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Report

Sox2 Acts through *Sox21* to Regulate Transcription in Pluripotent and Differentiated Cells

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Summary

***Sox2* is an important transcriptional regulator in embryonic and adult stem cells [1–4]. Recently, *Sox2* was identified as an oncogene in many endodermal cancers, including colon cancer [5–8]. There is great interest in how *Sox2* cooperates with other transcription factors to regulate stem cell renewal, differentiation, and reprogramming [9]. However, we still lack a general understanding of *Sox2* transcriptional action. To determine transcriptional partners of *Sox2* in adult cells, we generated mice where gene expression could be induced by an externally applied stimulus. We analyzed the consequences in the intestine where cell turnover is rapid. *Sox2* expression, but not *Oct4*, specifically increased the numbers of stem cells and repressed *Cdx2*, a master regulator of endodermal identity. In vivo studies demonstrated that *Sox21*, another member of the SoxB gene family, was a specific, immediate, and cell-autonomous target of *Sox2* in intestinal stem cells. In vitro experiments showed that *Sox21* was sufficient to repress *Cdx2* in colon cancer cells and in pluripotent stem cells. *Sox21* was also specifically induced by *Sox2* in fibroblasts and inhibition of *Sox21* blocked reprogramming to the pluripotent state. These results show that transcriptional induction of *Sox21* is a rapid and general mediator of the effects of *Sox2* on cell identity in a wide range of cell types.**

Results and Discussion

Inducing *Sox2* in the Adult Intestine

To study the function of *Sox2* in adult tissues, we employed an inducible overexpression strategy [10]. We focused on the intestine because the short life-cycle of differentiated cells allows rapid analysis of tissue homeostasis [11].

Morphological changes were observed in the intestine following 2-day *Sox2* induction (Figure 1A; see Figure S1A available online). When the bromodeoxyuridine (BrdU) analog ethynyl-2′-deoxyuridine (EdU) was administered for 3 hr on day 2 of DOX treatment, an increase in the number of proliferating cells was observed (Figures 1A, 1C′, and 1D). The majority of EdU labeled cells were Sox2-positive (Figure 1D) and crypts became morphologically expanded (Figures 1C–1C″; Figure S1A).

When a pulse of EdU labeling was followed by a 3-day chase, EdU-positive cells were found throughout the villi (Figures 1F and 1F′). EdU was observed in Villin$^+$ absorptive cells, DBA$^+$ goblet cells, Lysozyme$^+$ Paneth cells, and Serotonin$^+$ enteroendocrine cells (Figures S1F–S1I). These data suggest that *Sox2* stimulates the proliferation of precursor cells that subsequently differentiate into the major cell types of the intestine.

There are two populations of proliferating cells in the intestine, transit amplifying progenitors (TAP), and intestinal stem cells (ISCs) [12]. Prominin1 (Prom1) marks both populations whereas Lgr5 is expressed in ISCs [13, 14]. Upon *Sox2* induction, Prom1 expression was markedly extended toward the crypt-villus boundary (Figures 1G and G′). Lgr5-GFP positive cells were also dramatically expanded (Figures 1A, 1H, and 1H′). Neither Lgr5$^+$ nor Prom1$^+$ cells were detected in the villi. The coexpression of EdU and Sox2 in the nuclei of Lgr5-GFP$^+$ cells suggests that Sox2 directly affects the number and location of ISCs (Figure 1D).

Paneth cells provide niche signals for ISCs [15]. *Sox2*-activated, proliferating Lgr5-positive cells were seen that had no direct contact with Lysozyme$^+$ Paneth cells but these ectopic cells were restricted to the crypt (Figure 1E). These results show that the induction of *Sox2* is associated with a disruption in the signaling that normally restricts Lgr5 to the base of the crypt but differentiation in the villi is accompanied by downregulation of Lgr5.

Wnt signaling is important for maintaining stem and progenitor cells in the intestine [12, 16–18] and Lgr5 was recently identified as a coreceptor for Frizzled/Lrp Wnt receptor [19]. Wnt targets, including Lgr5, Myc, CD44, Ephb2, and cyclinD1, were upregulated on day 2 of *Sox2* induction (Figure S1B). CD44, which is normally restricted to the crypt [17], was present throughout the crypt-villus unit after induction of *Sox2* (Figure S1C). Olfm4, another marker of intestinal stem cells [20], was also dramatically upregulated (data not shown).

EphB2 and EphB3 receptors are expressed in the crypts, whereas the ephrin ligands are expressed in the villi [21, 22]. Upon Sox2 induction, EphB2 was upregulated in the villus and Ephrin B1 staining extended into the crypt (Figures S1D and S1E). Thus, *Sox2* induction disrupts some features of the crypt-villus organization but maintains the crypt as the main site of cell proliferation.

Distinct Effects of *Sox2* and *Oct4* on Intestinal Precursor Cells

Previous studies with TetON-*Oct4* mice showed increased proliferation in the intestine but did not specifically define the proliferating cells [10]. Consistent with published data, a clear proliferative response was seen after 2 days of DOX treatment in TetON-*Oct4* mice (Figures S2A–S2C). The presence of many EdU labeled cells in the neck of the crypt after *Oct4* induction and the elevated expression of Prominin1 (data not shown) suggest expansion of TAPs. Surprisingly, Lgr5 expression was not elevated and the Lgr5 cells remained restricted to the base of the crypt after *Oct4* induction (Figure 1A; Figures S2D–S2F).

Transcriptional Consequences of *Sox2* and *Oct4* Expression

Microarray data on gene expression patterns was used to define the specific transcriptional effects of *Sox2* compared

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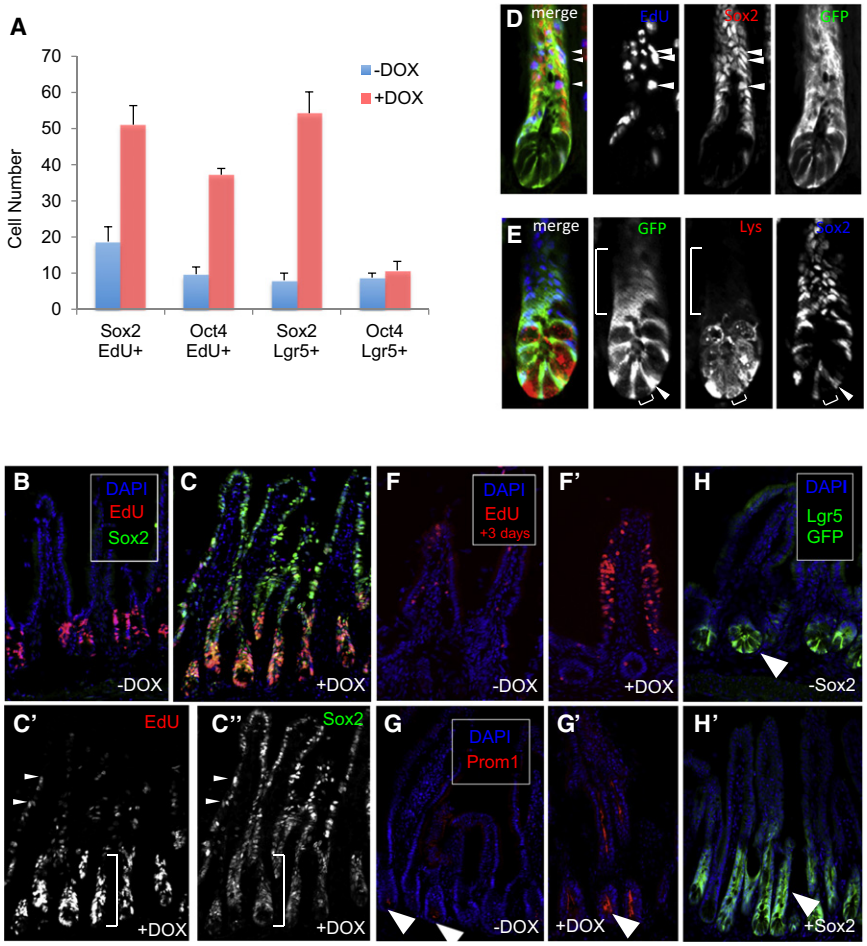

Figure 1. *Sox2* Overexpression Rapidly Expands Lgr5$^+$ Intestinal Stem Cells

(A) Quantification of numbers of EdU$^+$ and Lgr5$^+$ cells in 2-day DOX treated (+DOX) and untreated (−DOX) ileums of TetON-*Oct4* X *Lgr5*-GFP (Oct4) and TetON-*Sox2* X *Lgr5*-GFP (Sox2) mice. Error bars represent SD.

(B–C″) EdU and Sox2 staining of TetON-*Sox2* ileum; (B), −DOX, (C), +DOX for 2 days. Note increased numbers of proliferating Sox2$^+$ cells in the crypts (brackets in C′, C″), as well as their spreading into the villi (arrowheads in C′ and C″). (C′ and C″) Single-channel images of EdU and Sox2, respectively.

(D) EdU, Sox2, and GFP staining of ileum of 2-day DOX-treated *Lgr5*-GFP X TetON-*Sox2* animal. Arrowheads indicate Sox2$^+$EdU$^+$Lgr5$^+$ cells.

(E) Lysozyme, Sox2, and GFP staining of ileum of 2-day DOX-treated *Lgr5*-GFP X *Sox2* animal. Brackets indicate Lgr5$^+$ cells expanded from crypt base that have lost contact with Paneth cells. Arrowhead, Sox2$^+$Lgr5$^+$ crypt base cell; small bracket, adjacent Sox2$^-$ Paneth cell.

(F and F′) EdU staining of pulse-chased TetON-*Sox2* ileums: −DOX pulse (F); +DOX pulse (F′).

(G and G′) Prom1 staining of TetON-*Sox2* ileum: −DOX (G); +DOX (G′). Note expanded apical Prom1 staining upon *Sox2* induction compared to uninduced conditions (arrowheads).

(H and H′) GFP images of *Lrg5*-GFP X rtTA (H) and *Lgr5*-GFP X *Sox2* (H′) ileums (+DOX). Note the expansion of *Lgr5*-GFP$^+$ cells upon *Sox2* induction (arrowheads).

See also Figure S1.

to *Oct4*. Cluster analysis of the entire data set shows that the transcriptome of TetON-*Oct4* animals was clearly different from that of the *Sox2*-induced animals (Figure S2H). Interestingly, when TetON-*Sox2* and TetON-*Oct4* mice were crossed, the transcriptome (+DOX) of the double-induced animals showed marked similarity to the transcriptome of TetON-*Sox2* mice (Figures S2G and S2H). These results show that induction of Sox2 but not Oct4 increased Lgr5 expression.

Many intestinal genes, including *Vil1*, *Fabp2*, intestinal alkaline phosphatase (ALPI), peptide Y (*Pyy*), *Muc13*, *Muc3*, *Mucdhl*, and *Tff3*, were downregulated by Sox2 and many anterior genes were upregulated, including keratins (*Krt1-15*, *Krt1-13*, *Krt2-4*, *Krt2-6a*, *Krt1-23*, *Krt2-5*), *Pkp1*, and dermokine (*1110014F24Rik*) (Figures 2A and 2C; Figure S2K). Alongside *Sox2*, *Foxa2* is required for the differentiation of the anterior endoderm and is expressed in esophagus, trachea, lung, pancreas, and liver [23–25]. On *Sox2* induction, *Foxa2* became upregulated in the adult intestine (Figures 2A and 2C; Figure S2L).

During development, *Sox2* is expressed in the anterior endoderm and this expression is antagonized by the master regulator of posterior endoderm *Cdx2* [26]. Cdx2 expression was clearly reduced in the intestines of 2-day *Sox2*-induced animals (Figure 2B). Transcription factors regulated by *Cdx2* including *Klf4*, *HNF1a*, *Hnf4a*, and *Isx* were downregulated and anterior transcriptional regulators including *Pax9* and *Pitx1* were induced (Figure 2C; Figure S2K). *Oct4* did not repress *Cdx2* (Figure S2J), the *Cdx2* targets *Klf4*, *Hnf1a*, *Hnf4a*, and *Isx*, or intestinal markers *Vil1*, *Fabp2*, and *ALP*, nor did it upregulate anterior markers and keratins (data not shown). These results suggest that Sox2 induction reprograms the positional identity of cells in vivo.

Sox21 Induction Is a Specific and Cell-Autonomous Target of Sox2

Because *Sox2* is thought to be a transcriptional activator, we searched for *Sox2* activated repressors by comparing lists of DOX-induced genes. In TetON-*Sox2* intestines, mouse embryonic fibroblasts (MEFs), embryonic stem cells (ESCs), and neural stem cells, we found that *Sox21*, a SoxB family transcriptional repressor, was strongly upregulated by Sox2 (data not shown). *Sox21* induction was confirmed in the intestine as early as 16 hr after Dox treatment (Figures 2C and 2D), concomitant with repression of *Cdx2* and preceding the increase in cell proliferation, expansion of Lgr5$^+$ cells, or the induction of *Krt13* (data not shown). *Oct4* did not induce *Sox21* (Figure S2I).

For *Sox21* to act as an endogenous mediator of *Sox2* initiated transcriptional change, the two proteins must be expressed in the same cells. To study whether *Sox21* is a cell-autonomous target of *Sox2*, we specifically induced *Sox2* in Lgr5$^+$ cells using a combination of cre-dependent *tTA* and *Lgr5-EGFP-IRES-creER* alleles (see Supplemental Experimental Procedures). A low dose of tamoxifen was used to induce

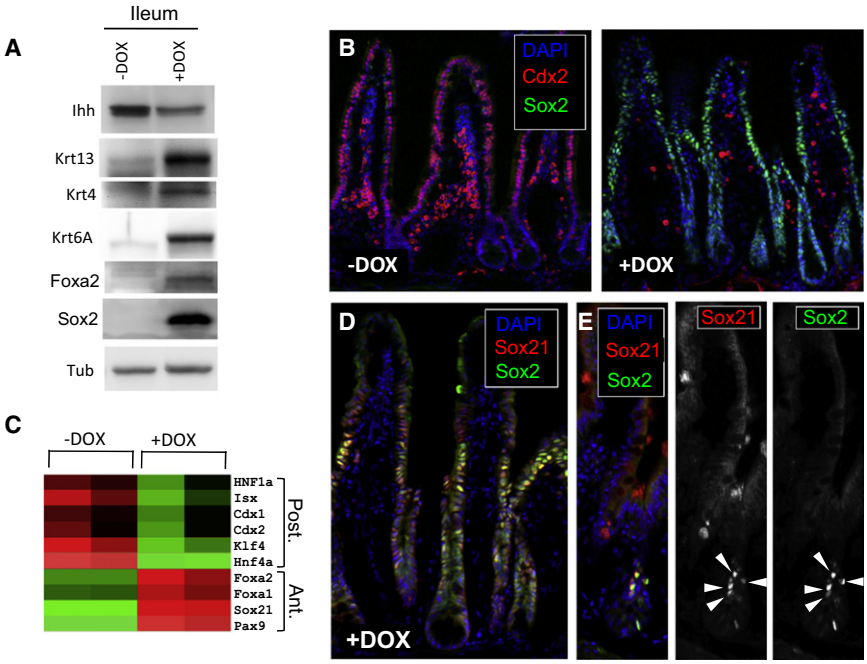

Figure 2. *Sox2* Represses *Cdx2* and Induces *Sox21*

(A) Western blot analysis of anterior/posterior markers in 2-day DOX-treated TetON-*Sox2* ileum.
(B) Cdx2 and Sox2 staining of TetON-*Sox2* ileums: −DOX (left); +DOX (right).
(C) Microarray analysis of anterior-posterior transcriptional regulators (labeled Ant and Post) in ileums of untreated (−DOX) and 2-day DOX-treated (+DOX) TetON-*Sox2* animals.
(D) Sox2 and Sox21 staining of TetON-*Sox2* ileums treated with DOX for 16 hr.
(E) Sox2 and Sox21 staining of Sox2$^+$ intestinal clones 4 days after Tamoxifen injection. Arrowheads indicate Sox21$^+$Sox2$^+$ cells.
See also Figure S2.

Sox2 in single Lgr5-EGFP-expressing cells. Four days after Tam injection, Sox2 expressing cells were seen in 10% of crypts. In all Sox2$^+$ crypts trains of Lgr5$^+$,Sox2$^+$ cells were seen extending into the neck of the crypt (Figure S2M). Aberrantly localized Lgr5$^+$ cells were never seen in normal, uninduced crypt-villus units (Figure S2N). One hundred percent of mislocalized Lgr5$^+$,Sox2$^+$ cells upregulated Sox21 (Figure 2E; Figure S2O), suggesting that *Sox2* cell-autonomously induces *Sox21*.

To test whether *Sox21* can repress *Cdx2*, we used the human colon cancer cell line Ls174T that expresses *Cdx2* but neither *Sox2* nor *Sox21* [27]. Cdx2 expression was diminished in cells stably transfected with Sox21 (Figure 3A). In these cells, chromatin immunoprecipitation (ChIP) demonstrated that Sox21 was present on the *Cdx2* enhancer [28] (Figure 3B). We compared the ability of *Sox2*, *Sox21*, and *Oct4* to repress *Cdx2* directly. As seen from Figure 3C, *Sox21*, but not *Sox2* or *Oct4*, strongly reduced the number of Cdx2$^+$ cells. These data show that *Sox21* induction is sufficient to repress *Cdx2*.

Sox21 Regulates the Expression of *Cdx2* in Pluripotent Cells

Sox2 and *Cdx2* also play important roles in the process of early embryonic lineage segregation [3, 29] and Cdx2 has been shown to repress *Sox2* [26]. Our data show that *Sox2* can repress *Cdx2* and suggest that this effect requires induction of *Sox21*. To ask whether *Sox21* is required for *Sox2* to repress *Cdx2* in pluripotent cells, we analyzed mouse ESCs containing the TetON-*Sox2* transgene. Sox21 protein was upregulated when Sox2 levels increased (Figure 3D; Figures 3A, and S3B), suggesting that *Sox2* induces *Sox21* in pluripotent cells.

BMP4 causes mouse ESCs to differentiate into Cdx2$^+$ cells [30, 31]. When mouse TetON-*Sox2* ESCs were treated with BMP4 for 4 days, Cdx2 expression was upregulated, but maintaining *Sox2* expression by treatment with DOX blocked Cdx2 induction (Figure 3E, left panel). Transfection of *Sox21* siRNA rescued Cdx2 expression (Figure 3E, right panel; Figures 3E, and S3F). Sox21 knockdown was efficient and did not affect levels of Sox2 (Figures S3C and S3D). These results show that *Sox21* is required for *Sox2* to repress *Cdx2* expression.

To confirm *Cdx2* repression by *Sox21* in physiological conditions, we performed Sox21 ChIP in human ESC-derived neurectodermal progenitors (Figure 3F). Sox21 was enriched at the *Cdx2* enhancer in neuroectodermal cells obtained by Noggin-SB431542 treatment, but not in BMP4-treated cells. Recently, Sox2 was found to bind the *Sox21* enhancer in ES cells undergoing neural differentiation [32]. We confirmed this result (Figure 3G), further strengthening our argument that *Sox2* directly induces *Sox21*.

Sox21 Induction by *Sox2* Is Required to Reprogram Cells

Sox2 is a critical factor that is required for cellular reprogramming so we asked whether *Sox21* induction is required for induced pluripotent stem cell (iPSC) generation from fibroblasts. Doxycycline treatment of TetON-*Sox2* MEFs rapidly upregulated *Sox21* (Figure 4A). Similar to intestine, *Oct4* did not induce *Sox21* (Figure 4B). *Sox21* induction by *Sox2* was unaffected by coexpression of *Oct4*, or the combination of *Oct4*, *Klf4*, and *c-Myc* (Figure 4C). During the reprogramming process we transfected Tet-*OSMK* MEFs [33] with *Sox21* siRNA or control siRNA. *Sox21* knockdown resulted in significantly reduced numbers of SSEA1$^+$ colonies compared to the control siRNA (Figure 4D). SSEA1$^+$ colonies established in the presence of *Sox21* and control siRNA became phenotypically normal iPSCs (Figures S4A–S4D). Therefore, induction of *Sox21* by *Sox2* is required to induce the pluripotent stem cell state.

Sox21 is widely expressed in the developing nervous system where it is often coexpressed with *Sox2* and these two genes are thought to have opposing roles in neurogenesis [34–39]. Our data suggest that the interaction of *Sox2* and *Sox21* should be assessed in the many studies analyzing the mechanisms of reprogramming and neuronal transdifferentiation [40–42].

In addition to endoderm, *Sox2* and *Sox21* intersect with Cdx2 in other systems, such as trophectoderm [43] and caudal neuroectoderm [44]. Our preliminary results show that expression of *Sox21* and *Cdx2* is mutually exclusive in human ESC-derived hindbrain cultures. There is also

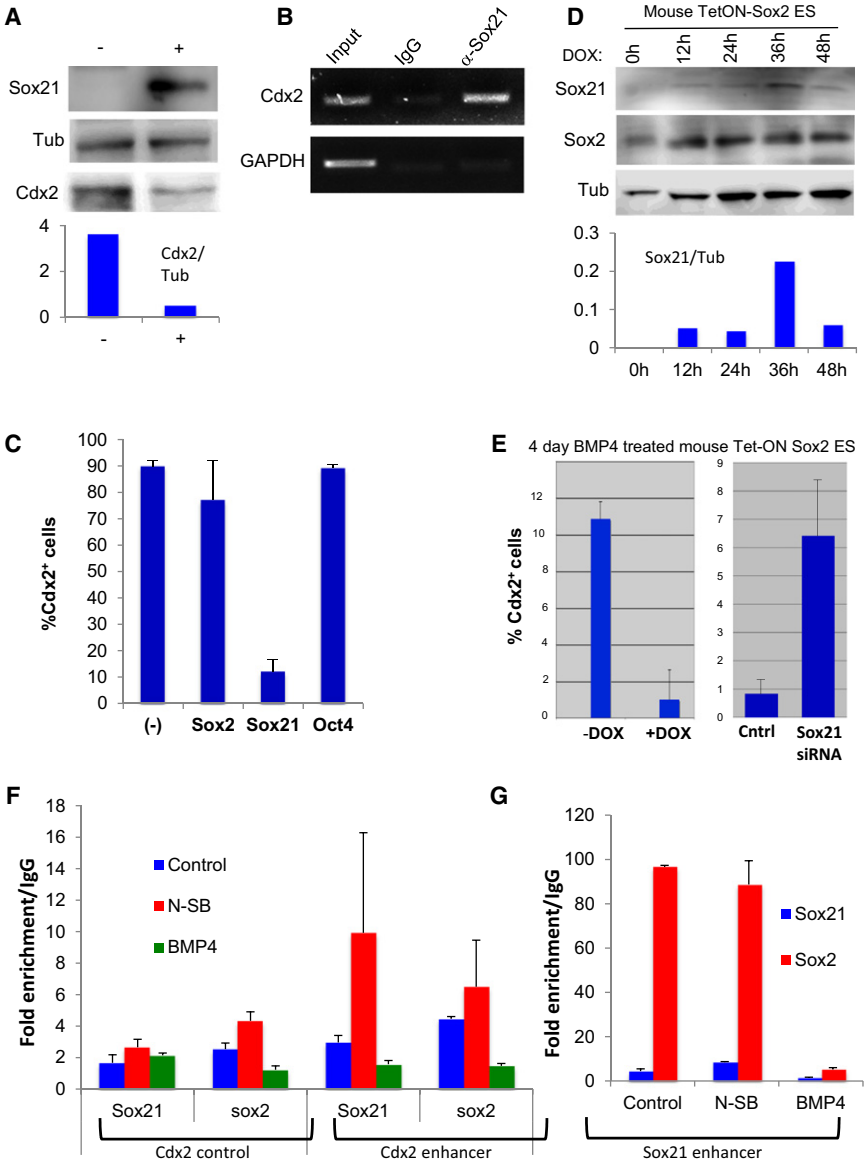

Figure 3. *Sox21* Represses *Cdx2*

(A) Western blot analysis of Ls174T cells stably transfected with *Sox21* cDNA (+) and untransfected Ls174T cells (−). Quantification of Cdx2 signal relative to Tubulin signal is shown below.

(B) Chromatin immunoprecipitation analysis of Sox21 binding to the *Cdx2* intestinal enhancer in Ls174T-*Sox21* cells.

(C) Transfection of *Sox21* but not *Sox2* or *Oct4* reduces numbers of Cdx2$^+$ Ls174T cells. Error bars represent SD.

(D) Western blot analysis of Sox21 induction in ESCs containing an inducible *Sox2* transgene (TetON-*Sox2*), treated with DOX for indicated periods of time. Quantification of Sox21 signal relative to Tubulin signal is shown below.

(E) Repression of *Cdx2* by *Sox2* requires *Sox21*. Left panel shows that TetON-*Sox2* mouse ESCs were treated with BMP4 for 4 days in the presence or absence of DOX and Cdx2$^+$ cells were counted. Right panel shows that prior to BMP4 treatment, TetON-*Sox2* ESCs were transfected with *Sox21* or control siRNA. Error bars represent SD.

(F) Quantitative PCR analysis of Sox21 and Sox2 chromatin immunoprecipitation of the *Cdx2* enhancer and control regions [45] in undifferentiated (Control), Noggin/SB431542-treated (N-SB), and in BMP4-treated hES cells. Error bars represent SD in three independant ChIP experiments per condition.

(G) Quantitative PCR analysis of Sox21 and Sox2 chromatin immunoprecipitation of the *Sox21* enhancer region [32] in undifferentiated (Control), Noggin/SB431542-treated (N-SB), and BMP4-treated human embryonic stem cells (hESCs). Error bars represent SD in three independent ChIP experiments per condition.

See also Figure S3.

increasing evidence that *Sox2* acts as an oncogene [5, 6, 8]. The data presented here suggest that it will be important to determine whether the oncogenic function of *Sox2* requires genetic or epigenetic silencing of the tumor suppressor *Sox21*.

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.07.013.

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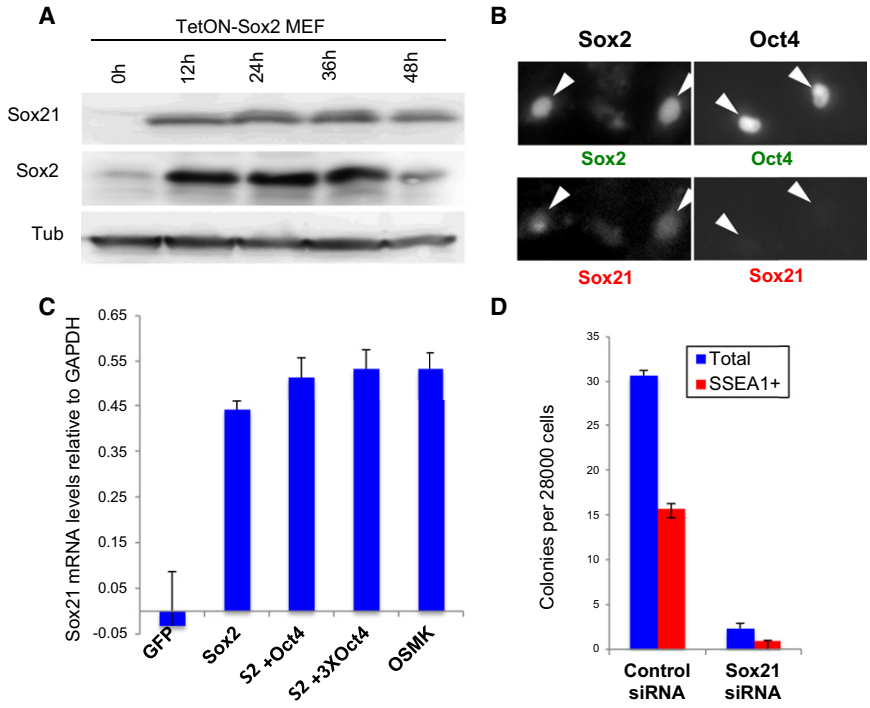

Figure 4. *Sox21* Is Required for Reprogramming

(A) *Sox2* rapidly induces Sox21 in MEFs. TetON-*Sox2* MEFs were treated with DOX for indicated periods of time and analyzed by western blotting.

(B) *Oct4* does not induce Sox21 in MEFs. MEFs were transfected with *Sox2* or *Oct4* and 2 days later costained for Sox21 and Sox2/Oct4. Arrowheads indicate transfected cells. Transfected plasmid is indicated on top of each panel, staining antibody is indicated on the bottom.

(C) Induction of *Sox21* by *Sox2* in MEFs is maintained in the presence of reprograming factors. MEFs were transfected with empty vector (GFP), *Sox2* alone (Sox2), *Sox2* in the presence of increasing amounts of *Oct4* (S2+Oct4 and S2+3XOct4), or with four reprograming factors (OSMK). Two days later *Sox21* messenger RNA levels were analyzed by qPCR. Error bars repesent SD.

(D) Small interfering RNA (siRNA)-mediated knockdown of *Sox21* during reprograming results in fewer reprogrammed colonies. Tet-*OSMK* MEFs were transfected with indicated siRNA every 3 days during DOX administration. On day 13, plates were live-stained with dye-conjugated SSEA1 antibody and numbers of total and SSEA1$^+$ colonies were counted. Error bars represent SD between three independent reprogramming experiments.

See also Figure S4.

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