Functional Equivalence of the SOX2 and SOX3 Transcription Factors in the Developing Mouse Brain and Testes

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ABSTRACT Gene duplication provides spare genetic material that evolution can craft into new functions. *Sox2* and *Sox3* are evolutionarily related genes with overlapping and unique sites of expression during embryogenesis. It is currently unclear whether SOX2 and SOX3 have identical or different functions. Here, we use CRISPR/Cas9-assisted mutagenesis to perform a gene-swap, replacing the *Sox3* ORF with the *Sox2* ORF to investigate their functional equivalence in the brain and testes. We show that increased expression of SOX2 can functionally replace SOX3 in the development of the infundibular recess/ventral diencephalon, and largely rescues pituitary gland defects that occur in *Sox3* null mice. We also show that ectopic expression of SOX2 in the testes functionally rescues the spermatogenic defect of *Sox3* null mice, and restores gene expression to near normal levels. Together, these *in vivo* data provide strong evidence that SOX2 and SOX3 proteins are functionally equivalent.

KEYWORDS SOXB1 genes; CRISPR/CAS9 mutagenesis; gene swap

ONE of the driving forces for the evolution of complex life is the duplication of genes, chromosomes, or entire genomes, providing the genetic material upon which natural selection can operate. Evolutionary theory predicts that having duplicated, a gene pair will be relieved from selective constraints, thereby enabling the accumulation of genetic alterations that can alter protein function (Force *et al.* 1999; Lynch and Conery 2000). The consequences of this are thought to favor loss of one copy (nonfunctionalization). Alternatively, gene functions can be divided between the paralogues (subfunctionalization), or one copy can acquire a novel advantageous function (neofunctionalization) while the other copy retains its original function (Force *et al.* 1999; Lynch and Conery 2000). Under this paradigm, it is expected that shared function within a given tissue will not be preserved by natural selection, and should therefore be lost over time. This stands in contrast to many observations of genetic redundancy that have emerged in the age of molecular genetics as gene deletions in seemingly important genes routinely yield no, or mild, phenotypes, and appear to be compensated for by paralogous partner genes (Wagner 2005). Estimates suggest that as many as 10–15% of mouse gene knockouts may have no or mild phenotypes (Barbaric *et al.* 2007). What forces allow the persistence of genetic redundancy are unclear, but genetic robustness that acts to maintain and bolster important processes may play a role (Force *et al.* 1999; Wagner 2005; Barbaric *et al.* 2007).

Persistent genetic redundancy is particularly striking in the *SoxB1* subfamily, which consists of *Sox1*, *Sox2*, and *Sox3*. These genes share highly similar sequences, both within and, to a lesser extent, outside of the DNA-binding HMG box. Several studies suggest that SOXB1 proteins have similar, if not identical, functional capabilities. For example, overexpression of chick or mouse *SoxB1* genes in chick neural tube results in inhibition of neural differentiation with cells retaining a progenitor identity (Bylund *et al.* 2003; Graham *et al.* 2003). Similarly, mouse *Sox1* and *Sox3* are able to replace *Sox2* for reprogramming of iPS cells (Nakagawa *et al.* 2008). Loss of function studies also generally support functional equivalence, particularly in the developing CNS where the

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SoxB1 genes exhibit extensive overlapping expression. For example, *Sox3* deletion in mice results in relatively mild neural defects, indicating that SOX2 and/or SOX1 can compensate for the absence of SOX3 in most neuroprogenitor contexts. However, one notable exception is the infundibulum, a ventral evagination of the ventral diencephalon that is responsible for induction of the anterior pituitary primordium (Rathke's Pouch). Despite coexpression of *Sox2* and *Sox3*, pituitary induction and development is severely compromised in *Sox2* and *Sox3* single mutants (Rizzoti *et al.* 2004; Kelberman *et al.* 2006). It is thought that this is due to reduced dosage of SOX2 or SOX3, as opposed to unique roles of these proteins (Zhao *et al.* 2012). However, to date, experimental approaches that distinguish between these possibilities have not been published.

Restricted zones of *SoxB1* expression outside of the nervous system have also been described, many of which are in stem/progenitor cells of developing organs. For example, *Sox3* is uniquely expressed in the spermatogonial stem/progenitor cells of the postnatal testes (Rizzoti *et al.* 2004; Raverot *et al.* 2005). Consistent with a model of limited subfunctionalization, more severe phenotypes occur in knockout mice at sites of unique expression. For example, *Sox3* null mice have spermatogenic defects likely due to the absence of *Sox1* and *Sox2* (Raverot *et al.* 2005). However, it is not known whether *SoxB1* genes are functionally interchangeable at these unique zones of expression.

Herein, we describe an *in vivo* gene swap experiment in which *Sox3* open reading frame (ORF) was deleted and replaced with *Sox2* ORF to investigate their functional similarities. We show that SOX2 can functionally replace SOX3 in both the developing pituitary and testes, thereby rescuing phenotypes associated with SOX3-null mice.

Materials and Methods

Generation of CRISPR/Cas9 modified mice

CRISPR gRNAs were designed either side of the *Sox3* ORF (5′-CCTGATGCGTTCTCTCGAGC-3′ and 5′-GACAGTTACGGC CAAACTTT-3′) using CRISPR Design tool (http://crispr.mit.edu/) and generated according to the protocol described in Wang *et al.* (2013). gRNA IVT was performed using HiScribe T7 Quick High Yield RNA Synthesis Kit. Cas9 mRNA was generated by IVT using the mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Ambion) from pCMV/T7-hCas9 (Toolgen) digested with *Xho*1. gRNAs and Cas9 mRNA were purified using a MEGAclear Transcription Clean-Up Kit (Ambion). Our previously published *Sox3* targeting vector (Hughes *et al.* 2013) was modified to replace *Sox3* ORF with *Sox2* ORF. Cas9 mRNA (100 ng/μl), gRNAs (50 ng/μl each) and donor plasmid (200 ng/μl) were injected into the cytoplasm of C57BL/6N zygotes using a FemtoJet microinjector, transferred to pseudo pregnant recipients, and allowed to develop to term. Homology directed repair (HDR) from the vector resulted in the $Sox3^{Sox2KI}$ mice carrying a neomycin resistance cassette 1 kb downstream from the *Sox2-KI* stop codon. The $Sox3^{Sox2KI}$ mice also contain a 2 bp deletion in the 5′UTR at the upstream gRNA site, and a 1 bp in the 3′UTR at the downstream gRNA site, presumably as a result of CRISPR/Cas9 recutting after HDR.

Microarray analysis

Microarray expression profiling was performed using Affymetrix GeneChip Mouse Gene 1.0 ST Arrays on three *Sox3* null and three $Sox3^{Sox2KI}$ 2-week testes. A total of six wild type age matched samples was included, comprising two groups of three matched to the same genetic background as the *Sox3* null and $Sox3^{Sox2KI}$ samples. Two way-ANOVA, using batch as a factor, was used to identify the significantly regulated genes. ANOVA was performed comparing to matched WT samples and comparing to pooled WT samples with similar results. We have presented data comparing two pooled WT samples.

Sperm counting

Cauda epididymis were isolated and minced in 1 ml of 37° DMEM media. Sperm were allowed to disperse for 10–15 min at 37°; 10 μl of the resuspension was diluted with 10 μl of 1 M Tris pH 9.5 solution to immobilize sperm before counting with a hemocytometer.

Data availability

Microarray data has been submitted to Gene Expression Omnibus (GEO) with accession number GSE96805. All other reagents can be made available upon request. Supporting data can be found in Supplemental Material, File S1.

Results

Generation of $Sox3^{Sox2KI}$ mice using CRISPR/Cas9 mutagenesis

To investigate the functional redundancy within the *SoxB1* subgroup, we replaced the *Sox3* ORF with that of *Sox2* while leaving the remaining native *Sox3* flanking sequences including the promoter and untranslated regions (UTR) intact. This mouse model, which we refer to as $Sox3^{Sox2KI}$, therefore lacks SOX3, and expresses SOX2 from the *Sox3* locus on the X-chromosome. To generate $Sox3^{Sox2KI}$ mice, we initially modified an existing *Sox3* KO targeting construct (Rizzoti *et al.* 2004; Hughes *et al.* 2013) by replacing the *Sox3* ORF with *Sox2*. Attempts to generate $Sox3^{Sox2KI}$ mice by conventional gene targeting in mouse ES cells failed to produce any chimeras despite multiple rounds of injections using germline competent cells (data not shown). To circumvent this issue, we employed CRISPR/Cas9 technology to generate $Sox3^{Sox2KI}$ mice by zygotic injection of Cas9 mRNA, as well as gRNA pairs targeting either side of the *Sox3* ORF, and the donor (targeting) plasmid (Figure 1A). PCR screening of 17 founders identified a $Sox3^{Sox2KI}$ female harboring the intended replacement event (Figure 1B). The other allele of this $Sox3^{Sox2KI}$ female, as well as seven other founders, lacked the entire *Sox3* ORF due to deletion of the interval between the two gRNA cuts. These were used to generate *Sox3*-null mice ($Sox3^{KO}$). No gross

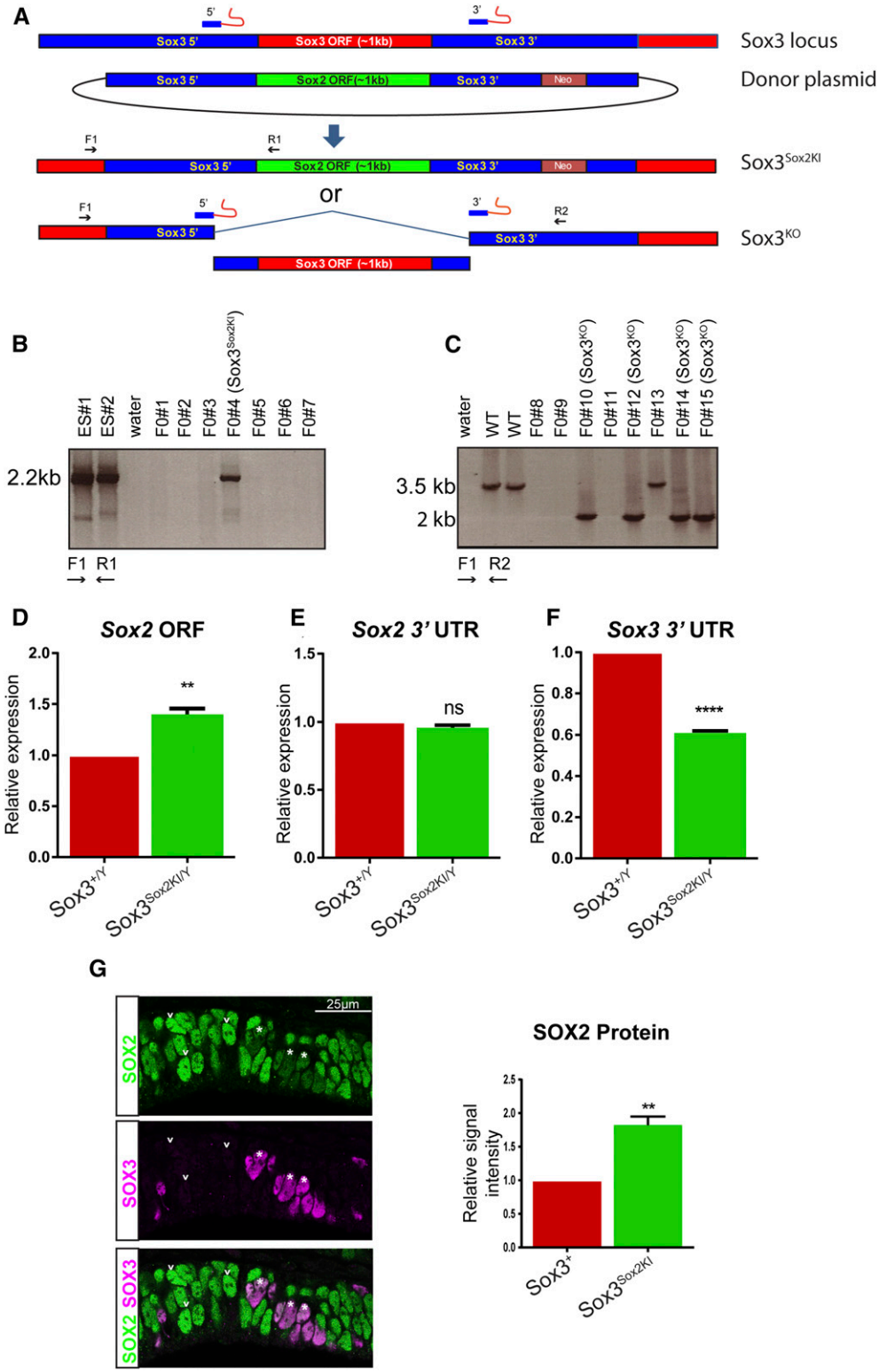

Figure 1 Generation of *Sox3*^{Sox2KI} and *Sox3*^{KO} mice with CRISPR mutagenesis. (A) Schematic showing the strategy for generation of the *Sox3*^{Sox2KI} mice. (B, C) PCR screening of founder mice. (D) *Sox2* transcript levels were measured in 11.5 dpc brains by qPCR with primers located in the *Sox2* ORF. (E) qPCR showing expression from the endogenous *Sox2* locus using primers located in the 3′UTR. (F) Transcription from the *Sox3* locus was reduced in *Sox3*$^{Sox2KI/Y}$ as determined by qPCR with primers located in the *Sox3* 3′UTR. (G) SOX3 and SOX2 coimmunostaining in the ventral diencephalon of 12.5 dpc *Sox3*$^{Sox2KI/+}$ embryos. Asterisks indicate SOX3 positive cells, and arrowheads indicate SOX3 negative cells. Quantification of SOX2 staining intensity in SOX3 positive and SOX3 negative cells (n = 3 embryos).

abnormalities were observed in *Sox3*^{Sox2KI} or *Sox3*^{KO} adult mice (data not shown), and their body weights were comparable with wild type (WT) littermates (Figure S1 in File S1).

To determine whether *Sox2* was expressed from the *Sox3* locus in the developing brain, we performed qRT-PCR using primers that amplify the *Sox2* ORF, the *Sox2* 3′UTR, and the *Sox3* 3′UTR. The level of *Sox2*-ORF-containing transcripts was significantly elevated in *Sox3*$^{Sox2KI/Y}$ embryos *vs.* *Sox3*$^{+/Y}$ (Figure 1D). *Sox2* 3′UTR-containing transcript levels were equivalent (Figure 1E), indicating that the lack of *Sox3* in *Sox3*^{Sox2KI} embryos does not result in a compensatory increase in *Sox2* transcript levels. Notably, the level of

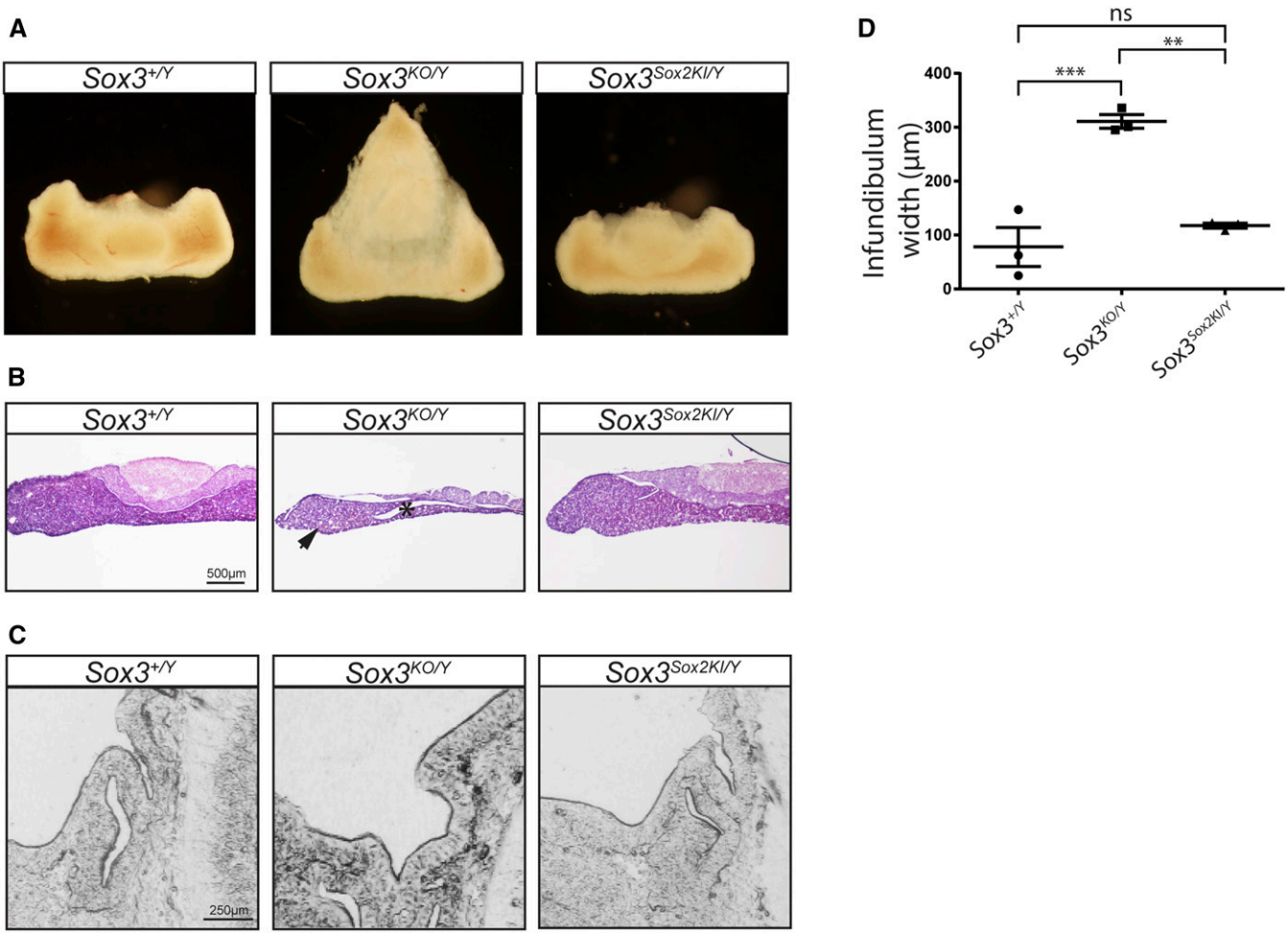

Figure 2 Increased expression of *Sox2* can rescue *Sox3* null pituitary defects. (A) Adult pituitaries showing dorsal displacement of the neural lobe in $Sox3^{KO/Y}$ mice. (B) Haemotoxylin and eosin staining of 8-week-old pituitary coronal sections. Asterisk highlights abnormal clefting. Arrowhead indicates hypoplastic anterior lobe. (C) Phase contrast images of 12.5 dpc developing pituitaries (saggital sections). (D) Quantification of infundibulum width of 12.5 dpc embryos (n = 3). One-way ANOVA using Tukey's Multiple comparison test. Mean ± SEM.

transcripts from the *Sox3* locus is lower in $Sox3^{Sox2KI}$ embryonic heads compared to $Sox3^{+/Y}$ heads (Figure 1F). This reduction may reflect small differences in transcription rate caused by transgene elements, or reduction in the stability of the $Sox3^{Sox2KI/Y}$ chimeric transcript.

To confirm that the observed increase in *Sox2* transcript levels in $Sox3^{Sox2KI/Y}$ embryos resulted in increased protein levels, we performed immunostaining on the ventral diencephalon, where SOX3 and SOX2 are coexpressed in the infundibulum (Wood and Episkopou 1999; Rogers *et al.* 2013). We examined $Sox3^{Sox2KI/+}$ embryos in which random X-inactivation results in a mixture of cells expressing either the *Sox2KI* or *Sox3* allele. SOX2 immunostaining intensity was significantly elevated in *Sox2KI* cells (SOX3 negative) in comparison to neighboring SOX3 positive cells, consistent with additional expression of *SOX2* from $Sox3^{Sox2KI}$ allele (Figure 1G). Collectively, these results demonstrate successful gene replacement in our $Sox3^{Sox2KI}$ mice, and show that *Sox3* has been removed and replaced with a copy of *Sox2* that is regulated in a *Sox3*-specific fashion.

Rescue of pituitary induction defect in $Sox3^{Sox2KI}$ mice

We next sought to assess whether SOX2 protein could functionally rescue *Sox3*-null phenotypes. Given that pituitary development is extremely sensitive to *SoxB1* gene dosage (Zhao *et al.* 2012), we examined adult pituitaries to determine whether the replacement of *Sox3* with *Sox2* was able to rescue *Sox3* null pituitary defects (Rizzoti *et al.* 2004). To assess rescue by *Sox2KI*, we collected pituitaries from 8-week-old mice. As expected, we found malformations in $Sox3^{KO/Y}$ pituitaries; indeed, these were even more severe than previously reported on a mixed genetic background (Figure 2A) (Rizzoti *et al.* 2004; Hughes *et al.* 2013). The dorsal aspect of *Sox3* null pituitaries was triangular, such that the neural lobe was rostrally displaced forming a tenuous connection with the anterior lobe. Sectioning revealed hypoplasia of the anterior lobe, and deep clefting of the residual lumen of Rathke's Pouch in $Sox3^{KO/Y}$ pituitaries, similar to previous reports (Figure 2B) (Rizzoti *et al.* 2004; Hughes *et al.* 2013). In contrast, the gross morphology of

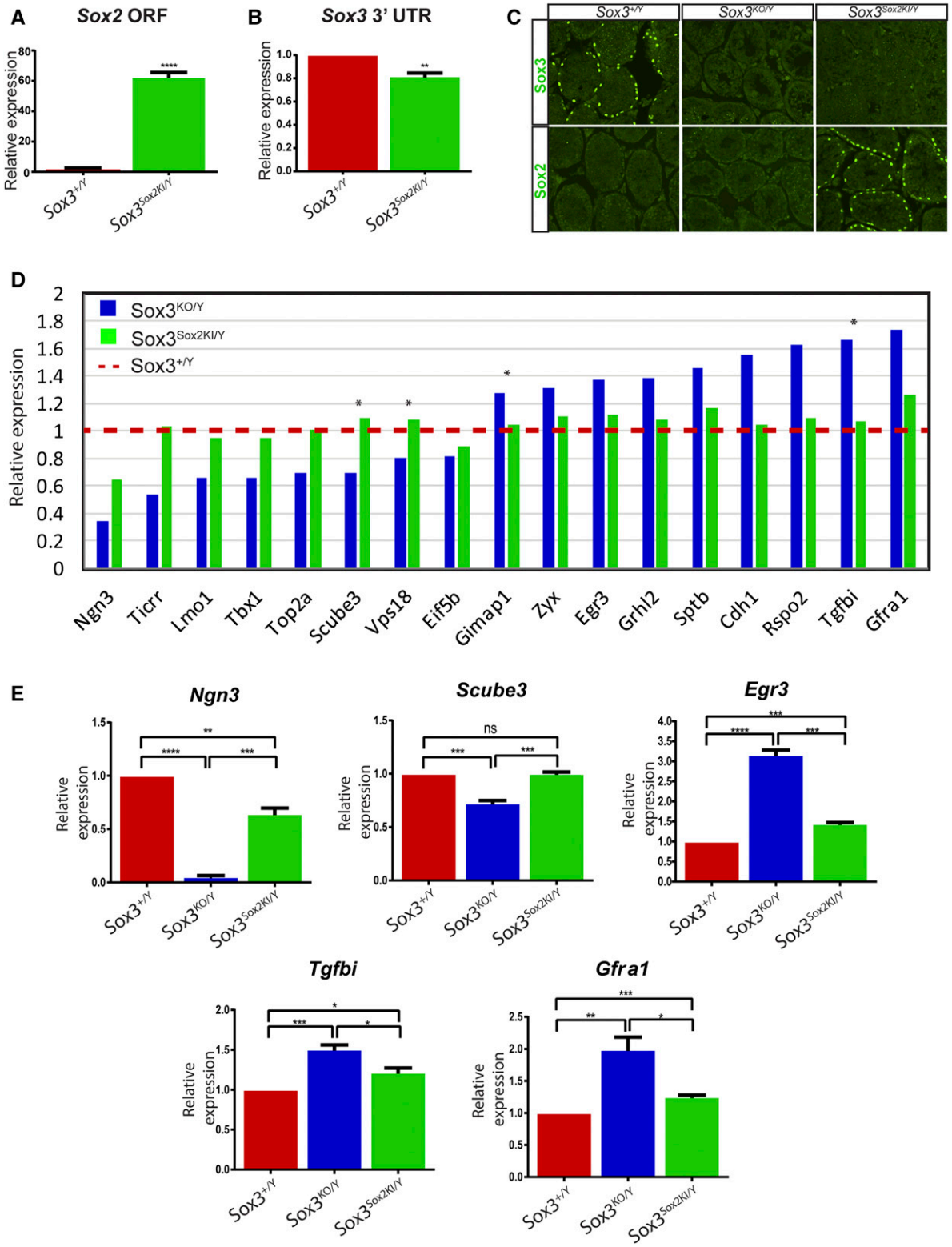

Figure 3 SOX2 regulates SOX3 target genes in the mouse testes. (A) *Sox2* qPCR analysis in 2 week $Sox3^{+/Y}$ and $Sox3^{Sox2KI/Y}$ testes [Student's two tailed unpaired *t*-tests (****<0.0001)]. (B) qPCR analysis of *Sox3* 3′ UTR in 2 week $Sox3^{+/Y}$ and $Sox3^{Sox2KI/Y}$ testes [Student's two-tailed unpaired *t*-tests (**<0.01)]. (C) SOX2 and SOX3 immunostaining on $Sox3^{+/Y}$, $Sox3^{KO/Y}$ and $Sox3^{Sox2KI/Y}$ 4-week-old testes. (D) Microarray analysis was performed on 2 week testes from $Sox3^{+/Y}$, $Sox3^{KO/Y}$ and $Sox3^{Sox2KI/Y}$, and two-way comparisons were performed between $Sox3^{+/Y}$ and either $Sox3^{Sox2KI/Y}$ or $Sox3^{KO/Y}$. Genes presented were significantly different between $Sox3^{+/Y}$ and $Sox3^{KO/Y}$ testes (Step-up *P* value <0.05). * <0.05 step-up *P* value between $Sox3^{KO/Y}$ and $Sox3^{Sox2KI/Y}$. (E) qPCR validation of SOX2 regulation of SOX3 target genes in 2 week testes. ANOVA multiple comparisons with Bonferroni's correction were performed using Graphpad Prism (****<0.0001, ***<0.001, **<0.01, *<0.05, ns, not significant).

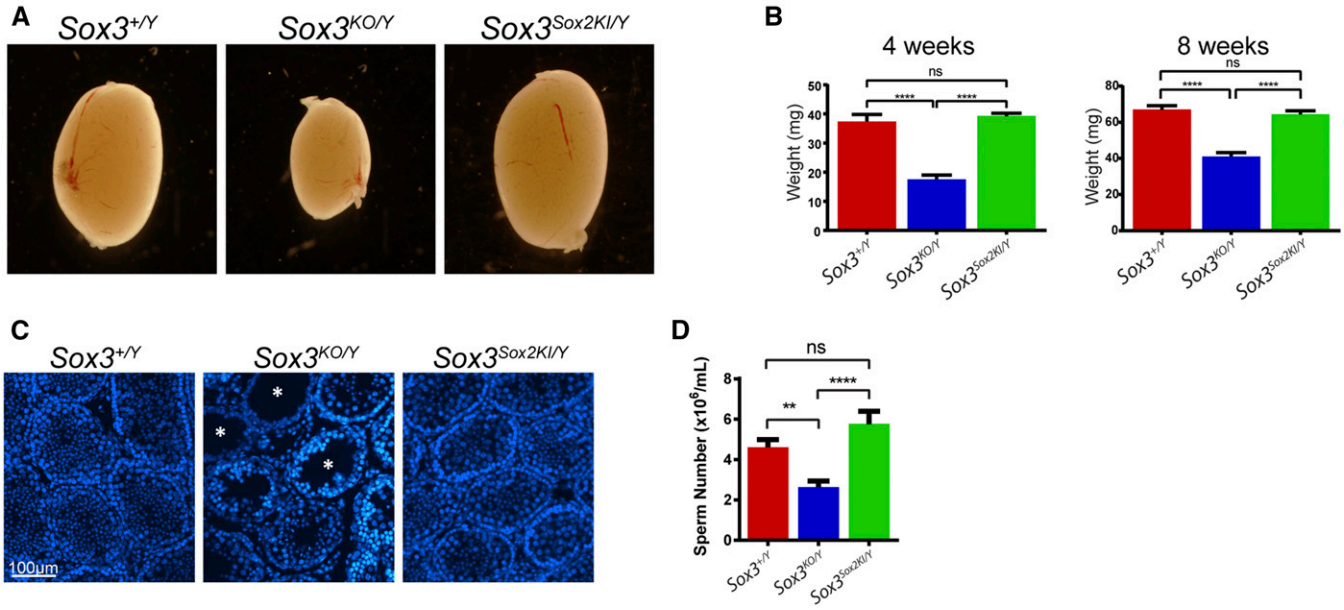

Figure 4 SOX2 can functionally replace SOX3 in the testes. (A) Gross morphology of *Sox3*$^{+/Y}$, *Sox3*$^{KO/Y}$ and *Sox3*$^{Sox2KI/Y}$ 4-week testes. (B) Quantification of testes weight at 4 and 8 weeks old [at least 18 testes were weighed for each genotype, and results were compared using one way ANOVA multiple comparisons with Bonferroni's correction (****<0.0001, ns, not significant)]. (C) DAPI staining of *Sox3*$^{+/Y}$, *Sox3*$^{KO/Y}$ and *Sox3*^{Sox2KI} 4 week old testes. Asterisks indicate empty tubules. (D) Sperm counts from 8-week *Sox3*$^{+/Y}$, *Sox3*$^{KO/Y}$ and *Sox3*$^{Sox2KI/Y}$ epididymis [at least 14 samples were counted for each genotype, and results were compared using one way ANOVA multiple comparisons with Bonferroni's correction (****<0.0001, **<0.01 ns, not significant)].

Sox3$^{Sox2KI/Y}$ pituitaries was indistinguishable from *Sox3*$^{+/Y}$ controls (Figure 2A), apart from very subtle clefting between the intermediate and anterior lobe (Figure 2, A and B). We next compared anterior pituitary induction in *Sox3*$^{KO/Y}$ and *Sox3*$^{KI/Y}$ embryos. *Sox3*$^{KO/Y}$ embryos had an infundibular recess that was significantly wider than *Sox3*$^{+/Y}$ embryos, and a dysmorphic Rathke's Pouch with extensive branching, some of which (three of four) had failed to detach from the oral cavity (Figure 2, C and D and Figure S2 in File S1). In contrast, the width of the infundibulum in *Sox3*^{Sox2KI} embryos was completely rescued. Furthermore, Rathke's Pouch was always separated from the oral cavity, although a mild branching phenotype was observed (Figure S2 in File S1). Together, this data indicates that SOX2 is able to functionally replace SOX3 in the developing brain.

Ectopic SOX2 in the testes can regulate SOX3 target genes

We next assessed whether SOX2 is able to replace SOX3 in a tissue where it is not normally expressed. For these experiments, we turned to the testes, where *Sox3* is the only *SoxB1* member expressed in spermatogonial stem/progenitor cells, and is functionally required for normal spermatogenesis (Raverot *et al.* 2005). After first confirming the absence of *Sox1* and *Sox2* in the testes by qRT-PCR (Figure S3 in File S1), we then examined testes from *Sox3*$^{Sox2KI/Y}$ mice to determine whether *Sox2* was expressed in a *Sox3*-specific manner. Robust expression of *Sox2* was detected by qRT-PCR in *Sox3*$^{Sox2KI/Y}$ testes, but not in *Sox3*$^{+/Y}$ testes (Figure 3A). To determine whether the level of *Sox2KI* expression in *Sox3*$^{Sox2KI/Y}$ mice was similar to endogenous *Sox3* expression in *Sox3*$^{+/Y}$ mice, we compared *Sox3* 3′ UTR transcript levels in testes (as *Sox3*$^{Sox2KI/Y}$ retains an intact *Sox3* UTR; Figure 1F). *Sox3* locus expression in *Sox3*$^{Sox2KI/Y}$ was slightly lower than levels seen in *Sox3*$^{+/Y}$, but was a closer match than that observed in the developing brain (Figure 1F and Figure 3B, respectively). In addition, SOX2 immunostaining could not be detected in *Sox3*$^{+/Y}$ and *Sox3*$^{KO/Y}$ testes, but was present in *Sox3*$^{Sox2KI/Y}$ testes in undifferentiated spermatogonia (Figure 3C). SOX3 immunostaining was present in *Sox3*$^{+/Y}$ testes in undifferentiated spermatogonia, but, as expected, not in *Sox3*$^{KO/Y}$ and *Sox3*$^{Sox2KI/Y}$ testes (Figure 3C).

Since SOX2 and SOX3 bind and regulate target genes via highly similar HMG-box DNA binding domain (Bergsland *et al.* 2011), we reasoned that SOX2 could be capable of regulating SOX3 target genes in the testes of *Sox3*$^{Sox2KI/Y}$ mice. To test this possibility, we performed microarray analysis comparing *Sox3*$^{+/Y}$, *Sox3*$^{KO/Y}$ and *Sox3*$^{Sox2KI/Y}$ 2-week testes. A total of 17 genes (excluding *Sox3*) were significantly altered when comparing *Sox3*$^{KO/Y}$ to *Sox3*$^{+/Y}$ (step-up P value ≤ 0.05), of which nine were upregulated and eight were downregulated. These genes included *Ngn3*, which has previously been shown to be downregulated in *Sox3* null testes (Raverot *et al.* 2005). Expression levels of all 17 genes were statistically returned to *Sox3*$^{+/Y}$ levels in *Sox3*$^{Sox2KI/Y}$ testes (Figure 3D), although only four were significantly different in *Sox3*$^{KO/Y}$ *vs.* *Sox3*$^{Sox2KI/Y}$ testes. As step-up P values are known to be a very strict measure of microarray changes, and often underestimate changes in order to avoid high false discovery rates (Benjamini and Hochberg 1995), we sought

to validate the degree of expression rescue using qPCR on independent biological samples. Five of the 17 genes were selected for validation by qPCR, and fold changes were all in the same direction as the microarray data (Figure 3E). Notably, all five genes assessed by qPCR were statistically closer to *Sox3*$^{+/Y}$ levels in *Sox3*$^{Sox2KI/Y}$ in comparison to *Sox3*$^{KO/Y}$, but, in four of five cases fell short of complete restoration, with only *Scube3* showing no significant difference between *Sox3*$^{+/Y}$ and *Sox3*$^{Sox2KI/Y}$. Collectively, this analysis indicates that ectopically expressed SOX2 is able to regulate SOX3 genes in the testes, and can largely restore normal gene expression patterns. However, a small subset of those genes most heavily deregulated by the loss of *Sox3* fail to achieve complete rescue in *Sox3*$^{Sox2K/Y}$ mice, likely due to the slightly decreased levels of SOX2 expression in the testes.

Sox2 can functionally replace Sox3 in the testes

Given the high degree of expression restoration in *Sox3*$^{Sox2KI/Y}$ testes, we next sought to assess whether this correlated with functional rescue of testes size and sperm production, which has previously been reported for *Sox3*$^{KO/Y}$ animals (Rizzoti *et al.* 2004; Raverot *et al.* 2005). *Sox3*$^{KO/Y}$ mice exhibited significantly reduced testes size and weight at 4 and 8 weeks (Figure 4, A and B). Empty seminiferous tubules were observed in sections of 4-week-old *Sox3*$^{KO/Y}$ testes (Figure 4C), and *Sox3*$^{KO/Y}$ mice had a significantly reduced sperm count at 8 weeks of age (Figure 4D). In contrast, *Sox3*$^{Sox2KI/Y}$ males had normal testes size and sperm count with no evidence of empty tubules (Figure 4, A–D). Thus, testes morphology and function were completely rescued in *Sox3*$^{Sox2KI/Y}$ mice. These data provide unequivocal evidence of *SoxB1* functional redundancy *in vivo*.

Discussion

Gene swap experiments provide an ideal approach to investigate functional overlap of related genes under physiological conditions. To our knowledge, this is the first report describing the use of CRISPR/Cas9-assisted mutagenesis to perform an *in vivo* gene-swap of two closely related genes. By cutting either side of *Sox3* in the presence of a *Sox2*-containing repair template, we were able to simultaneously remove *Sox3* and insert the *Sox2* ORF in its place. From a practical standpoint, this provides a feasible and rapid pathway to generate gene-swap mice. It should be noted that the efficiency of generation was lower than anticipated based on previous reports of CRISPR/Cas9-assisted insertional mutagenesis (Yang *et al.* 2013), with only a single *Sox3*^{Sox2KI} founder generated from a total of 17 live born mice. Generation of null alleles was more efficient, with almost half of the founders having a large deletion extending between the two CRISPR cut sites. The reason for the low efficiency of *Sox3*^{Sox2KI} alleles most likely reflects low efficiency of HDR in comparison to nonhomologous end joining, as noted by others (Yang *et al.* 2013). In the future, it will be interesting to test whether strategies to promote HDR, such as knockdown of the Ku protein, can be used to increase knock-in efficiency (Basu *et al.* 2015). Any future optimization should be sure to allow the coincidental production of KO alleles, as this enables simultaneous assessment of KI and KO phenotypes, and, as such, circumvents the significant confounding influence of genetic background. Indeed, we have illustrated the importance of this consideration with our description of a more severe pituitary phenotype in *Sox3* null mice on a C57Bl/6 background in comparison with the previously published phenotype on a mixed genetic background (Rizzoti *et al.* 2004).

The testes provide a particularly tractable setting for this investigation, as SOX3 is the only SOXB1 protein to be expressed in this tissue, and its absence results in a relatively severe phenotype. Importantly, we have shown that the morphogenetic and spermatogenic defects that result from *Sox3* deletion were completely rescued when *Sox2* was ectopically expressed in its place. At the molecular level, these data indicate that ectopic SOX2 is able to bind and regulate SOX3 target genes in spermatogonial stem/progenitor cells. This finding is consistent with published ChIP-seq studies showing that the binding sites of these proteins overlap extensively in cultured neural progenitor cells (Bergsland *et al.* 2011). While a modest difference in spermatogonial marker expression remained in *Sox3*$^{Sox2KI/Y}$ testes, it seems likely this results from the slightly lower level of *Sox3*^{Sox2KI} mRNA compared with wild type *Sox3*. However, potential differences in the affinity of SOX2 and SOX3 antibodies make it near impossible to directly compare levels of SOX3 and SOX2 protein in *Sox3*$^{+/Y}$ and *Sox3*$^{Sox2KI/Y}$ testes, respectively. Therefore, we cannot completely exclude the possibility that the residual differences in *Sox3*$^{Sox2KI/Y}$ testes are due to slightly different functions of SOX3 and SOX2 proteins. It is also conceivable that functional differences may be exposed under nonlaboratory conditions as observed in *HoxA1/B1* gene swap mice (Ruff *et al.* 2015). Nevertheless, these gene swap data provide the most compelling evidence to date that SOX2 and SOX3 are functionally interchangeable proteins. Given the complete rescue of SOX3 morphological defects by SOX2, we predict that this functional redundancy should operate in both directions, such that SOX3 should be equally well suited to replacing SOX2 if expressed at the correct time and place. Thus, it would be interesting to perform the complementary gene swap to determine whether, for example, *Sox3* expression from the *Sox2* locus is capable of rescuing the early lethality phenotype of *Sox2* null mice.

Unlike the testis, where a single SOXB1 protein (SOX3) is expressed in a small population of cells, all three SOXB1 proteins are expressed in neural progenitors across the developing CNS. Direct comparison of SOX3 and SOX2 expression in the embryonic brain has shown that virtually all *SOX3*+ cells also express SOX2 (Rogers *et al.* 2013; Cheah and Thomas 2015). Given our gene swap data showing SOX2/3 functional redundancy in the testes, as well as overexpression data indicating their functional equivalence (Bylund *et al.* 2003; Graham *et al.* 2003), it is not surprising that most of the CNS develops normally in *Sox3* null mice.

However, a notable exception is the developing ventral diencephalon where *Sox3* loss-of-function results in CNS/pituitary defects in both mice and humans despite expression of *Sox2*. Here, we show that these *Sox3* null defects are almost completely rescued by expression of *Sox2* from the *Sox3* locus, indicating that SOX2 and SOX3 proteins are functionally interchangeable in pituitary induction. It is likely that the residual minor clefting in the $Sox3^{Sox2KI}$ adult pituitary is due to a decreased level of $Sox3^{Sox2KI}$ mRNA, reinforcing that pituitary induction is extremely sensitive to *Sox2/3* levels, which, in $Sox3^{Sox2KI/Y}$ brains, are reduced by only ~16% compared to wild type (see File S1). Alternatively, these minor abnormalities may be due to a slight difference in the functionality of SOX3 and SOX2 in pituitary development.

In the mouse, only a handful of examples exist in which paralogous gene function has been examined using a KI gene swap approach. In these examples, the degree of functional equivalence has been complete (*Otx1/2*, *En1/2*, *Osr1/2*, and *HoxA3/D3*) (Hanks *et al.* 1995; Greer *et al.* 2000; Acampora *et al.* 2003; Gao *et al.* 2009), partial (*Sox8/10* and *HoxA1/B1*) (Kellerer *et al.* 2006; Ruff *et al.* 2015), or limited (*Phox2a/2b*)(Coppola *et al.* 2005). Given the profound rescue of testes defects in $Sox3^{Sox2KI}$ mice, we suggest that SOX3 and SOX2 proteins are biochemically equivalent. The observed differences in their null mutant phenotypes therefore likely reflects unique zones of expression, presumably generated through evolutionary subfunctionalization. However, why two biochemically equivalent proteins have retained widespread overlapping expression within the developing brain across an extensive evolutionary period remains an intriguing question. This does not appear to be an accident of evolution, as ancestral SOX B proteins in *Drosophila* (SoxNeuro and Diachete) exhibit similar partial redundancy, and active conservation of binding sites that allow both paralogues to bind (Carl and Russell 2015). One explanation is that it may be difficult to fully disentangle regulatory elements that direct *Sox3* expression in the ventral diencephalon from other neuroprogenitor zones. Further analysis of *SoxB1* gene regulation, for example, though CRISPR/Cas9-mediated enhancer deletion, may reveal insights into this interesting biological phenomenon.

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Author contribution: F.A., J.H. and P.T. conceived the project. F.A. performed all the experiments apart from the quantitative SOX2 immunofluorescence (performed by D.P.) and the SoxB1 qPCR comparison (performed by D.M.). J.H., P.T., and F.A. drafted the manuscript, which was reviewed and edited by all authors.

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