected (compare panels A–B with panels E–F).

Figure 3.

Cre-mediated apoptosis in engineered conditional lines containing from 2 to 6 *loxP* sites.

Lysotracker Red staining for apoptosis (Zucker et al, 1999) in E10.5-E11 limb buds.

Rosa26LacZ-hom;AP2Cre+ embryos (C) show no increase in apoptosis compared to either

wild-type (A) or AP2Cre+ embryos (B). *Shh^{FL/+}*;AP2Cre+ embryos with 2 *loxP* sites (D)

display an increased level of apoptosis compared to *Shh^{+/-}*;AP2Cre+ (C) and control

embryos (A,B), whereas *Rosa26LacZ-hom*;AP2Cre+ embryos (E) with 4 *loxP* sites have

similar levels of apoptosis compared to either wild-type (A) or AP2Cre+ embryos (B).

However, *Rosa26LacZ-hom*; *Shh^{FL/+}*;AP2Cre+ embryos (F), having 4 additional *loxP* sites

more than *Shh^{FL/+}*, display an increase in the level of apoptosis compared to *Shh^{FL/+}*;AP2Cre + embryos (compare panel F to panels D, E).

Figure 4. Sustained apoptosis in Cre⁺ embryos carrying transgenes containing inverted *loxP* sites

A: Diagram of CAGG-Brainbow-2.1 transgene, modified from CMV-Brainbow-2.1 (Livet *et al.*, 2007), containing two pairs of inverted *loxP* sites flanking fluorescent protein genes. **B-M:** Lysotracker Red staining for apoptosis in E10.5–E13.5 hindlimb buds. CAGG-Brainbow-2.1;AP2Cre⁺ embryos (**D**, **G**, **J**, **M**) display a sustained, increased level of apoptosis compared to either wild-type (**B**, **E**, **H**, **K**) or AP2Cre⁺ embryos (**C**, **F**, **I**, **L**). Note that apoptosis lessens by E13.5 (compare panel **M** to panels **D**, **G**, **J**), while a consequent digit phenotype is apparent by this stage (**M**).

Figure 5. Skeletal phenotypes of α ACrys-loxp-TAg;AP2cre (E16.5) and Col2-loxp-Hoxd12;AP2cre embryos (E17.5). All three limb skeletal components are shortened in α ACrys-loxp-TAg;AP2cre forelimbs compared to cre negative control littermates (left panels). More severe phenotypes, including marked long bone shortening, complete loss of radius and the loss of anterior digits, are invariably seen in Col2-loxp-Hoxd12;AP2cre forelimbs (right panels). Note that AP2Cre is not expressed in scapula progenitors, which are spared.

Col2-loxp-Hoxd12; AP2Cre

Cre-;p53^{+/-}Cre+;p53^{-/-}Cre+;p53^{+/-} **Figure 6.**

Skeletal phenotypes of Col2-loxP-Hoxd12;AP2cre embryos generated in a p53^{+/-} or p53^{-/-} background. E17.5 forelimbs are shown in all the panels. Note that severe phenotypes such as missing long bone elements and loss of anterior digits (lower panel) are absent from p53^{-/-} embryos, although a milder long bone shortening phenotype remains (middle panel).