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The *Wnt1-Cre2* transgene is active in the male germline

Colin J. Dinsmore, Chen-Yeh Ke, Philippe Soriano*

Department of Cell, Development, and Regenerative Biology, Icahn School of Medicine at Mount Sinai, New York, NY 10029

Abstract

The *Wnt1-Cre* transgenic mouse line is widely used to express the Cre recombinase in neural crest lineages, but it overexpresses *Wnt1* itself, which can cause undesired phenotypes. To address this, we and others previously developed a *Wnt1-Cre2* line based on the same regulatory elements as *Wnt1-Cre* but without ectopic *Wnt1* expression. However, while *Wnt1-Cre2* exhibits normal activity when transmitted from female mice, it exhibits unexpected activity in the male germline. The *Wnt1-Cre2* transgene was previously mapped to the *E2f1* locus. Several genes in this genomic region exhibit significant expression in spermatogonia or spermatocytes, suggesting that local regulatory elements may be driving ectopic transgene expression. The *Wnt1-Cre2* line can therefore be used both as a neural crest specific and a general deleter, and care should be taken when setting up genetic crosses.

Keywords

Wnt1-Cre; Wnt1-Cre2; mouse genetics; germline

Introduction

Wnt1-Cre is a transgenic *Cre* line used extensively to drive recombination in the dorsal neural tube and neural crest (Danielian et al., 1998; Jiang et al., 2000). However, the original *Wnt1-Cre* line resulted not only in *Cre* expression but also ectopic expression of *Wnt1* itself (Lewis et al., 2013). Ectopic expression of *Wnt1* caused several unintended consequences, including alterations in neural tube patterning and overgrowth of the midbrain. To circumvent this, we previously generated a second *Wnt1-Cre* transgenic line, *Wnt1-Cre2*, using the same regulatory elements as the original line but without the possibility of ectopic *Wnt1* expression (Lewis et al., 2013). Crosses to reporter lines indicated an identical expression pattern as the original *Wnt1-Cre* but *Wnt1* itself was expressed at normal levels.

*Corresponding author: philippe.soriano@mssm.edu.

Author Contributions

CJD, CYK, and PS designed the study. CJD and CYK analyzed crosses. CJD performed expression analysis and testis staining. CJD, CYK, and PS wrote the manuscript.

Competing Interests Statement

The authors declare no competing financial or non-financial interests.

We subsequently received reports and sometimes observed ourselves evidence of germline recombination using *Wnt1-Cre2*. We therefore set up a series of crosses to determine the penetrance of this phenotype and whether it was due to activity in the male germline, female germline, or sporadic activity in the epiblast itself, any of which might result in complete recombination of a conditional allele in the subsequent generation. We found that *Wnt1-Cre2* is highly expressed in the male germline, but not in the female germline or epiblast. We confirmed that *Wnt1-Cre2* drives reporter expression in spermatogonia of adult male mice. Further, we identify several genes near the *Wnt1-Cre2* transgene insertion site that are highly expressed in the male germline. Their regulatory elements likely contribute to the ectopic germline expression of *Wnt1-Cre2*.

We caution researchers using this line to take the possibility of germline recombination into account when setting up crosses and always utilize genotyping primers that can detect wild type, conditional, and recombined () alleles to best manage their colony and accurately genotype embryos.

Results and Discussion

To determine the extent of germline activity in the *Wnt1-Cre2* transgenic line, we first crossed *Wnt1-Cre2^{Tg/+}* mice to *ROSA26^{mTmG/mTmG}* mice, generating G1 *Wnt1-Cre2^{Tg/+}; ROSA26^{mTmG/+}* double heterozygous mice of both sexes. *ROSA26^{mTmG}* mice constitutively express membrane targeted tdTomato but switch to expressing membrane targeted EGFP in cells (and their progeny) where *Cre* is expressed (Muzumdar et al. 2007). These G1 mice were then crossed to wild type mice and the resulting G2 embryos were subject to genotyping and characterization by fluorescence microscopy. We found that 100% of embryos that inherited the *ROSA26^{mTmG}* and *Wnt1-Cre2* alleles together through the female lineage displayed the expected pattern of recombination, i.e. completely tdTomato positive in *Cre*-negative embryos (n=15) and a GFP-positive neural crest lineage in *Cre*-positive embryos (n=14) (Figure 1, top). On the other hand, 100% of embryos that inherited the *ROSA26^{mTmG}* allele from a sire also positive for *Wnt1-Cre2* showed recombination throughout the embryo (100% GFP-positive), whether they inherited the *Wnt1-Cre2* allele (n=28) or not (n=25), indicative of significant CRE activity in the male germline (Figure 1, bottom). Genotyping results are summarized in Table 1.

To test the possibility that a paternally-inherited *Wnt1-Cre2* allele could act in trans and recombine a female-inherited *flox* allele, either through residual CRE protein associated with the male pronucleus or activation of the gene during early embryogenesis, we also analyzed several embryos resulting from male *Wnt1-Cre2^{Tg/+}* mice crossed to female *ROSA26^{mTmG/mTmG}* mice. The resulting embryos all showed the expected fluorescence pattern for their genotype, ruling out this possibility (data not shown).

To confirm that CRE activity is already present in adult male mice, we examined the testes of three adult male G1 *Wnt1-Cre2^{Tg/+}; ROSA26^{mTmG/+}* mice. All three showed extensive GFP signal in the seminiferous tubules, with particularly high expression in the maturing spermatocytes (Figure 2). As a control, we also examined two adult male G1 *Wnt1-Cre^{Tg/+}; ROSA26^{mTmG/+}* mice, which did not show GFP signal in this tissue (Figure

2, bottom). Somatic tissue remained unrecombined (red) in both lines, indicating CRE activity specifically in the germline of male *Wnt1-Cre2* mice.

Because the original *Wnt1-Cre* transgene did not exhibit germline recombination, we suspected that regulatory elements near the *Wnt1-Cre2* insertion site were responsible for this behavior. *Wnt1-Cre2* has been previously mapped to the *E2f1* locus and the nature of the insertion extensively characterized (Goodwin et al., 2019). The insertion resulted in a complicated rearrangement in which a genomic segment was duplicated and inverted (Figure 3A, green stars) and a smaller section within the duplication was deleted (Figure 3A, magenta stars). 1–3 copies of the *Wnt1-Cre2* cassette inserted at this deletion site. The final *Wnt1-Cre2* allele likely disrupts the *E2f1* gene (Figure 3A, bottom).

In addition to *E2f1*, the genes *Necab3*, *Actl10*, *1700003F12Rik*, and *Cbfa2t2* are near the *Wnt1-Cre2* insertion site. We first assessed the literature to determine whether any of the genes are implicated in testis biology in the mouse. Indeed, *E2f1* knockout mice display testicular atrophy, among other phenotypes (Field et al., 1996; Yamasaki et al., 1996) and *Cbfa2t2* mutants have defective male and female germline development (Tu et al., 2016). However, *Actl10* and *1700003F12Rik* mutant mice have not been linked to germline biology and *Necab3* mice have not been studied (Dickinson et al., 2016). We also examined published expression datasets to assess if these genes are enriched in testis or have significant expression in the spermatogonial lineage (Cardoso-Moreira et al., 2019; Gan et al., 2013). *Actl10* and *1700003F12Rik* were both enriched in testis at the tissue level (Figure 3B). In sorted cells, *E2f1* and *Cbfa2t2* showed moderate expression in spermatogonia. *Necab3* and *Actl10* were moderately expressed and *1700003F12Rik* highly expressed in spermatids (Figure 3C). Endogenous *Wnt1* was not expressed in these tissues or cells (Figure 3B–C). Altogether, these results indicate that *Wnt1-Cre2* integrated into a region of the genome harboring several genes important for male germline development and/or with significant expression in that lineage. While we cannot link *Wnt1-Cre2* male germline activity to a particular regulatory element, it seems likely that one or more elements from nearby genes drive ectopic *Cre* expression in the male germline. Alternatively, sequence within the transgene may support germline expression but only in certain permissive chromatin environments or the insertion itself may have generated a novel binding site for a testis-enriched transcription factor.

The pattern of *Wnt1-Cre2* recombinase activity was also examined by Jackson Labs using the *ROSA26^{LacZ}* reporter allele (Mouse Genome Informatics, informatics.jax.org). They also report recombination in spermatogonia, but the observed activity is more sporadic than we find in our crosses. It is possible that the degree of germline recombination varies by mouse strain, age, or the specific conditional allele. With this in mind, although we did not detect female germline recombination in our crosses, we cannot rule out that this would ever occur with a low frequency that might vary by gene or mouse strain.

Male germline activity may have important implications for researchers using the *Wnt1-Cre2* line. Because many breeding schemes involve a *Cre*-positive sire crossed to homozygous reporter dams, the reporter will behave as expected and germline activity will go undetected. If the male also carries a conditional *lox* allele, this will be inherited

as a recombined () allele (Figure 4B–C). Additionally, some null alleles retain a floxed selection marker, such as *NeoR*, used for genotyping. If genotyping primers are not designed in a way to detect wild type, *flox*, and alleles, then allele could go undetected in a colony. Importantly, in the common breeding scheme where a *Cre/+; flox/+* sire is crossed to a *flox/flox* dam, conditional knockout remains in the intended tissue only (Figure 4B). *Cre*-negative tissue, however, is */flox*, not *flox/flox*, so this could be an issue if the conditional gene has phenotypes in the heterozygous state. This could alternatively be advantageous if researchers are interested in studying timepoints shortly after *Cre* induction, as the need to only recombine a single floxed allele may lead to faster depletion of gene product and yield earlier or stronger phenotypes. Recommendations for future use of *Wnt1-Cre2* (or any *Cre* with undesired male germline activity) are summarized in Figure 4.

A potential benefit of the unintended germline activity is the ability to use the line not only for conditional recombination in the neural crest, but also as a general deleter to intentionally generate alleles or remove floxed selection cassettes from newly generated mouse lines without the need to maintain a separate general deleter *Cre* line.

Transgenesis as a method for expression of exogenous genes such as *Cre* is widely used in the mouse, despite the many recognized drawbacks to this method. These include damage due to injection, toxicity caused by *Cre* expression, variable expression, methylation-induced silencing over time, and ectopic expression including in the male and female germlines (Clark et al., 2020; Song & Palmiter, 2018). Indeed, *Wnt1-Cre2* joins several other transgenic *Cre* lines recently found to have ectopic male germline activity (de Lange et al., 2008; He et al., 2017; McLeod et al., 2020). The ease of transgenesis has historically been viewed as a large enough advantage to overcome the many drawbacks and justify use of the technique. However, given advances in knock-in technology such as using CRISPR-Cas and the identification of multiple safe-harbor loci in the mouse, it may now be preferable to generate *Cre* lines by knock-in to the endogenous locus or ectopically at a safe harbor site. On the other hand, unanticipated germline activity can also be found using knock-in *Cre* lines (Shi et al., 2016). Therefore, in addition to screening for a desired recombination pattern by crossing a *Cre* mouse with a reporter, a second generation cross using double heterozygous mice of both sexes crossed back to wild type is recommended, regardless of the method used to generate the *Cre* line (Song & Palmiter, 2018). Even when such crosses are reported, the expression pattern may change over successive generations due to a shift in genetic background or phenomena such as epigenetic silencing (Shimshek et al., 2002), so individual researchers should remain attentive to how *Cre* lines behave in their own colonies.

In summary, we report significant male germline activity using the *Wnt1-Cre2* transgene. While we believe it retains its intended benefit over the original *Wnt1-Cre* due to the normal expression levels of *Wnt1*, researchers should adjust their breeding schemes or genotyping protocols accordingly.

Methods

Animal Husbandry

All animal experimentation was conducted according to protocols approved by the Institutional Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. Mice were kept in a dedicated animal vivarium with veterinarian support. They were housed on a 13hr-11hr light-dark cycle and had access to food and water *ad libitum*.

Mouse Models

The following previously described mouse lines were used: *H2az2^{Tg(wnt1-cre)}11Rth* referred to as *Wnt1-Cre* (Danielian et al., 1998), *E2f1^{Tg(Wnt1-cre)}2Sor* referred to as *Wnt1-Cre2* (Lewis et al., 2013), *Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}* referred to as *R26R^{mTmG}* (Muzumdar et al., 2007). All mice were maintained on a 129S4 co-isogenic background until the final *Wnt1-Cre2^{Tg/+}; ROSA26^{mTmG/+}* x wild type cross, where either 129S4 or C57Bl/6J wild type mice were used. In both cases, any germline recombination would have happened on a pure 129S4 background. Genotyping primers are available in Supplementary Table 1.

Whole-mount DAPI staining

Timed matings were set up and noon at the day of vaginal plug discovery was designated E0.5. Embryonic day E9.5 embryos were dissected in ice-cold PBS and subsequently fixed in 4% formaldehyde in PBS at 4° C for one hour, rinsed in PBS, and stained in 5 ug/ml DAPI in PBS overnight at 4° C on an orbital shaker. The next day embryos were rinsed in PBS and imaged using #1.5 coverglass-bottom dishes (Ibidi) on a Zeiss AxioObserver microscope with 2.5x objective. Z-stacks were projected using the wavelets function in Zeiss Zen software. Images were then identically thresholded using the FIJI implementation of ImageJ.

Testis sectioning

Adult male mice were euthanized by carbon dioxide inhalation followed by cervical dislocation. Testes were immediately dissected and fixed in 4% formaldehyde in PBS at 4° C for two hours. Following fixation, the tissue was rinsed in PBS, cryoprotected overnight in 30% sucrose, embedded in OCT, and frozen in a dry ice 100% ethanol bath. Cryosections were cut at 10 um thickness using a Leica cryostat. Slides were rinsed in PBS to remove OCT, briefly blocked with PBS supplemented with 1% bovine serum albumin, 5% calf serum, 0.1% Tween-20 and then stained with 5 ug/ml DAPI and 1:400 Phalloidin-Alexa647 in fresh blocking solution for thirty minutes at room temperature. Finally, sections were rinsed in PBS at room temperature, mounted in Slowfade Diamond under a #1.5 coverglass held in place with clear nail polish, and imaged on an inverted Zeiss AxioObserver microscope. Z-stacks were maximally projected and level adjustments made in the FIJI implementation of ImageJ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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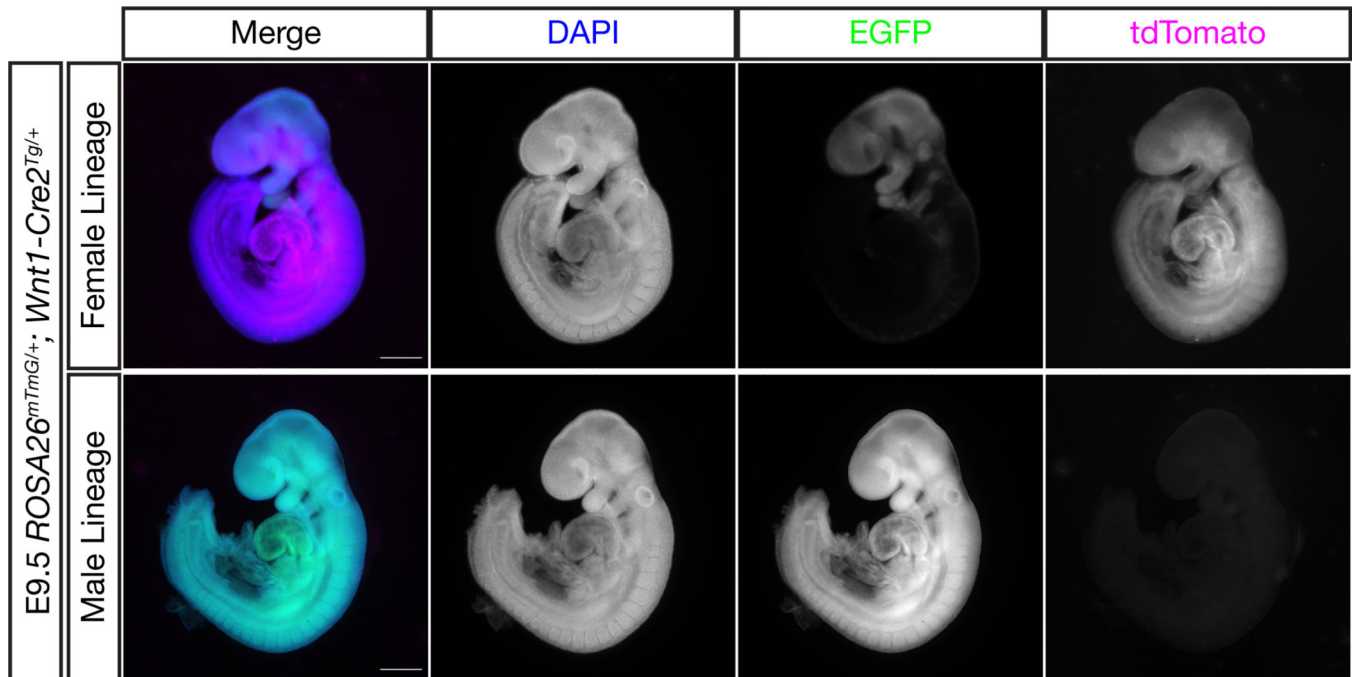


Figure 1.

Wnt1-Cre2 is active in the male germline

Male or female ROSA26^{mTmG/+}; Wnt1-Cre2^{Tg/+} mice were crossed to wild type mice and the resulting embryos were analyzed for their recombination patterns. Here, embryos with identical genotypes show either an expected pattern of recombination in the neural crest or complete recombination throughout the embryo depending on whether the genes were inherited through the female or male lineage, respectively. Scale bars represent 500 μ m.

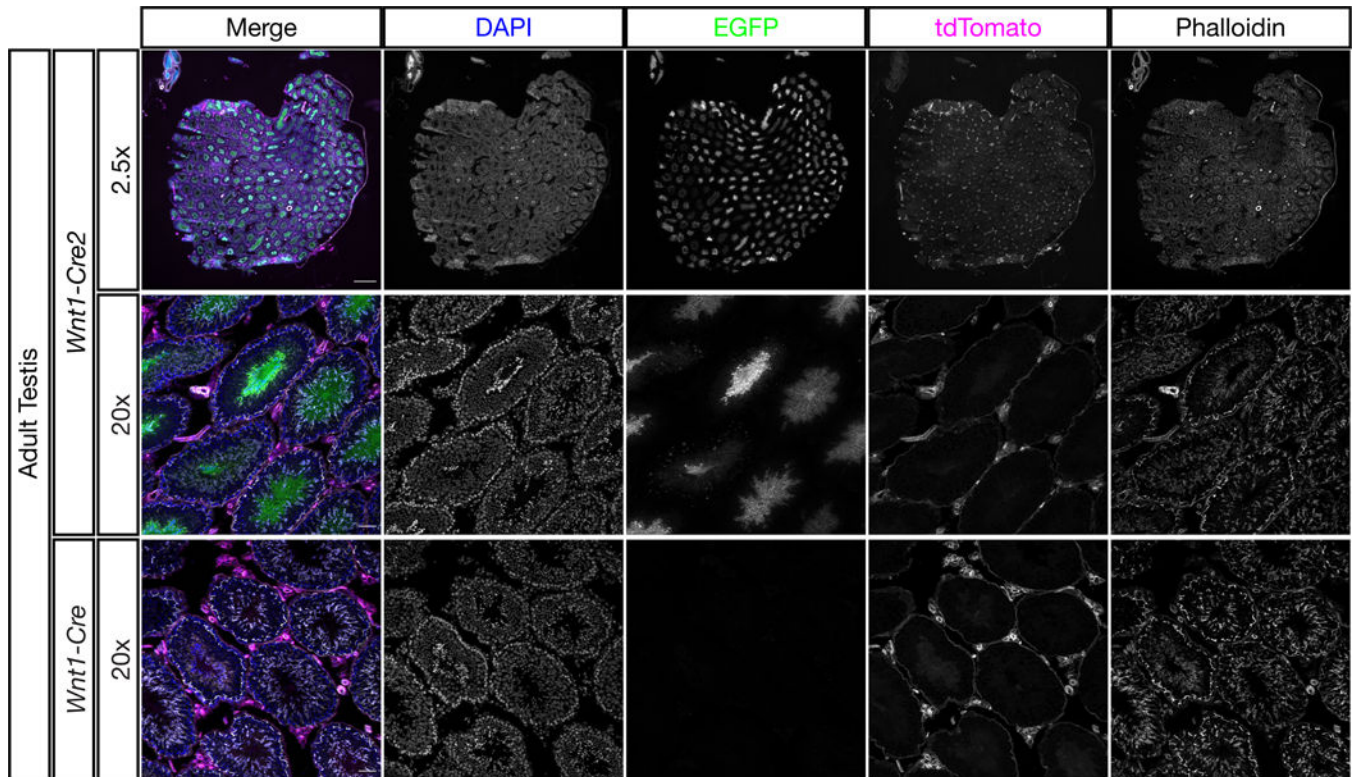
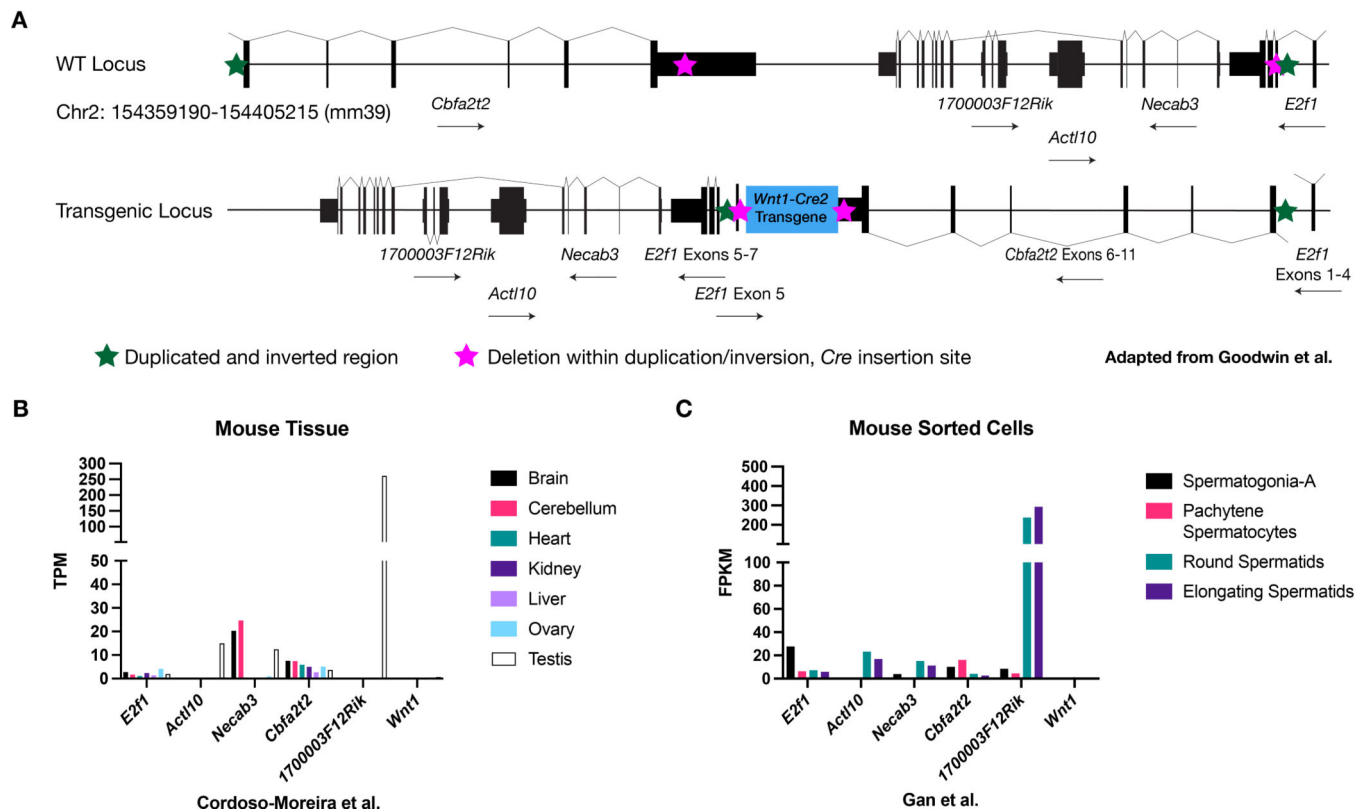


Figure 2.

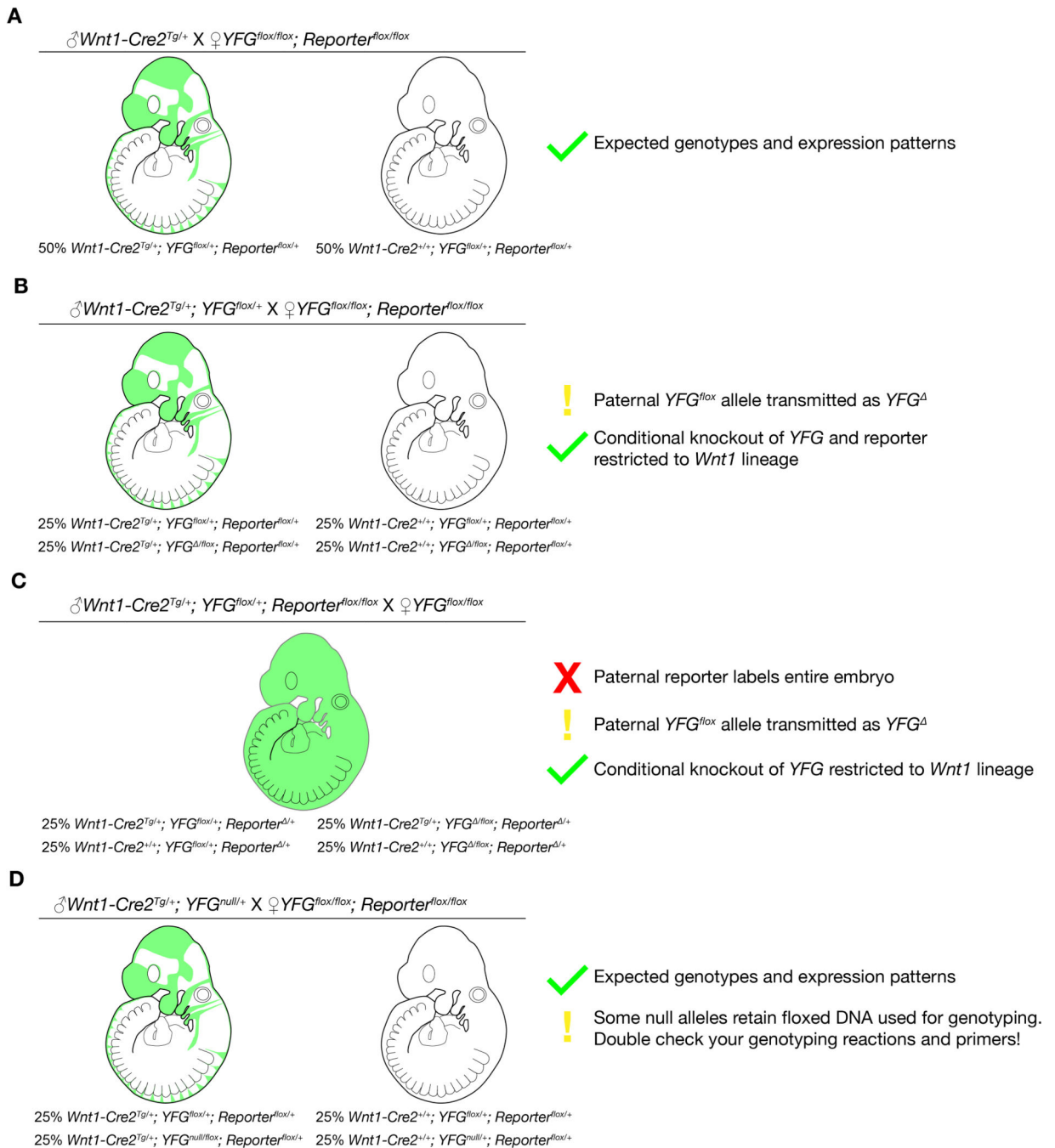
Wnt1-Cre2 activity is detectable in adult male germ cells

Testes from adult male *ROSA26^{mTmG/+}; Wnt1-Cre2^{Tg/+}* and *ROSA26^{mTmG/+}; Wnt1-Cre^{Tg/+}* mice were sectioned and stained with DAPI and phalloidin. EGFP signal indicating CRE activity was found extensively within the seminiferous tubules of *Wnt1-Cre2* but not *Wnt1-Cre* mice. Somatic tissue outside the tubules remained negative for EGFP and positive for tdTomato, indicating that *Wnt1-Cre2* is active in the adult male germline but not somatic tissue within the testis. At 2.5x scale bars represent 500 μm . At 20x scale bars represent 50 μm .

**Figure 3.**

The Wnt1-Cre2 transgene inserted among genes expressed in the male germline

(A) *Wnt1-Cre2* inserted at the *E2f1* locus in a complicated rearrangement involving a large duplication and inversion (green stars) enclosing a smaller deletion (magenta stars) where 1–3 copies of the transgene itself inserted. The *Wnt1-Cre2* transgenic locus is depicted at bottom. This panel is based on Figure 3 from Goodwin et al. (B) Several genes near the insertion site are expressed or enriched in the testis relative to other organs. (C) *E2f1* expression is enriched in spermatogonia and nearby *Act110* and *1700003F12Rik* are enriched in spermatids.

**Figure 4.**

Advice for coinheritance of $Wnt1-Cre2$ and other alleles from the male lineage.

Depicted are four breeding schemes involving a conditional allele of Your Favorite Gene (YFG), a floxed reporter, and a paternal allele of $Wnt1-Cre2$. The cross and genotype ratios of the offspring are listed, and reporter activity is depicted in E9.5 embryos. (A) When no floxed allele is inherited from the sire, results are as expected. (B) When a floxed allele of YFG is present with $Wnt1-Cre2$ in the sire, it will transmit as a recombinant () allele. This can complicate interpretation of results if heterozygous animals exhibit phenotypes.

(C) Similarly, a reporter present in the sire will be fully recombined and be expressed ubiquitously in the next generation. (D) Coinheritance of *Cre* and a null allele will not affect results. Of note, many genotyping assays for conditional and null alleles rely on floxed sequences and are not able to detect a recombined () allele. This can lead to mis-genotyping of embryos and breeders, so primers that detect wild type, flox, and alleles should always be used.

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Table 1*Genotyping results and recombination patterns for Wnt1-Cre2 reporter crosses*

♂ROSA26^{mTmG/+}; Wnt1-Cre2^{Tg/+} × ♀Wild Type n=6 Sires					
Genotype	Expected Pattern	Germline Pattern	Total Embryos	# Expected Pattern (%)	# Germline Pattern (%)
<i>ROSA26^{+/+}; Wnt1-Cre2^{+/+}</i>	Not Fluorescent	Not Fluorescent	34	34 (100)	N/A
<i>ROSA26^{+/+}; Wnt1-Cre2^{Tg/+}</i>	Not Fluorescent	Not Fluorescent	29	29 (100)	N/A
<i>ROSA26^{mTmG/+}; Wnt1-Cre2^{+/+}</i>	All tdTomato+	All EGFP+	25	0 (0)	25 (100)
<i>ROSA26^{mTmG/+}; Wnt1-Cre2^{Tg/+}</i>	EGFP+ Neural Crest	All EGFP+	28	0 (0)	28 (100)
♂Wild Type x ♀ROSA26^{mTmG/+}; Wnt1-Cre2^{Tg/+} n=6 Dams					
Genotype	Expected Pattern	Germline Pattern	Total Embryos	# Expected Pattern (%)	# Germline Pattern (%)
<i>ROSA26^{+/+}; Wnt1-Cre2^{+/+}</i>	Not Fluorescent	Not Fluorescent	18	18 (100)	N/A
<i>ROSA26^{+/+}; Wnt1-Cre2^{Tg/+}</i>	Not Fluorescent	Not Fluorescent	13	13 (100)	N/A
<i>ROSA26^{mTmG/+}; Wnt1-Cre2^{+/+}</i>	All tdTomato+	All EGFP+	15	15 (100)	0 (0)
<i>ROSA26^{mTmG/+}; Wnt1-Cre2^{Tg/+}</i>	EGFP+ Neural Crest	All EGFP+	14	14 (100)	0 (0)

When *Wnt1-Cre2* and a floxed reporter coexist in the male line, 100% germline recombination is observed in *ROSA26^{mTmG/+}* offspring, even when *Wnt1-Cre2* is not co-inherited (25/25 *Cre⁻* embryos and 28/28 *Cre⁺* embryos), indicating germline activity for this strain. However, germline activity is not observed when the genes are inherited through the female lineage.