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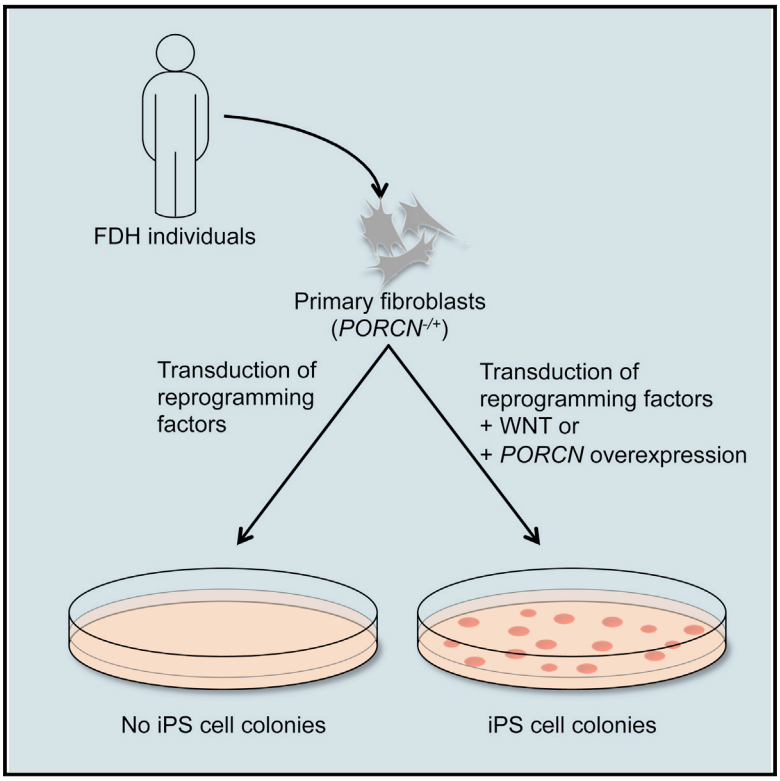
A Rare Human Syndrome Provides Genetic Evidence that WNT Signaling Is Required for Reprogramming of Fibroblasts to Induced Pluripotent Stem Cells

Graphical Abstract



Highlights

Reprogramming of *PORCN* mutant fibroblasts requires ectopic WNT signaling

Low levels of WNT signaling maintain undifferentiated human pluripotent stem cells

Aberrant WNT signaling promotes chromosomal instability

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In Brief

WNT signaling is required for embryonic development, maintenance of stem cells in an undifferentiated state, and homeostasis of adult tissues. Here, Ross et al. provide genetic evidence that this signaling pathway is also essential to the process of reprogramming of somatic cells to an induced pluripotent state. These studies also reveal an unexpected link between aberrant WNT signaling and chromosomal instability.

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A Rare Human Syndrome Provides Genetic Evidence that WNT Signaling Is Required for Reprogramming of Fibroblasts to Induced Pluripotent Stem Cells

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SUMMARY

WNT signaling promotes the reprogramming of somatic cells to an induced pluripotent state. We provide genetic evidence that WNT signaling is a requisite step during the induction of pluripotency. Fibroblasts from individuals with focal dermal hypoplasia (FDH), a rare genetic syndrome caused by mutations in the essential WNT processing enzyme PORCN, fail to reprogram with standard methods. This blockade in reprogramming is overcome by ectopic WNT signaling and *PORCN* overexpression, thus demonstrating that WNT signaling is essential for reprogramming. The rescue of reprogramming is critically dependent on the level of WNT signaling: steady baseline activation of the WNT pathway yields karyotypically normal iPSCs, whereas daily stimulation with Wnt3a produces FDH-iPSCs with severely abnormal karyotypes. Therefore, although WNT signaling is required for cellular reprogramming, inappropriate activation of WNT signaling induces chromosomal instability, highlighting the precarious nature of ectopic WNT activation and its tight relationship with oncogenic transformation.

INTRODUCTION

The process of converting, or reprogramming, a mature cell type to an embryonic stem cell-like state requires the establishment of a transcriptional regulatory network comprised of transcription factors, including POU5F1/OCT4, SOX2, and NANOG (Boyer et al., 2005; Cole et al., 2008). In human and mouse embryonic stem cells, these factors maintain each other's expression, and hence the pluripotent state, through regulatory feedback mechanisms. Disruption of this regulatory circuit causes cells to exit the pluripotent state and differentiate. Extracellular signals, such as FGF2 in human embryonic stem cells (hESCs) and LIF in mouse embryonic stem cells, influence and regulate the pluripotent state. In addition, the WNT signaling pathway critically influences the pluripotent state of embryonic stem cells (Blauwkamp et al., 2012; Jiang et al., 2013; Lyashenko et al., 2011; Sato et al., 2004; ten Berge et al., 2011; Wray et al., 2011; Yi et al., 2011). Although establishment of the OCT4-NANOG-SOX2 transcriptional regulatory network is clearly critical for the generation of induced pluripotent stem cells (iPSCs), the role of extracellular signals, such as WNTs, in this process has not been examined extensively.

WNT and the WNT/β-catenin signaling pathway (also known as the canonical WNT signaling pathway) have been implicated in iPSC generation; however, significant controversy surrounds their specific role in this process. First, in the original iPSC studies, β-catenin was found to promote reprogramming; however, it was eliminated from the final reprogramming factor cocktail (Takahashi and Yamanaka, 2006). Second, addition of WNT proteins influences the induction of the pluripotent state (Aulicino et al., 2014; Ho et al., 2013; Marson et al., 2008; Zhang et al., 2014); however, one study found that WNT/β-catenin signaling was stimulatory (Zhang et al., 2014), whereas other studies found that it was inhibitory during early stages of reprogramming (Aulicino et al., 2014; Ho et al., 2013). Third, small molecules that inhibit GSK3—and hence activate WNT/β-catenin signaling—stimulate reprogramming efficiencies (Li et al., 2009; Silva et al., 2008) and can promote reprogramming with OCT4 as the only reprogramming factor (Li et al., 2011). However, GSK3 inhibitors, as well as purified WNT proteins, potently promote mesendodermal differentiation of hESCs (Bakre et al., 2007; Davidson et al., 2012), creating a conundrum over how prodifferentiation factors can also promote the induction of the pluripotent state. Finally, despite these established links between WNT signaling and the generation of iPSCs, a strict requirement for WNT signaling in this process has not been demonstrated. In this study, we employ fibroblasts from patients harboring mutations in an essential WNT processing enzyme, called PORCN, to establish that endogenous WNT signaling is required during the process of inducing a pluripotent stem cell state from fibroblasts.

The *PORCN* gene encodes an integral membrane-resident endoplasmic reticulum protein that regulates processing of

WNT proteins by catalyzing the covalent attachment of a lipid moiety to the WNT polypeptide backbone (Barrott et al., 2011; Biechele et al., 2011; Galli et al., 2007; Herr and Basler, 2012; Kadowaki et al., 1996; Proffitt and Virshup, 2012; van den Heuvel et al., 1993; Zhai et al., 2004). This lipid modification is essential for WNT activity and, as demonstrated by the X-ray crystal structure of a WNT protein in complex with its receptor, is directly involved in receptor binding (Janda et al., 2012). Given the high degree of homology among members of the *WNT* gene family, it is generally accepted that disruption of PORCN activity, either by mutation or with small molecule inhibitors, impairs processing of all WNT proteins. Therefore, PORCN dysfunction will produce an "all-WNT" mutant phenotype.

Porcn knockout mice are early embryonic lethal and fail to enter early stages of embryonic induction as indicated by the absence of *Bry/T* expression at E6.5 (Barrott et al., 2011; Biechele et al., 2011; Liu et al., 2012), a nearly identical phenotype to that observed in *Wnt3* knockout mice (Liu et al., 1999). In humans, *PORCN* mutations lead to a rare pleiotropic disorder called Focal Dermal Hypoplasia (FDH; also known as Goltz syndrome) (Grzeschik et al., 2007; Wang et al., 2007), characterized by skin lesions and defects of the gastrointestinal, cardiovascular, and central nervous system (Temple et al., 1990). In affected females, the severity of disease is influenced by the nature of the *PORCN* mutation and the extent to which the mutant *PORCN* allele is on the active X chromosome. Males carrying *PORCN* mutations are rare and have acquired somatic mutations during embryonic development and hence are mosaic with respect to the *PORCN* mutation and PORCN function. Because the mammalian genome encodes a single *PORCN* gene that specifically modifies WNT proteins, it offers a natural bottleneck in WNT processing to explore the requirement of WNT proteins in any biological process.

In this study, we show that fibroblasts from individuals carrying mutations in the *PORCN* gene fail to reprogram to an induced pluripotent state. This blockade to iPSC formation is readily overcome through the ectopic activation of WNT signaling or by overexpression of *PORCN*. Additionally, we show that precise dosing of WNT activity is critical to generating karyotypically normal iPSCs, an observation that has important implications for WNT's role in promoting genomic instability and hence in cancer progression.

RESULTS

Focal dermal hypoplasia (FDH) is a rare genetic syndrome with many pleiotropic consequences caused by mutations in the X-linked gene *PORCN*. To study this complex disorder, we sought to develop a disease-in-a-dish by first reprogramming fibroblast cultures from FDH patients to iPSCs (Figure 1A). To this end, we established fibroblast cultures from skin punch biopsies from individuals with clinical hallmarks of FDH and confirmed the presence of *PORCN* mutations by sequencing (summarized in Table S1). One of the mutations (FDH2) altered the highly conserved GT splice donor site after exon 2, allowing us to demonstrate by RT- PCR that the fibroblast culture expressed undetectable to very low levels of the wild-type (WT) *PORCN* gene (Figures S1A and S1B). Interestingly, in contrast to WT fibroblasts, FDH fibroblasts failed to yield iPSC colonies (Figures 1B and 1C; Table S1). Because *PORCN* encodes an essential WNT processing enzyme, we reasoned that a defect in WNT secretion contributed to the observed reprogramming defect. Consistent with this hypothesis, FDH fibroblasts (FDH2 and FDH3) failed to secrete WNT proteins at levels observed for WT fibroblasts (an age-matched control FDH6 and FDH7), as monitored by secretion of the WNT protein encoded by the *WNT5A* gene (Figures S1C and S1D). (Note that other WNT proteins may be secreted by WT cells; however, we do not currently have a sensitive detection assay for other WNT proteins.) Treatment of WT fibroblasts with the PORCN inhibitor IWP2 (IWP) (Chen et al., 2009) greatly reduced levels of WNT5A protein in the CM, confirming that WNT secretion is dependent on PORCN function (Figure S1D).

Importantly, treatment with Wnt3a after transduction of the reprogramming factors restored iPSC generation from FDH fibroblasts (Figures 1B and 1C; Table S1), suggesting that endogenous WNT secretion and signaling are required during the process of reprogramming from a fibroblast to an iPSC. Reprogramming of FDH fibroblasts required exogenous Wnt3a regardless of whether *MYC*, a known WNT/β-catenin target in several cell types (He et al., 1998; Zhang et al., 2012), was included as one of the reprogramming factors (KMOS versus KOS; Table S1). The fact that inclusion of *MYC* as a reprogramming factor fails to yield FDH-iPSCs indicates that transcriptional activation of *MYC* by WNT is insufficient in promoting reprogramming and that other downstream WNT targets are involved. In addition, we found that a GSK3 inhibitor, CHIR98014 (CHIR), rescued FDH fibroblast reprogramming (Table S1), suggesting that WNT acts through the stabilization of its key mediator, β-catenin. These data indicate that WNT acts through β-catenin (i.e., the canonical WNT pathway) to promote reprogramming to the induced pluripotent state.

The observation that exogenous Wnt3a rescues reprogramming of FDH fibroblasts implies that the mutant PORCN protein fails to properly process endogenously expressed WNT proteins. To address this point, we transduced a *PORCN* transgene along with the KMOS reprogramming factors. As expected, overexpression of *PORCN*, like ectopic Wnt3a treatment, rescued the reprogramming defect of FDH fibroblasts (Figures 1D and 1E). Isolated PORCN-rescued FDH iPSCs carried the *PORCN* transgene (Figure S1E). Therefore, in FDH fibroblasts, PORCN is an essential reprogramming factor, along with KMOS.

Consistent with this genetic evidence that PORCN function is required for reprogramming, we found that treatment of WT fibroblast cultures undergoing standard reprogramming assays with the PORCN inhibitor IWP significantly reduced the number alkaline phosphatase-positive (AP+) colonies (Figure 1F). This reduction in reprogramming rates was previously reported by others (Ho et al., 2013). As with FDH fibroblasts, this blockade on reprogramming was overcome by addition of exogenous Wnt3a protein, suggesting that PORCN processing of endogenously produced WNT proteins is required for cellular reprogramming.

Isolation and expansion of iPSC colonies generated from FDH fibroblasts required continuous ectopic stimulation of

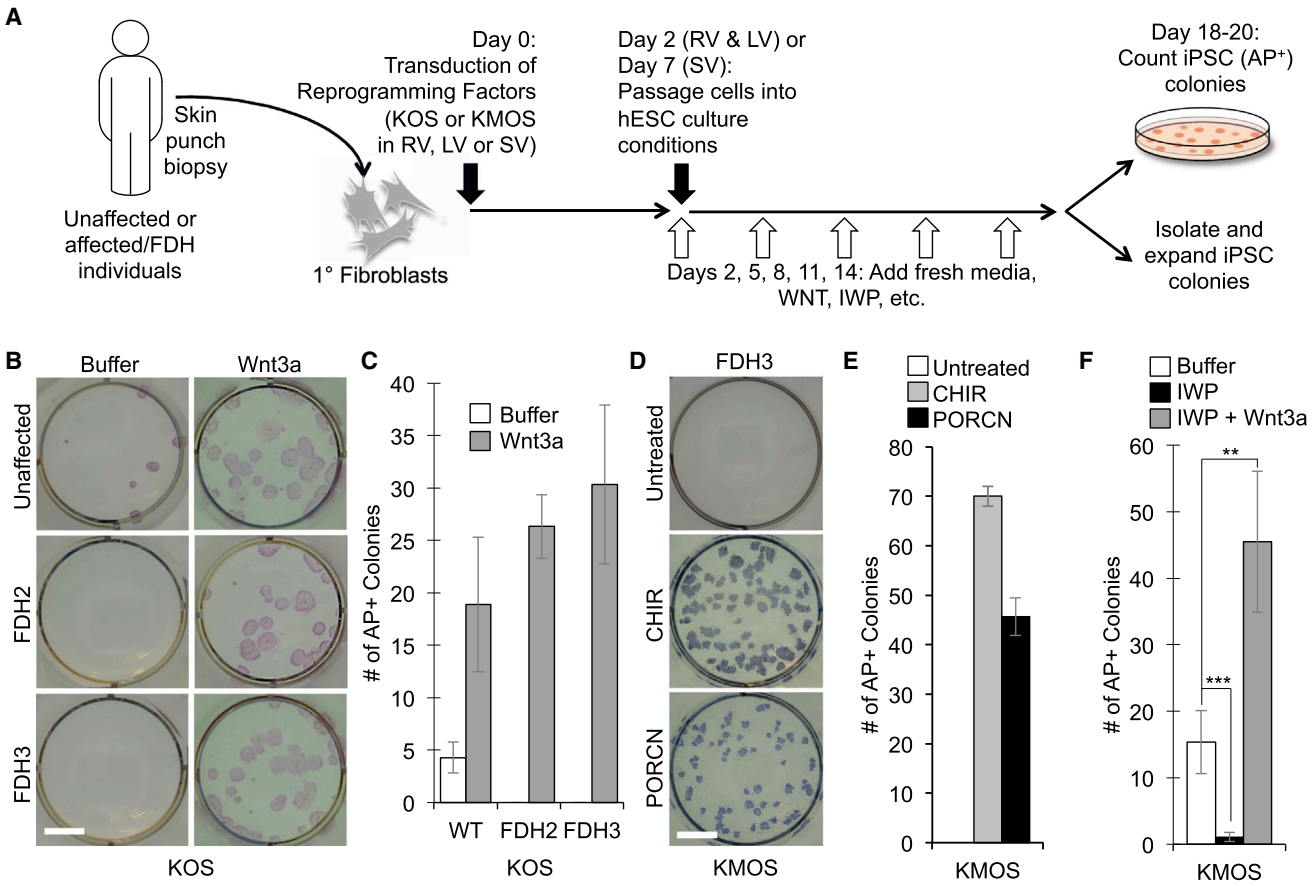

Figure 1. Ectopic WNT Signaling and PORCN Overexpression Restores Reprogramming of *PORCN* Mutant Fibroblasts

(A) Flow chart of reprogramming experiments. Fibroblast cultures established from unaffected and affected FDH individuals were transduced with reprogramming factors (*KLF4*, *MYC*, *OCT4*, and *SOX2* [*KMOS*] or *KLF4*, *OCT4*, and *SOX2* [*KOS*]) via viral infections with either retro- (RV), lenti- (LV) or Sendai-viral (SV) vectors. After passaging to hESC culture conditions, culture media were supplemented with compounds that perturb WNT signaling. Once colonies were visible by eye, cultures were fixed and stained for Alkaline Phosphatase (AP) to quantify colony numbers per condition or individual colonies were isolated and expanded as iPSC lines.

(B) *PORCN* mutant fibroblasts (FDH2 and 3) fail to reprogram in the absence of exogenous WNT activation. Addition of Wnt3a during reprogramming restores generation of AP+ colonies. Buffer, WNT storage buffer. Shown are representative images of AP-stained cultures. Scale bar represents 10 mm.

(C) Quantitation of reprogramming experiments in FDH fibroblasts. (Mean ± SD of four biological replicates for WT and of three biological replicates for FDH2 and 3.)

(D) Overexpression of *PORCN* in FDH3 fibroblasts, as well as treatment with the GSK3 inhibitor CHIR98014 (CHIR), rescues the reprogramming defect of *PORCN* mutant cells. Scale bar represents 10 mm.

(E) Quantitation of reprogramming experiments in FDH fibroblasts. (Mean ± SD of three biological replicates for FDH3.)

(F) A PORCN inhibitor blocks reprogramming. Fibroblasts undergoing reprogramming in the presence of the PORCN inhibitor IWP fail to yield AP-positive (AP+) colonies. This inhibitory effect is overcome by addition of Wnt3a protein. (Mean ± SD of three biological replicates. **$p < 0.005$; ***$p < 0.001$.)

See also Figure S1 and Table S1.

WNT signaling. When cultured in media containing CHIR at low concentrations (20 nM), FDH-iPSCs expressed the pluripotency markers *NANOG* and *POU5F1/OCT4* at levels comparable to WT iPSCs (Figure 2A). In the absence of CHIR, *NANOG* and *POU5F1/OCT4* expression levels were unaffected in WT iPSCs but decreased in FDH-iPSCs, indicating that an endogenous WNT signaling loop maintains optimal expression levels of these pluripotency regulators. As expected, at high CHIR concentrations (200 to 1,000 nM), *NANOG* and *POU5F1/OCT4* expression levels declined dramatically, indicative of cells exiting the pluripotent state. In addition, in the presence of low CHIR concentrations (20 nM), FDH-iPSCs displayed morphologies most closely resembling those of human pluripotent stem cells (Figure 2B). Withdrawal of CHIR from FDH-iPSCs led to marked reduction in cell-surface staining of the pluripotency markers SSEA4 and TRA1-81 (Figure 2C). Similar dosage effects were observed when Wnt3a protein was used in place of CHIR (Figure S2). Therefore, endogenous WNT signaling, as exists in WT iPSCs, or a low level of exogenous WNT signal activation with CHIR in FDH iPSCs is required to maintain normal expression of pluripotency-associated genes.

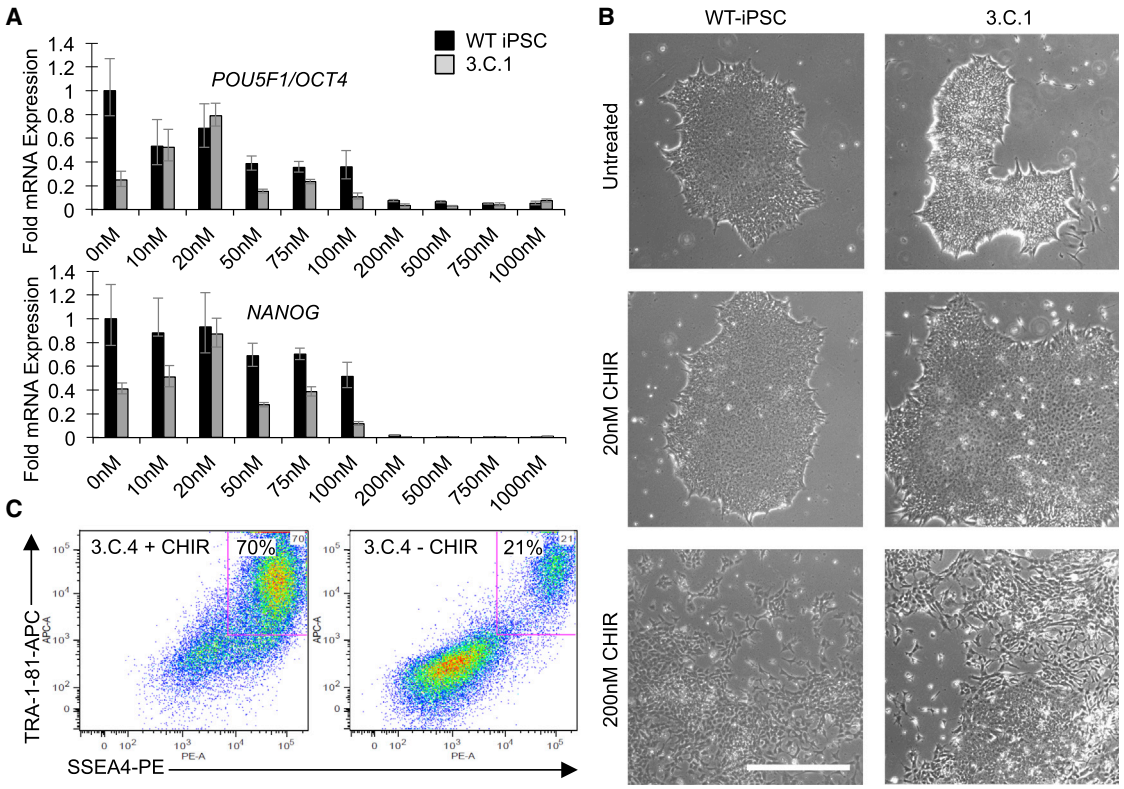

Figure 2. WNT Signaling Is Required to Maintain the Pluripotent State

(A) WT iPSCs or FDH iPSCs (clone 3.C.1) were cultured for 5 days with increasing doses of CHIR98014 (CHIR), and expression of *POU5F1/OCT4* and *NANOG* was determined by qRT-PCR. See also Figure S2.

(B) Clone 3.C.1 requires low levels of WNT stimulation to maintain an optimal hPSC morphology. In the absence of CHIR, WT iPSCs exhibit a normal hPSC morphology, whereas FDH-iPSCs fail to grow with the characteristic morphology associated with hPSCs. At CHIR concentrations of 20 nM, FDH iPSC colonies most closely resemble WT iPSCs. At concentrations of 200 nM, both cell populations assume a cell morphology indicative of differentiation. Scale bar represents 500 μM.

(C) FDH iPSCs (Clone 3.C.4) were cultured for 5 days in the presence (left) or absence (right) of CHIR (20 nM), stained for the cell-surface markers SSEA4 and TRA1-81, and analyzed by flow cytometry. Significantly higher cell-surface staining for these two pluripotency-associated markers is observed in the presence of CHIR (70%) than in the absence of CHIR (21%).

Additional characterization confirmed that FDH-iPSCs exhibit properties associated with bona fide pluripotent stem cells, including the characteristic morphology associated with human embryonic stem cells (Figure 3A), and expression of pluripotency markers, such as *OCT4*, *NANOG* (Figure 3B), TRA-1-81 (Figure 3C), and SSEA4 (Figure S3A). In addition, we confirmed their pluripotency by directing them to differentiate via embryoid bodies (EB) into cell populations of the three major germ layers: endo-, ecto-, and mesoderm. EBs from FDH-iPSC clone 3.C.1 expressed genes indicative of definitive endoderm (DE, *SOX17*, *FOXA2*, and *CXCR4*; Figures 3D, S3B, and S3C), mesoderm (*BRY/T*, *MESP1*, and *ACTC1*; Figures 3E and S3D), and ectoderm (*PAX6*, *SOX1*, and *TUBB3*; Figure 3F). Therefore, the FDH-iPSCs retain their ability to differentiate into cell types representative of the three major germ layers.

Surprisingly, FDH-iPSC clones isolated in the presence of Wnt3a exhibited dramatically altered karyotypes, with cells containing more than 60 chromosomes and other chromosomal aberrations, including insertions, deletions, translocations, and inversions (Figures 4A and S4A–S4C). Because the FDH fibroblasts were euploid (Figure 4B), the acquisition of aberrant chromosome numbers likely occurred during reprogramming. Importantly, iPSCs derived from WT fibroblasts (BJ) in the presence of Wnt3a were karyotypically normal (Figure 4C), indicating that changes in chromosome numbers upon reprogramming are associated with ectopic WNT signaling only in a PORCN-deficient context. Karyotypic abnormalities in Wnt3a-derived FDH-iPSCs did not represent clonal expansions because individual metaphase spreads from the same iPSC line exhibited distinct chromosome numbers (Figures 4E and S4A–S4C). Therefore, reprogramming of FDH fibroblasts (and hence WNT-deficient cells) in the presence of Wnt3a is detrimental to genomic integrity.

Because purified WNT proteins are highly unstable (Dhamdhere et al., 2014; Green et al., 2013), we reasoned that daily addition of Wnt3a protein may produce nonphysiological spikes in WNT signaling activity that may contribute to this chromosomal instability. Previous studies have linked aberrant WNT signaling to chromosomal instability (Aoki et al., 2007; Fodde et al., 2001; Hadjihannas and Behrens, 2006; Hadjihannas et al.,

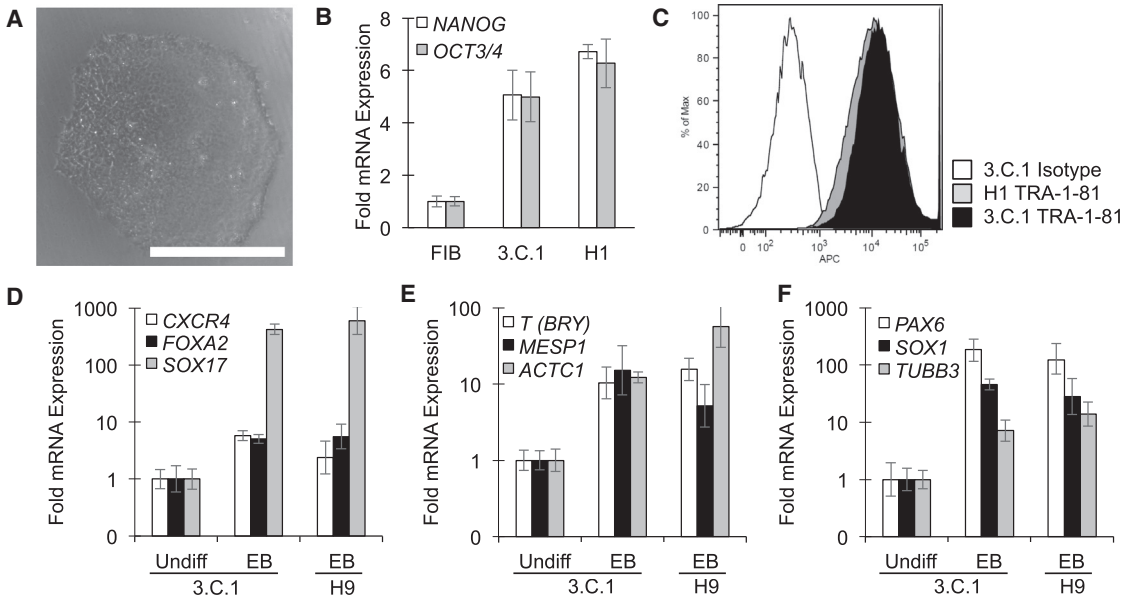

Figure 3. Characterization of a FDH-iPSC Clone

(A) A phase contrast image of a FDH-iPSC clone (3.C.1) derived in the presence of CHIR exhibits characteristics of human pluripotent stem cells. Note that, as shown in Figure 4D, these cells have a normal number of chromosomes. Scale bar represents 500 μm.

(B) qRT-PCR confirms expression of the pluripotency markers *NANOG* and *OCT4/POU5F1* at levels comparable to a human embryonic stem cell line (H1/WA01).

(C) Flow cytometry confirms presence of the cell-surface pluripotency markers TRA1-81 at levels comparable to those found on H1 cells.

(D–F) Upon differentiation via embryoid body (EB) formation, cells express markers associated with endodermal (D), mesodermal (E), and ectodermal (F) lineages. See also Figure S3.

2006; Kaplan et al., 2001; Tighe et al., 2007). To circumvent this problem, we isolated FDH-iPSC colonies from reprogrammed cultures derived in the presence of CHIR, which can be carefully dosed and is more stable over longer periods than Wnt3a protein (Figure S4D). Importantly, iPSC clones isolated, expanded, and maintained in the presence of low CHIR concentrations that do not promote differentiation of hESCs exhibited normal chromosome numbers (Figures 4D, 4E, and S4C), indicating that a continuous and low level of WNT signaling activity, as provided by CHIR, is essential for the generation of euploid iPSC colonies from *PORCN* mutant fibroblasts.

To gain a better understanding of the role of the WNT signaling pathway during reprogramming, we perturbed the pathway using a number of manipulations. Addition of Wnt3a protein during reprogramming significantly increased the number of AP+ colonies (Figure 5A), consistent with findings by other groups (Ho et al., 2013; Marson et al., 2008; Zhang et al., 2014). IPSCs derived from WT fibroblasts in the presence of Wnt3a displayed all the hallmarks of bona fide iPSCs, including a normal karyotype of 46 chromosomes (Figure 4C), the characteristic cell morphology of hPSCs (Figure S5A), and expression of the pluripotency markers *OCT4*, *NANOG* (Figure S5B), TRA-1-81 (Figure S5C), and SSEA4 (Figure S5D). In addition, these iPSCs were pluripotent, as determined by their ability to differentiate into endodermal (expression of *FOXA2* and *SOX17*; Figure S5E and CXCR4 (Figures S5E and S5F), ectodermal (expression of *PAX6*, *SOX1*, and *MAP2*; Figure S5G), and mesodermal lineages (expression of *T* (*BRY*), *MESP1*, and *ACTC1*; Figure S5H).

The enhancement of AP+ colonies was particularly pronounced in the absence of MYC (KOS versus KMOS; Figure 5A versus 5B). Likewise, addition of the GSK3 inhibitor BIO increased the number of iPSC colonies (Figure 5A). Overexpression of constitutively active β-catenin (β-catenin 4A) increased reprogramming efficiencies by 1.4-fold (Figure 5C), thus confirming the findings by several other groups, including the original description of iPSC generation (Takahashi and Yamanaka, 2006). Addition of RSPO1, a WNT agonist and LGR4/5/6 ligand (Carmon et al., 2011; de Lau et al., 2011; Ruffner et al., 2012), sensitized cells to low levels of exogenously added Wnt3a (Figure 5D). In the absence of exogenous Wnt3a, RSPO1-treated cells exhibited a subtle and reproducible increase in reprogramming efficiency. Because RSPO1 itself fails to activate WNT/β-catenin signaling and only acts to augment WNT signaling, potentially through WNT receptor turnover by ZNRF3 (Hao et al., 2012), this result is consistent with the model that an endogenous WNT signaling pathway is present and is uncovered by RSPO1.

Several recent publications examined at which stage WNT signaling most significantly influenced the rate of reprogramming. These studies reached opposite conclusions, with two studies demonstrating that early WNT activation inhibited reprogramming rates (Aulicino et al., 2014; Ho et al., 2013) and another study demonstrating that early WNT activation stimulated reprogramming rates (Zhang et al., 2014). We do not have an explanation for this discrepancy; however, our findings strongly argue that WNT signaling not only enhances

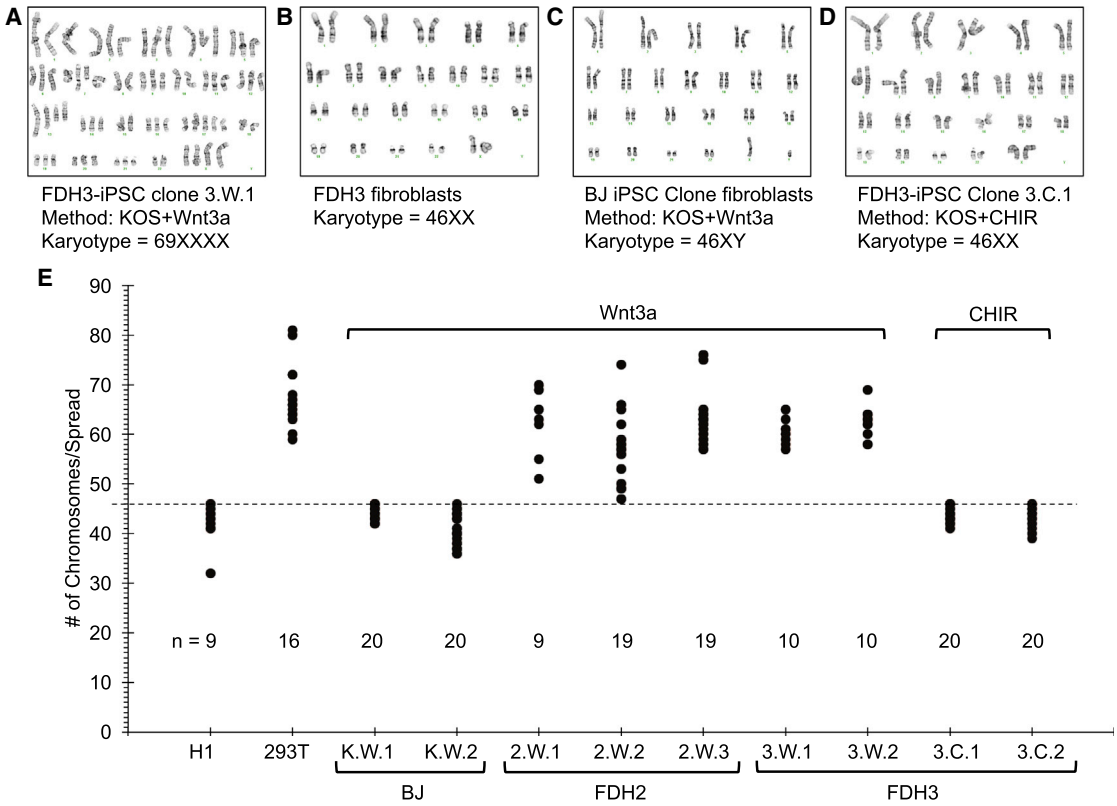

Figure 4. Ectopic Wnt3a Stimulation Yields Karyotypically Abnormal iPSCs

(A) A FDH3-iPSC derived in the presence of exogenous Wn3a (clone 3.W.1) exhibits an abnormal chromosome number. Additional images of abnormal chromosome spreads highlighting chromosomal anomalies, such as insertions, deletions, translocations, and inversions, are provided in the Supplemental Experimental Procedures (Figure S4).

(B) FDH3 fibroblasts exhibit a normal karyotype.

(C) An iPSC derived from unaffected fibroblasts (BJ, ATCC CRL-2522) in the presence of Wnt3a is euploid. Additional characterization of this iPSC clone is provided in the Supplemental Experimental Procedures (Figure S5).

(D) A FDH-iPSC (clone 3.C.1) derived in the presence of the GSK3 inhibitor CHIR98014 (CHIR) is euploid.

(E) Quantitation of chromosome numbers in FDH-iPSCs. Chromosome numbers were obtained by counting condensed chromosomes of metaphase-arrested cells (for representative images, see the Supplemental Experimental Procedures and Figure S4C). The dashed line marks 46 chromosomes. Like HEK293T (293T) cells, FDH-iPSCs derived in the presence of exogenous Wnt3a carried significantly increased numbers of chromosomes. The hESC line H1, BJ-iPSCs derived in the presence of Wnt3a (K.W.1 and K.W.2), and FDH-iPSCs derived in the presence of CHIR (clones 3.C.1 and 3.C.2) have 46 chromosomes. Note that this method to quantify chromosome numbers is only reliable in determining the maximum number of chromosomes; metaphase spreads with less than 46 are most likely due to loss of chromosomes during the preparation of the samples.

See also Figure S4.

reprogramming but is also required for reprogramming. In addition, in our system, which differs from the other studies in that it is human and does not involve the use of secondary iPSCs, we find that early activation of WNT signaling promotes reprogramming rates, whereas late activation has no impact (Figure 5E). Treatment with IWP during any stage of reprogramming diminishes the number of AP+ colonies. We therefore speculate that WNT signaling is required early to establish the pluripotent state.

In contrast to activation of WNT signaling, inhibition of the WNT signaling pathway reduced reprogramming efficiencies. Like IWP (Figures 1F and 5E), blocking WNT/β-catenin signaling with IWR (Chen et al., 2009), which acts to stabilize AXIN (a component of the β-catenin degradation complex), reduced reprogramming efficiencies (Figure 5F). Consistent with the effect of IWR, overexpression of AXIN by lentiviral transduction reduced reprogramming efficiencies (Figure 5G). Treatment with purified Wnt5a, which antagonizes WNT/β-catenin signaling (Ishitani et al., 2003; Mikels and Nusse, 2006), also interfered with reprogramming (Figure 5H), indicating that this noncanonical WNT signaling pathway blocks reprogramming. Therefore, canonical, rather than noncanonical WNT signaling promotes reprogramming.

Several studies have implicated the WNT receptor encoded by the *FZD7* gene to be critical to the maintenance of the pluripotent stem cell state (Fernandez et al., 2014; Melchior et al., 2008). Furthermore, *FZD7* is the most abundantly expressed *FZD* gene (the mammalian genome encodes 10 *FZD* genes) in hESCs (Fernandez et al., 2014). We therefore examined to which extent knockdown of *FZD7* expression affected reprogramming rates. Lentiviral transduction of a FZD7-specific short

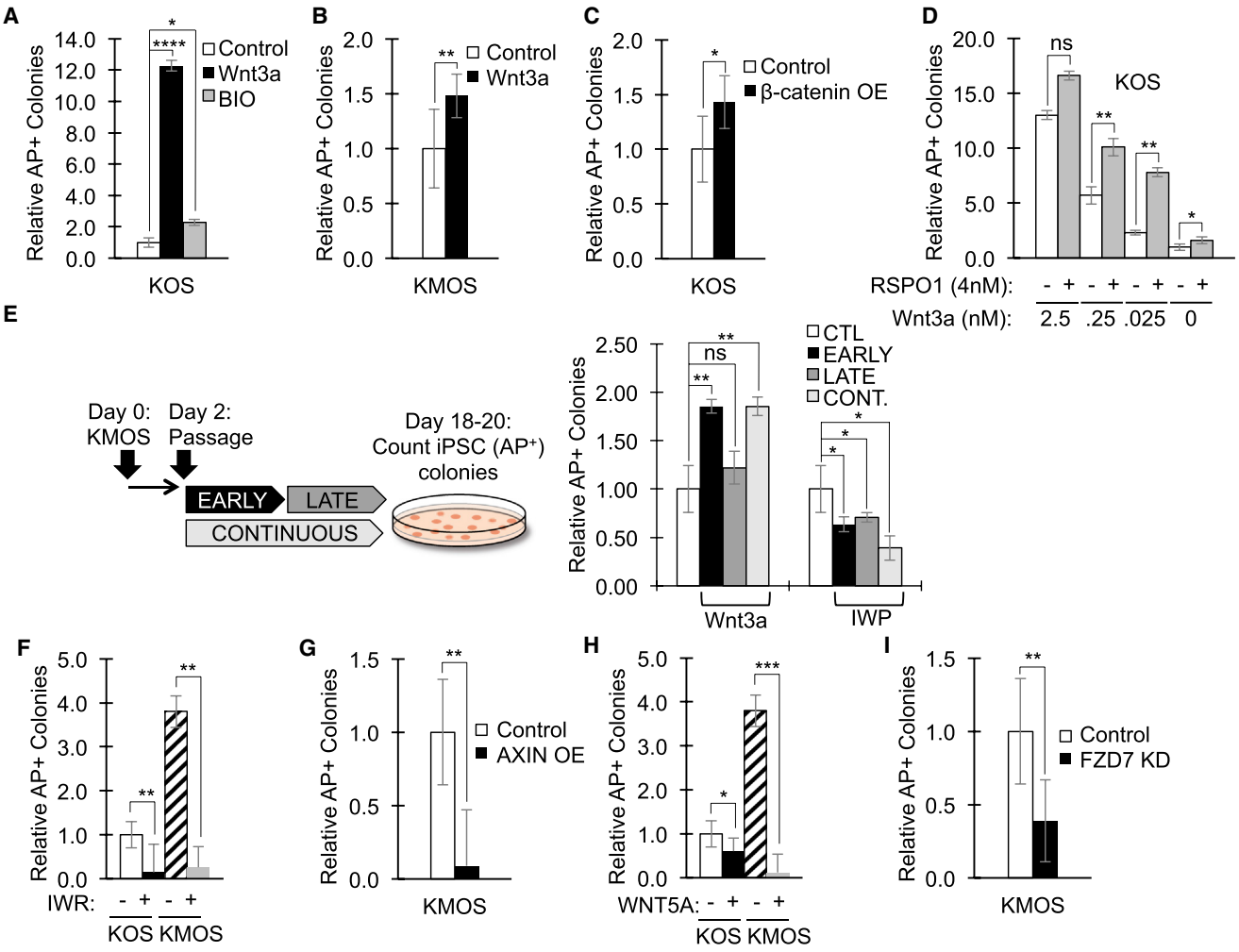

Figure 5. Modulation of WNT Signaling Influences Reprogramming Efficiencies

(A) Wnt3a and the GSK3 inhibitor BIO increase KOS reprogramming of wild-type fibroblasts. Fibroblasts were reprogrammed with factors KOS alone or in the presence of Wnt3a or the GSK3 inhibitor BIO. (Quantification represents mean number of AP+ colonies ± SD, normalized to untreated condition [CTL]; n = 7 for CTL, n = 6 for Wnt3a, and n = 3 for BIO; *p ≤ 0.05; ****p ≤ 0.0001.)

(B) Wnt3a increases KMOS reprogramming of wild-type fibroblasts. Fibroblasts were reprogrammed with factors KMOS in the presence or absence of Wnt3a. (Quantification represents mean number of AP+ colonies ± SD, normalized to untreated condition [CTL]; n = 23 for CTL and n = 6 for Wnt3a; **p ≤ 0.01.)

(C) Overexpression (OE) of constitutively active β-catenin (β-catenin 4A) increases reprogramming efficiencies. Fibroblasts were reprogrammed with factors KOS in the presence or absence of β-catenin 4A OE. (Quantification represents mean number of AP+ colonies ± SD, normalized to untreated condition [no Wnt3a and no RSPO1]; n = 13 for KOS, n = 3 for all other conditions; *p ≤ 0.05.)

(D) RSPO1 sensitizes cells to low levels of Wnt3a. Fibroblasts were reprogrammed with factors KOS in the presence or absence of RSPO1 with increasing concentrations of Wnt3a. (Quantification represents mean number of AP+ colonies ± SD, normalized to untreated condition [no Wnt3a and no RSPO1]; n = 13 for KOS, n = 3 for all other conditions; p values are between corresponding Wnt3a concentrations ± RSPO1; ns, not significant [p value for 2.5 nM Wnt3a data points = 0.061], *p ≤ 0.05 [p value for 0 nM Wnt3a data points = 0.022], **p ≤ 0.01.)

(E) Addition of Wnt3a increases AP+ colony numbers when added early during the reprogramming process, whereas addition of IWP reduces AP+ colony numbers when added early or late. Fibroblasts were reprogrammed with factors KMOS. Wnt3a or IWP were added either early, late or continuously during reprogramming, as indicated in the diagram. (Quantification represents mean number of AP+ colonies, normalized to untreated condition [CTL]; n = 3 for all conditions.) CONT., continuous treatment with Wnt3a or IWP.

(F) The Tankyrase inhibitor IWR, which induces AXIN stabilization, reduces numbers of AP+ colonies. Fibroblasts were reprogrammed with factors KOS or KMOS in the presence or absence of IWR. (Quantification represents mean number of AP+ colonies ± SD, normalized to untreated KOS condition; n = 7 for KOS, n = 5 for KOS+IWR, n = 13 for KMOS, and n = 3 for KMOS+IWR; **p ≤ 0.01.)

(G) *AXIN* overexpression (OE) by lentiviral gene transduction reduces numbers of AP+ colonies. Fibroblasts were reprogrammed with factors KMOS in the presence or absence of AXIN OE. (Quantification represents mean number of AP+ colonies ± SD, normalized to KMOS condition [CTL]; n = 23 for CTL and n = 9 for AXIN; **p ≤ 0.01.)

(H) Treatment of reprogramming cultures with Wnt5a, which antagonizes WNT/β-catenin signaling, reduces numbers of AP+ colonies. Fibroblasts were reprogrammed with factors KOS or KMOS in the presence or absence of WNT5A. (Quantification represents mean number of AP+ colonies ± SD, normalized to untreated KOS condition; n = 7 for KOS, n = 4 for KOS+Wnt5a, n = 23 for KMOS, and n = 4 for KMOS+Wnt5a; *p ≤ 0.05, ***p ≤ 0.001.)

(legend continued on next page)

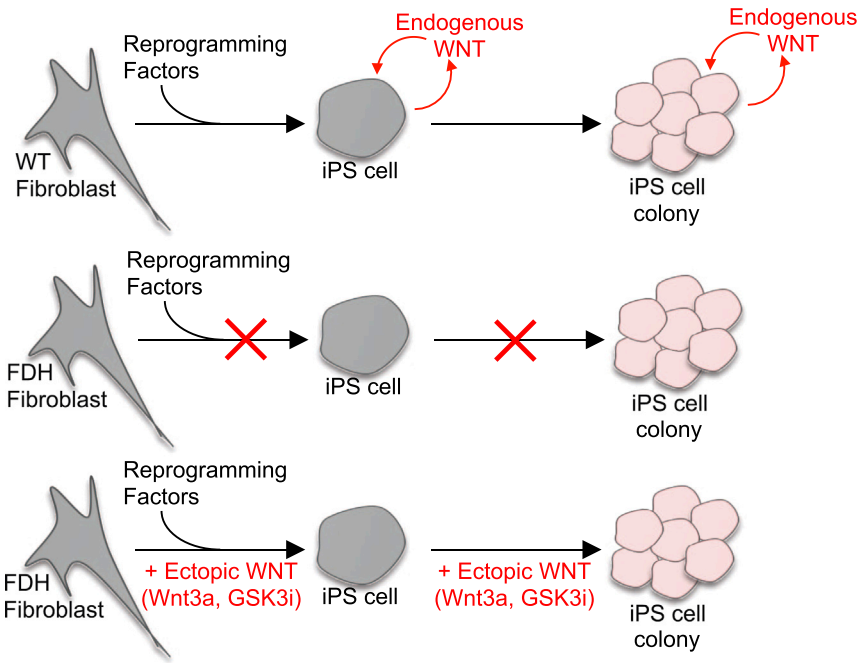

Figure 6. A Model Summarizing the Role of WNT Signaling during the Reprogramming Process

An endogenous WNT signaling loop is required for acquisition of the pluripotent state and for the expansion of iPSCs.

hairpin RNA (shRNA) significantly reduced reprogramming rates (Figure 5I), suggesting a potential role for FZD7 in reprogramming. However, because an endogenous WNT-FZD signaling loop is essential to the maintenance of the pluripotent state, it is difficult to distinguish whether this negative effect of FZD7 knockdown is due to an effect on the reprogramming process or maintenance and expansion of iPSCs. Nonetheless, these WNT signal pathway perturbations provide strong evidence that reprogramming requires an active endogenous WNT/FZD7/β-catenin signal.

DISCUSSION

Here, we show that WNT signaling is required for the generation of iPSCs from fibroblasts. Mutation of the *PORCN* gene or chemical inhibition of this essential WNT processing enzyme potently blocks iPSC generation, a blockade that is readily overcome through the ectopic activation of the WNT/β-catenin signaling pathway. This effect of WNT signaling can be attributed to two possible, and not necessarily mutually exclusive, modes of action (summarized in Figure 6). First, activation of WNT signaling is a requisite step to convert a differentiated cell to an iPSC. Second, continuous WNT signaling is required to promote expansion of a single iPSC to an iPSC colony.

Our experiments, as well as studies by several other groups, indicate that endogenous WNT signaling is required to maintain the pluripotent state. Blocking endogenous WNT signaling with PORCN inhibitors, such as IWP2, leads to a decrease in expression of WNT target genes *AXIN2* and *SP5* and a concomitant reduction in expression of the pluripotency regulators *NANOG* and *OCT4* (Fernandez et al., 2014). Consistent with these observations, WNT signal activation is required to generate iPSCs in which endogenous WNT signaling is disrupted through PORCN mutation or inhibition. Interestingly, expression of pluripotency genes is sensitive to the level of WNT signal activation in FDH-iPSCs, but not in wild-type iPSCs, again consistent with the notion that WNT signaling is required to maintain expression of pluripotency components. We also found that the WNT cofactor RSPO1, which augments WNT signaling activity, increases the efficiency of reprogramming, suggesting that it uncovers an endogenous WNT signaling activity necessary to promote the reprogramming process. Together, these data support the model that endogenous WNT signaling is required to maintain pluripotency and consequently is essential to induce pluripotency in fibroblasts.

Our experiments to address the timing requirement for WNT signaling during reprogramming indicate that WNT signal activation is more critical during the early, rather than later, stages of reprogramming, consistent with the results of others (Zhang et al., 2014). However, other groups found that activation of WNT signaling during early stages inhibits reprogramming, whereas inhibition of WNT signaling during early stages promotes reprogramming (Aulicino et al., 2014; Ho et al., 2013). We currently do not have an explanation for this discrepancy. However, it is important to stress that WNT signaling exhibits distinct dosage effects, with low levels promoting the pluripotent state, as discussed above, and high levels promoting mesendodermal differentiation (Bakre et al., 2007; Davidson et al., 2012). Treatment of undifferentiated human embryonic stem cells with Wnt3a or a GSK3 inhibitor is an established method to direct definitive endoderm differentiation (Brafman et al., 2013; D'Amour et al., 2005; Kroon et al., 2008; Schulz et al., 2012). Therefore, overstimulation of WNT signaling may drive pluripotent stem cells into a mesendodermal lineage, which will lack expression of pluripotency-associated genes and hence will not be observed as iPSCs.

(I) FZD7 knockdown (KD) by lentiviral transduction of a FZD7-specific shRNA reduces numbers of AP+ colonies. Fibroblasts were reprogrammed with factors KMOS. Simultaneously, one set of cells was transduced with a lentiviral vector expressing a FZD7-specific shRNA. (Quantification represents mean number of AP+ colonies ± SD, normalized to standard condition [CTL]; n = 13 for CTL and n = 8 for FZD7 knockdown, $^{**}p \leq 0.01$.)
See also Figure S5.

The dosing of WNT activity not only affects the choice between self-renewal and differentiation but also influences genomic integrity. In our reprogramming experiments we found that ectopic WNT signal activation with Wnt3a promoted genomic instability during reprogramming of *PORCN* mutant cells. The association between ectopic WNT signaling and chromosomal instability had been described previously but only in cells with compromised genomic integrity, such as HCT116, SW480, and HeLa cells (Hadjihannas and Behrens, 2006; Tighe et al., 2007). Additional studies found that mouse embryonic stem cells harboring mutations in the *APC* gene, a negative regulator of WNT/β-catenin signaling, exhibit defects in chromosome segregation, leading to highly variable chromosome numbers (Aoki et al., 2007; Fodde et al., 2001; Kaplan et al., 2001). We extend these studies by showing that cells harboring *PORCN* mutations, and are consequently deficient for endogenous WNT protein processing, are exquisitely sensitive to the level of exogenous WNT signaling activity, with low and stable levels of WNT activation promoting iPSC generation without inducing genomic instability. In contrast, higher levels of WNT signal activation invariably yielded iPSCs with grossly aberrant chromosome numbers. These findings on WNT's effects on genomic integrity serve as a cautionary note for the manipulation of WNT signaling in the generation of cell populations intended for cell replacement therapies because such genomic aberrations may promote tumorigenesis. Our observation that ectopic WNT signaling promotes chromosome missegregation and results in aneuploidy may be related to WNT's function as a locally acting polarity ligand as previously demonstrated with bead-immobilized WNT protein (Habib et al., 2013).

The process of reprogramming a mature cell type to an embryonic stem cell-like state shares similarities with the processes of cellular and tissue regeneration, especially as it occurs in vertebrates with high regenerative potential, such as axolotl and zebrafish. As these organisms repair damaged tissues, such as an amputated limb, a cell population, called the blastema, is formed that orchestrates the regenerative process. Formation of the blastema is thought to involve cellular dedifferentiation in a manner that may resemble cellular reprogramming and the formation of iPSCs. *WNT* genes are required for blastema formation (Kawakami et al., 2006; Stoick-Cooper et al., 2007). In addition, several studies have linked WNT signaling to tissue repair and wound healing, including in skin and bone (Chen et al., 2007; Chua et al., 2011; Fathke et al., 2006; Lim et al., 2013; Whyte et al., 2013). It is tempting to speculate that the observed requirement for WNT signaling during the induction of the pluripotent state represents a cell culture equivalent to these in vivo regenerative processes. Additional studies are needed to address the extent to which WNT's role in reprogramming resembles its role in regenerative processes, such as wound healing.

EXPERIMENTAL PROCEDURES

Collection of FDH Patient Biopsies and Establishment of Fibroblast Cultures

Skin punch biopsies were collected from consented individuals under protocol H-21291 ("Pathogenesis of Focal Dermal Hypoplasia or Goltz Syndrome and Related Disorders and the Role Of PORCN in Focal Dermal Hypoplasia"), which was approved by the Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals. Fibroblast cultures were established as described in the Supplemental Experimental Procedures.

Cells and Culture Conditions

Cells were cultured as described in the Supplemental Experimental Procedures. HESC lines (H1/ WA01/NIH registration number 0043; H9/ WA09/NIH registration number 0062; HUES 9/NIH registration number 0022) and iPSC lines were cultured as described in Chen et al. (2011). Experiments involving human embryonic and induced pluripotent stem cells were approved by the UCSD Human Research Protections Program (Principal Investigator: Karl Willert, protocols #120799: "A model for focal dermal hypoplasia using human induced pluripotent stem cells," and #100210: "Role of Wnt signaling in human ES cell proliferation and differentiation").

Plasmids, Recombinant Proteins, Small Molecules, and Antibodies

Sources of plasmids, small molecules, and antibodies are provided in the Supplemental Experimental Procedures. WNT proteins were purified as previously described (Willert, 2008). RSPO1 was purified as previously described (Wei et al., 2007) with the modifications described in the Supplemental Experimental Procedures.

Reprogramming and Differentiation Protocols

Details of the reprogramming protocols, quantitation of iPSC colonies, and differentiation into EBs or specific lineages are provided in the Supplemental Experimental Procedures.

Gene Expression Analysis

Gene expression was analyzed by quantitative (qRT-PCR) with the protocols and primers provided in the Supplemental Experimental Procedures.

Flow Cytometry

Flow cytometry was performed in the UCSD Human Embryonic Stem Cell Core Facility with experimental details and antibodies provided in the Supplemental Experimental Procedures.

Karyotyping

Karyotyping with G-banding was performed by Cell Line Genetics. Chromosome counts shown in Figures 4E and S4C were performed as described in the Supplemental Experimental Procedures.

WNT Protein Detection

WNT5A protein was detected by immunoblotting of Blue Sepharose precipitates of fibroblast-conditioned media, as shown in Figure S1D. A detailed protocol is provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.049.

AUTHOR CONTRIBUTIONS

K.W. and J.R. designed the research; J.R., J.B., E.M., D.N., A.S., D.B., and K.W. performed the research; V.R.S. and I.V. contributed new reagents and analytic tools; and K.W. and J.R. analyzed data and wrote the paper.

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