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Non-parallel recombination limits Cre-LoxP-based reporters as precise indicators of conditional genetic manipulation

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Abstract

Cre/LoxP-mediated recombination allows for conditional gene activation or inactivation. When combined with an independent lineage-tracing reporter allele, this technique traces the lineage of presumptive genetically modified Cre-expressing cells. Several studies have suggested that floxed alleles have differential sensitivities to Cre-mediated recombination, which raises concerns regarding utilization of common Cre-reporters to monitor recombination of other floxed loci of interest. Here, we directly investigate the recombination correlation, at cellular resolution, between several floxed alleles induced by Cre-expressing mouse lines. The recombination correlation between different reporter alleles varied greatly in otherwise genetically identical cell types. The chromosomal location of floxed alleles, distance between LoxP sites, sequences flanking the LoxP sites, and the level of Cre activity per cell all likely contribute to observed variations in recombination correlation. These findings directly demonstrate that, due to non-parallel recombination events, commonly available Cre reporter mice cannot be reliably utilized, in all cases, to trace cells that have DNA recombination in independent-target floxed alleles, and that careful validation of recombination correlations are required for proper interpretation of studies designed to trace the lineage of genetically modified populations, especially in mosaic situations.

Keywords

Mosaic analysis; lineage tracing; cell autonomous; Cre detection; non-parallel recombination

In mouse genetic studies, Cre recombinase mediates DNA recombination between LoxP sequences to allow for manipulation of gene activity. Cre-based lineage tracing alleles are often utilized to determine the consequence of genetic manipulation at other floxed loci within individual cells, or populations of cells. However, these systems assume that activation of reporter allele indicates recombination at other floxed loci (Herrera et al., 1998; Kawaguchi et al., 2002; Gu et al., 2003; Dzierzak and Speck, 2008; Fox et al., 2008; Spence et al., 2009; Kretzschmar and Watt, 2012; Lao et al., 2012). However, because the chromosomal location of LoxP sites (Vooijs et al., 2001), distances between LoxP sites (Collins et al., 2000; Koike et al., 2002; Zong et al., 2005), and cell type specific epigenetic context of floxed loci (Hameyer et al., 2007; Long and Rossi, 2009) all affect recombination efficacy, the occurrence of recombination in one allele may not predict recombination in another within the same cell. Here, we directly demonstrate such nonparallel recombination with commonly utilized Cre-reporter alleles using several Cre/CreERT mouse lines (Table 1).

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First, we derived *Ngn3*$^{B\text{-}Cre}$; *R26R*$^{Ai9/eYFP}$ neonatal mice, wherein the reporter alleles at the *Rosa26* locus are *R26R*^{ai9} and *R26R*^{eYFP}, and examined the extent of parallel reporter activation in endocrine and exocrine pancreatic lineages. Progenitor cells that express high levels of *Ngn3* become endocrine islet cells, whereas cells that express low levels of *Ngn3* become exocrine cells (Schonhoff et al., 2004)(Wang et al., 2010). These properties allowed us to assess the influence of differential *Ngn3*$^{B\text{-}Cre}$-driven promoter activity on the recombination correlation between *R26R*^{ai9} and *R26R*^{eYFP} (express RFP (tDT) or eYFP, respectively). Most, if not all, endocrine islet cells (recognizable as tightly-packed cell clusters) in *Ngn3*$^{B\text{-}Cre}$; *R26R*$^{Ai9/eYFP}$ neonatal pancreas produced both reporters. In contrast, many acinar and duct cells only produced a single reporter, indicating non-parallel recombination (Fig 1a–c). These above findings suggest that 'high *Ngn3*-expressing cells' produced high Cre levels sufficient to recombine both alleles within individual cells, whereas low-*Ngn3*-expressing cells recombine one allele but not the other.

To assess the incidence of non-parallel recombination under mosaic experimental conditions, we used a *Sox9*^{CreERT2} transgene to drive CreERT2 [a tamoxifen (TM)-inducible Cre], to recombine a Cre reporter (*R26R*^{eYFP}) and a floxed Pdx1 allele (*Pdx1*^{FLOX}) whose recombination results in a null mutation. All pancreatic progenitor cells express *Sox9* and a low level of *Pdx1* (Pdx1^{Lo}). When pancreatic progenitor cells differentiate into beta cells, *Pdx1* expression is upregulated (Pdx1^{Hi}), while Sox9 becomes inactivated (Fujitani et al., 2006; Kopp et al., 2011). Therefore, any Sox9^{+} pancreatic progenitor cell that has inactivated *Pdx1* will be incapable of becoming a Pdx1^{Hi}Sox9^{-} cell. We administered 0.3 mg/mouse TM to plugged females at E12.5 to activate CreERT2 in *Pdx1*$^{FLOX/-}$; *Sox9*^{CreERT2}; *R26R*^{eYFP} in mosaic fashion, and scored YFP^{+} individuals for Sox9 and Pdx1 expression status. Three days after TM administration, about half of the eYFP^{+} cells retained Pdx1 production, with a portion of these cells displaying a high Pdx1 signal (Fig. 1d–g), demonstrating that the *Pdx1*^{FLOX} allele is not inactivated even though recombination in the *R26R*^{eYFP} allele had occurred in some cells. Together, the above findings demonstrate that different levels of Cre influence the efficiency with which one can recombine two independent floxed alleles in an individual cell.

Several available reporters including *R26R*^{eGFP}, *R26R*^{ai9}, *R26R*^{mTmG}, and *R26R*^{eYFP} are derived by Rosa26-based targeting, and contain different stop signals and reporter genes (Table 1). Conversely, Z/EG reporter is an insertion based-transgene (Lobe et al., 1999). Recombination events in *R26R*^{ai9}, *R26R*^{mTmG}, *R26R*^{eYFP}, and *Z/EG* lines activate a downstream fluorescence reporter only, whereas recombination in *R26R*^{eGFP} results in an IRES-based bi-cistronic mRNA that produces both rtTA and eGFP (Fig. 2a). Thus, *R26R*^{eGFP} produces lower levels of eGFP compared to other reporters after recombination. Yet the eGFP expression pattern in *R26R*^{eGFP} faithfully identifies cells that have undergone recombination (Belteki et al., 2005). In order to evaluate, within a linear range, the level of Cre required to activate each reporter gene, we took advantage of a *Pdx1*^{CreERT} line that maintains a low level of Cre activity in pancreatic progenitor cells (Gu et al., 2002) in the absence of TM (see below). No TM-independent recombination, scored by reporter expression, was observed in *Pdx1*^{CreERT}; Z/EG pancreatic cells (Fig. 2b). Similarly, less than 0.1% of all pancreatic cells of *Pdx1*^{CreERT}; *R26R*^{eGFP} underwent recombination (n=6. Fig. 2c). Both *Pdx1*^{CreERT}; *R26R*^{eYFP} and *Pdx1*^{CreERT}; *R26R*$^{mT/mG}$ mice displayed between 0.4–2.7 % pancreatic cells with recombination (n=6–8. Fig. 2d and e). Surprisingly, over one-third of all pancreatic cells in *Pdx1*^{CreERT}; *R26R*^{ai9} mice recombined to express RFP (Fig. 2f. n=5). None of the reporter mice express detectable FPs in the absence of the Cre-expressing transgene (Fig. 2g and data not shown). To confirm that the lack of reporter gene expression was not a result of gene silencing after recombination, we examined DNA recombination in *R26R*$^{ai9/mTmG}$; *Pdx1*^{CreERT} (with two reporter alleles at the *Rosa26* locus) pancreas by PCR analysis. Recombinant DNA product was detected from the *R26R*^{ai9} allele

but not from *R26R*^{mTmG} (Fig. 2h). As a positive control for PCR detection, recombinant products were detected at *R26R*^{ai9} and *R26R*^{mTmG} loci in *R26R*$^{ai9/mTmG}$; *Pdx1*^{Cre} pancreas (Fig. 2i). Taken together, these data demonstrate differential recombination efficiencies between select reporter alleles in a model for low level Cre activity.

The above findings strongly suggest that experimental lineage tracing results may vary in a reporter line dependent fashion. We tested this possibility by following the lineage of *Ngn3* expressing cells using different reporter alleles, in order to reconcile observations suggesting in one case that Ngn3^{+} cells only give rise to endocrine islet cells (Gu et al., 2002), and in another case that Ngn3^{+} cells also give rise to exocrine pancreatic cells (Schonhoff et al., 2004; Wang et al., 2010). We derived *Ngn3*$^{B\text{-}Cre}$; *Z/EG* and *Ngn3*$^{B\text{-}Cre}$; *R26R*^{eYFP} adult pancreata to examine eGFP or eYFP expression. Most of the identifiable EGFP^{+} cells in *Ngn3*$^{B\text{-}Cre}$; *Z/EG* pancreas were localized in cell clusters producing endocrine hormones (Fig. 3a–c). Several eGFP-producing cells were found singularly and within the duct of *Ngn3*$^{B\text{-}Cre}$; *Z/EG* pancreas, yet these lineage-traced cells also expressed endocrine hormones (inset in Fig. 3b). In contrast, a large number of eYFP^{+} cells were found in pancreatic duct and acinar tissue of *Ngn3*$^{B\text{-}Cre}$; *R26R*^{eYFP} mice (Fig. 3d–f). These findings suggest that reporter selection in Cre-reporter-based lineage tracing influence experimental outcomes.

Finally, we investigated whether high levels of Cre activity can normalize the observed differential sensitivities of *R26R*^{eGFP} and *R26R*^{eYFP} reporters. Both *Pdx1*^{Cre}, *and* *Pdx1*^{CreERT} under high TM dose conditions were used to produce high Cre activities. One milligram of TM was administered into E14.5 pregnant female mice to activate CreERT in *Pdx1*^{CreERT}; *R26R*^{eYFP} or *Pdx1*^{CreERT}; *R26R*^{eGFP} embryos, respectively. Reporter expression was characterized in newly born neonates (n=3). Over a third of pancreatic cells in *Pdx1*^{CreERT}; *R26R*^{eYFP} mice expressed eYFP (Fig. 4a), whereas less than 8% of pancreatic cells in *Pdx1*^{CreERT}; *R26R*^{eGFP}mice expressed eGFP (Fig. 4b). Similarly, *Pdx1*^{Cre} induced *eYFP* expression in more than 85% of *Pdx1*^{Cre}; *R26R*^{eYFP} pancreatic cells at E11.5 (Fig. 4c), but only 35% of pancreatic cells activated eGFP expression in *Pdx1*^{Cre}; *R26R*^{eGFP} pancreata at the same stage (Fig. 4d). By E14.5, over 93% of pancreatic cells in *Pdx1*^{Cre}; *R26R*^{eYFP} mice activated eYFP expression (Fig. 2e), whereas only 72% pancreatic cells activated eGFP expression in *Pdx1*^{Cre}; *R26R*^{eGFP} mice (Fig. 4f) (n=3). These data suggest that increased Cre activity is not sufficient to normalize the differential sensitivity detected for these two floxed reporter alleles.

Our studies demonstrate that *R26R*^{ai9} is the most sensitive reporter of Cre-mediated recombination, whereas *Z/EG* is the least sensitive reporter. Our studies also directly demonstrate that Cre-mediated recombination in one floxed allele does not necessarily report recombination at another allele within the same cell. While this phenomenon has been implicated in other experimental settings, it has not been demonstrated directly (Vooijs et al., 2001)(Hameyer et al., 2007; Schmidt-Supprian and Rajewsky, 2007; Long and Rossi, 2009). Importantly, observed non-parallel recombination is particularly severe in cells with low levels of Cre activity, which can occur in many experimental settings, such as weak promoter-driven Cre or TM-inducible CreER activation for mosaic analysis.

Our data further implies that reporter sensitivity inversely correlates with the distance between LoxP sites in the R26-reporter transgenes (table 1). This implication also seemed applicable to the *Z/EG* line, which has the greatest inter-LoxP distance and lowest sensitivity to recombination, albeit with the additional variable that the *Z/EG* cassette is located on a different chromosome from the R26-based reporters. Furthermore, one unexpected finding from our analysis is that *R26R*^{eYFP} and *R26R*^{eGFP} have vastly different recombination sensitivities despite identical floxed stop signals. This observation suggests that sequences outside the LoxP sites may influence Cre-based recombination efficiencies.

At least two mechanisms could contribute to this difference. It is likely that the different sequences downstream the LoxP sites of *R26R*^{eYFP} and *R26R*^{eGFP} result in different methylation status, which has been suggested to affect recombination efficiency (Long and Rossi, 2009). Alternatively, the different sequences surrounding LoxP sites could form different nucleasomal structures that affect the accessibility of LoxP sites to Cre enzyme. Our current data do not allow us to differentiate these two possibilities. Future efforts to unravel these possibilities will likely facilitate engineering floxed alleles with specific recombination efficiencies.

Methods

Mouse lines *R26R*^{EYFP}, *R26R*^{Ai9}, *Z/EG*, and *R26R*^{eGFP} (see Table 1 for details) were from Jackson Laboratory (Bar Harbor, ME). *Ngn3*$^{B\text{-}Cre}$ was a gift from A. Leiter (Schonhoff et al., 2004). *Sox9*^{CreERT2} was a gift from M. Sander (Kopp et al., 2011). *Pdx1*^{CreERT}, *Pdx1*^{Cre}, and *Pdx1*^{FLOX} were previously reported (Gu et al., 2002; Wang et al., 2007a; Gannon et al., 2008).

Tissue collection and section preparation followed published methods (Wang et al., 2008). Antibodies utilized were: Chicken anti-GFP (Aves Labs, Inc., Tigard, OR), 1:500. Rabbit anti-Sox9 (Millipore, Billerica, MA). Guinea pig anti-insulin, guinea pig anti-glucagon, rabbit anti-SS, goat anti-PP, FITC-conjugated Donkey anti-rabbit, FITC-conjugated Donkey anti-guinea pig, Cy3-conjugated Donkey anti-rabbit, Cy3-conjugated Donkey anti-guinea pig, Cy3-conjugated Donkey anti-goat, Cy5-conjugated Donkey anti-rabbit, Cy5-conjugated Donkey anti-goat, and FITC-conjugated streptavadin were all from Jackson Immunoresearch. Biotin-DBA was from Sigma Aldrich. Antibody detection followed standard protocols (Wang et al., 2007b). All antibodies were utilized at a 1:1000 dilution ratio, or as noted. Conventional PCR (31 cycles) was utilized to detect the genomic sequences derived from recombination of *R26R*^{ai9} and *R26R*^{mTmG} alleles. Control oligos to amplify genomic DNA: 5′-ccatgcatatgcctggtgcttgt and gggttaggattaagagttttagt-3′. Oligos for detecting the recombination product P1: 5′-ggttcggcttctggcgtgtgac-3′, P2: 5′-gcaccttgaagcgcatgaactc-3′ and P3: 5′-acgctgaacttgtggccgtttac-3′ (see Fig. 2a).

Both confocal imaging and epifluorescence microscopy were used. For semi-quantification, a quarter (embryonic pancreata) to one tenth (postnatal pancreata) of the pancreas, in 10–20 μm sections, was analyzed. Images (usually low magnification to image a large area, but high enough to discern single cells properly) were captured at >20 microscopic views/slide. Cell numbers were quantified with Image J.

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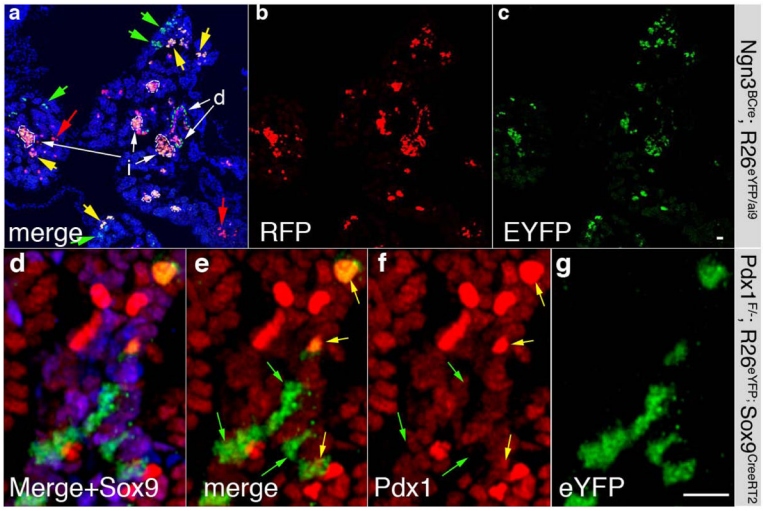

Figure 1. Recombination in multiple floxed alleles are independent events

(a–c) Reporter activation in neonatal *Ngn3*^{BCre}*; R26R*$^{eYFP/ai9}$ pancreas. RFP (tDT), eYFP, and a merged channel are presented. Islet cells, broken-lined circle (Panel a). Green arrows, eYFP only cells. Red arrows, RFP only cells. Yellow arrows indicate cells expressing both fluorescent proteins. (d–g) E15.5 pancreatic sections from *Pdx1*$^{FLOX/-}$*; Sox9*^{CreERT2}; *R26R*^{eYFP} embryos (0.3 mg TM injected at E12.5) with immunodetection for Pdx1, Sox9, and eYFP. Two merged images [d (Pdx1, Sox9, and eYFP) and e (Pdx1 and eYFP)] and two individual channels [f (Pdx1) and g (eYFP)] are shown. Yellow arrows, Pdx1^{+}eYFP^{+} cells. Green arrows, Pdx1^{-}eYFP^{+} cells. Bars=20 μm.

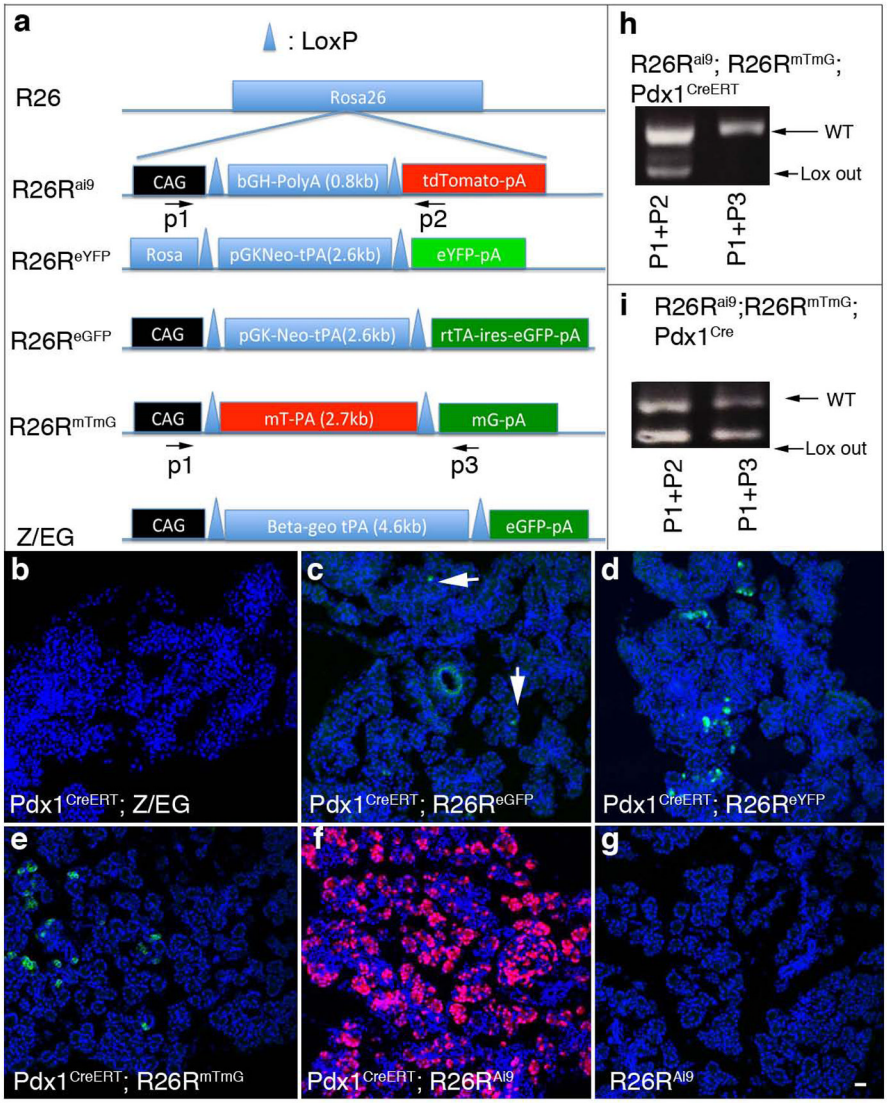

Figure 2. Cre-reporter alleles have different sensitivity to Cre-induced recombination

(a) Diagrammatic representations of the reporter alleles utilized in this study. (b–g) Representative neonatal pancreatic sections of *Pdx1*^{CreERT} transgene in combination with reporter lines: *Z/EG, R26R*^{eYFP}*, R26R*^{eGFP}*, R26R*^{TG}, and *R26R*^{Ai9} without TM administration. Natural fluorescence of the expressed FPs is shown. White arrows in panel c point to two eGFP^{+} cells. An image of *R26R*^{ai9} pancreatic section (without *Pdx1*^{CreERT}) is also included, to show that its reporter activation depends on the presence of CreERT (g). DAPI marks all nuclei, including non-pancreatic mesenchymal cells that do not express Cre. (h and i) PCR-based detection of DNA products after expected recombination in *R26R*^{ai9}*; R26R*^{mTmG}*; Pdx1*^{CreERT} (h) and *R26R*^{ai9}*; R26R*^{mTmG}*;Pdx1*^{Cre} (i) pancreas, respectively. The locations of utilized oligos, P1, P2, and P3 are noted in panel (a). A wild control band (WT, a fragment in the Myt1 locus) was utilized as PCR control (450bp). The Lox out band were only detectable after Cre mediated recombination (250bp). Bar=20 μm.

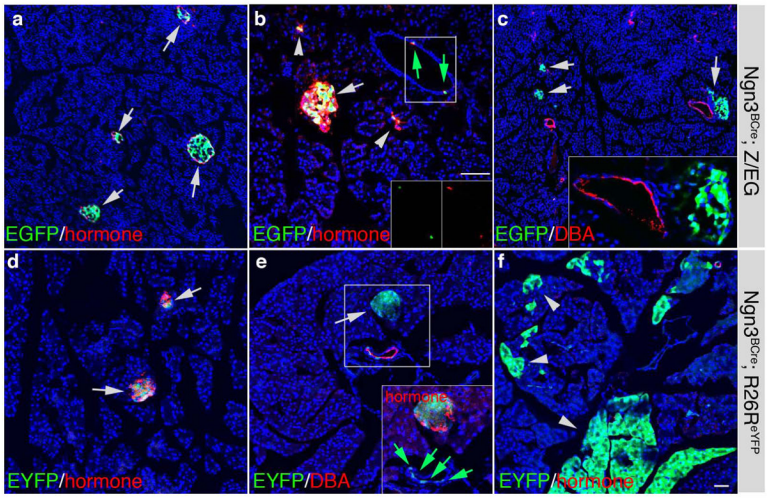

Figure 3. Cre-reporter allele sensitivity can lead to different lineage tracing outcomes

Ngn3^{BCre}-mediated recombination of the *Z/EG* (a–c) and *R26R*^{eYFP} (d–f) reporter alleles. Sections from 2 months old pancreata were utilized [counter-stained with DAPI (blue)]. Islets are marked with arrows. In sections of *Ngn3*^{BCre}*; Z/EG* pancreata, GFP^{+} cells did not react with DBA lectin, which specifically marks pancreatic ducts (c). Note that eGFP^{+} cells observed in duct (green arrows) expressed endocrine hormones (insets in b). Inset in c, a higher magnification image to show lack of green cells in a duct section. In *Ngn3*^{BCre}*; R26R*^{eYFP} mice, significant numbers of duct (e, green arrows) and acinar cells (f, white arrowhead) expressed eYFP reporter. In e, the inset shows hormone staining within the boxed area. Also note that acinar labeling in pancreatic tissues are not randomly distributed, so that some microscopic fields do not have eYFP^{+} exocrine cells [I don't get the previous sentence] (d and e). Bar=50 μm.

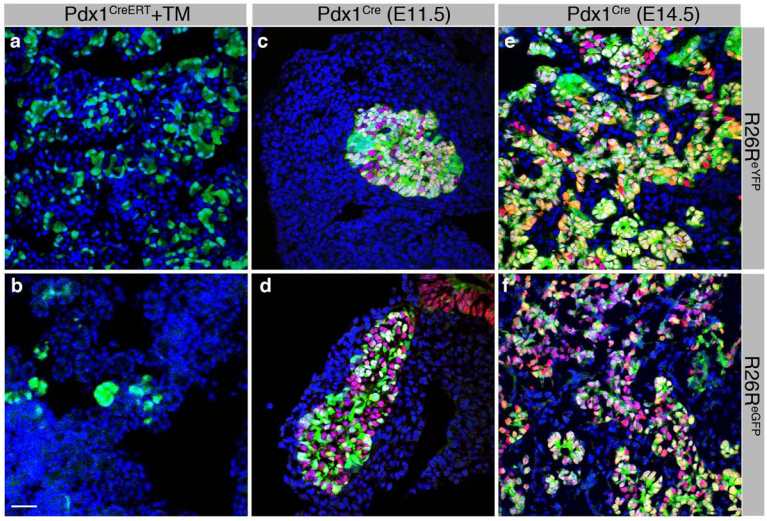

Figure 4. Increased Cre activity cannot normalize the variable sensitivity of different floxed alleles

Neonatal pancreatic sections of different reporter mice when activated by increased levels of Cre with *Pdx1*^{CRETM} plus TM administration or *Pdx1*^{Cre}. In the presence of TM, *Pdx1*^{CreERT} induced over a third of pancreatic cells to activate reporter expression in *R26R*^{eYFP} (a). Less than 8% pancreatic cells activated eGFP in *R26R*^{eGFP} mice with TM+ Pdx1^{CreERT} (b). Similarly, *R26R*^{eYFP} also showed higher recombination than *R26R*^{eGFP} when a *Pdx1*^{Cre} line was analyzed at E11.5 and E14.5 (c–f). Note the red only cells (Pdx1 staining to visualize the pancreatic cells) in panels c–f, which indicate the cells that failed to undergo recombination. DAPI, blue. Bar=50 μm.

Table 1

Reporter alleles, Cre drivers, and conditional alleles used in this study.

Reporter Name	Official strain name	Floxed sequences/trans-gene	References
R26R^{Ai9}	*(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$*/J	3XpolyA	(Madisen et al., 2010)
R26R^{eYFP}	*Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$*/J	Pgk-Neo-3XpolyA	(Srinivas et al., 2001)
R26R^{EGFP}	Gt(Rosa)26Sor$^{<tm1(rtTA,EGFP) Nagy>}$/J	Pgk-Neo-3XpolyA	(Belteki et al., 2005)
R26R^{mTmG}	*Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$*/J	Membrane-tagged td-Tomato-PolyA	(Muzumdar et al., 2007)
Z/EG	Tg(CAG-Bgeo/GFP)21Lbe/J	LacZ-polyA	(Novak et al., 2000)
Ngn3^{B-Cre}	Tg(Neurog3Cre)C1Able/J	Cre	(Schonhoff et al., 2004)
Pdx1^{Cre}	Tg(Pdx1-Cre)89.1	Cre	(Gu et al., 2002)
Pdx1^{CreERT}	Tg(Pdx1-cre/Esr1*)35.10Dam	CreERT	(Gu et al., 2002)
Pdx1^{FLOX}	Pdx1-tm4Cvw	Conditional LOF allele	(Gannon et al., 2008)
Sox9^{CreERT2}	Tg(Sox9-cre/ERT2)1Msan	CreERT2	(Kopp et al., 2011)