Jarid2 Coordinates Nanog Expression and PCP/Wnt Signaling Required for Efficient ESC Differentiation and Early Embryo Development

Highlights

- ESCs lacking Jarid2 show constitutive Nanog expression
- ESCs lacking Jarid2 have reduced PCP/Wnt signaling
- Co-culture of Jarid2-null and WT ESCs restores differentiation capability
- Jarid2-null ESCs form more than one ICM upon injection to E3.5 mouse blastocysts

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In Brief

Landeira et al. show that Jarid2-null ESCs have reduced Wnt9a/Prickle1/Fzd2 and low β-catenin activity, resulting in altered adhesion, constitutive expression of Nanog, and failure to differentiate. Their experiments identify a non-canonical function for Jarid2 in regulating the balance between ESC self-renewal and differentiation.
Jarid2 Coordinates Nanog Expression and PCP/Wnt Signaling Required for Efficient ESC Differentiation and Early Embryo Development

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SUMMARY

Jarid2 is part of the Polycomb Repressor complex 2 (PRC2) responsible for genome-wide H3K27me3 deposition. Unlike other PRC2-deficient embryonic stem cells (ESCs), however, Jarid2-deficient ESCs show a severe differentiation block, altered colony morphology, and distinctive patterns of deregulated gene expression. Here, we show that Jarid2−/− ESCs express constitutively high levels of Nanog but reduced PCP signaling components Wnt9a, Prickle1, and Fzd2 and lowered β-catenin activity. Depletion of Wnt9a/Prickle1/Fzd2 from wild-type ESCs or overexpression of Nanog largely phenocopies these cellular defects. Co-culture of Jarid2−/− with wild-type ESCs restores variable Nanog expression and β-catenin activity and can partially rescue the differentiation block of mutant cells. In addition, we show that ESCs lacking Jarid2 or Wnt9a/Prickle1/Fzd2 or overexpressing Nanog induce multiple ICM formation when injected into normal E3.5 blastocysts. These data describe a previously unrecognized role for Jarid2 in regulating a core pluripotency and Wnt/PCP signaling circuit that is important for ESC differentiation and for pre-implantation development.

INTRODUCTION

Jarid2 (Jumonji, AT-rich interactive domain 2) is the founding member of the Jumonji family of proteins that can demethylate histones, although Jarid2 itself is unable to do so (Klose et al., 2006; Landeira and Fisher, 2011). Jarid2 was discovered in 1995 as a regulator of neural development in a gene trap screen in mice (Takeuchi et al., 1995), and Jarid2 deficiency was later shown to result in a range of phenotypes with variable severity and onset with defects in heart and neural tube formation and hy- poplasia of liver, spleen, and blood tissues (Jung et al., 2005; Landeira and Fisher, 2011; Takeuchi et al., 2006). In humans, mutations in Jarid2 have been linked to congenital defects including nonsyndromic cleft lip, spina bifida, and congenital heart abnormalities (Scapoli et al., 2010; Volcik et al., 2004), and haploinsufficiency is linked to intellectual disability and brain dysfunction including schizophrenia (Barea et al., 2013; Celes- tino-Soper et al., 2012; Pedrosa et al., 2007; Ramos et al., 2012). At the cellular level, Jarid2 is known to inhibit myogenic differentiation in mouse and human cells (Walters et al., 2014), is important for the scheduled proliferation of epidermal stem and progenitor cells (Mejetta et al., 2011), and is thought to input into cell-cycle control by regulating cyclin D1 (Nakajima et al., 2011; Toyoda et al., 2003). In embryonic stem cells (ESCs), Jarid2 is particularly abundant and has been implicated as a hub component of transcriptional networks that underpin pluripotency (Assou et al., 2009; Kim et al., 2008; Loh et al., 2006; Sun et al., 2008; Zhou et al., 2007). ESCs lacking Jarid2 remain viable and pluripotent but are unable to efficiently differentiate in culture and show alterations in growth kinetics and in colony morphology (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Shen et al., 2009).

Jarid2 is a critical component of the Polycomb Repressor complex 2 (PRC2), implicated in guiding the deposition of H3K27me3 across the genome (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). In ESCs, Jarid2 binds to a similar set of genomic sites as core PRC2 components, such as Embryonic ectoderm development (Eed), Suppressor of Zeste 12 (Su(z)12), and Enhancer of Zeste 2 (Ezh2) (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2009). In Drosophila, Jarid2 also binds to sites that broadly overlap with other core PRC2 members, albeit at slightly different levels and with some exceptions.
(Herz et al., 2012). Although these results support a role for Jarid2 in PRC2 recruitment, in concert with co-factors such as Aebp2, Pcl1, 2, 3, and esPRC2p48 (Hunkapiller et al., 2012; Kalb et al., 2014; Kim et al., 2009; Li et al., 2010; Sarma et al., 2008; Walker et al., 2010; Zhang et al., 2011), the consequence of Jarid2 loss for H3K27me3 levels is unclear, as both increased (Peng et al., 2009; Shen et al., 2009) and decreased H3K27me-thylation have been reported (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010). In addition, recent studies of X-inactivation have shown that, while Jarid2 is necessary for efficient PRC2 recruitment, Jarid2 binds to Xist RNA-associated chromatin domains independently of PRC2 (da Rocha et al., 2014).

Jarid2 also binds to other non-coding RNAs in ESCs (Kaneko et al., 2010; Pasini et al., 2010). In addition, recent studies of X-inactivation have been reported (Landeira et al., 2010; Li et al., 2010; Sarma et al., 2014) and has been shown to bind nucleosomes directly (Son et al., 2013). These findings suggest that Jarid2 may have multiple functions in pluripotent ESCs and could act as an intermediate in the chromatin events that occur as pluripotent cells move toward differentiation (da Rocha et al., 2014). Consistent with this view, Jarid2-null ESCs grow and self-renew robustly in culture but are severely compromised in their capacity to generate mesoderm, endoderm, or ectoderm lineages in vitro (Landeira et al., 2010; Shen et al., 2009). In vivo, Jarid2-null mouse embryos develop normally until E10.5 or beyond (Jung et al. et al., 2005; Takeuchi et al., 2006), suggesting that Jarid2-null ESCs may be capable of differentiation but fail to do so in standard in vitro cultures.

Here, we examined the properties of Jarid2-null ESCs derived from different sources, as well as the factors that were consistently de-regulated in these cells. Our results show that Jarid2-null ESCs express constitutively high levels of Nanog proteins, indicative of a “naïve pluripotent state,” together with dramatically reduced planar cell polarity (PCP) and Wnt signaling components. In particular, expression of Prickle1, Fzd2, Wnt9a, E-cadherin, and β-catenin activity were all reduced in Jarid2-null ESCs, while Wnt antagonists such as Sfrp1 were upregulated. Remarkably, this inverse correlation between PCP/Wnt signaling and Nanog expression was replicated in wild-type ESCs depleted of Prickle1/Fzd2/Wnt9a, or engineered to overexpress Nanog. Co-culture of Jarid2-null ESCs with wild-type partners allowed the mutant cells to regain variable Nanog expression and β-catenin activity and restored their capability for differentiation. Surprisingly, we showed that disruption of PCP/Wnt signaling early in mouse embryogenesis, by introducing Jarid2-null ESCs (but not Eed-null ESCs), resulted in multiple ICM formation. This emphasizes the importance of the Jarid2-Nanog-PCP/Wnt core circuit for normal development and revealed a non-canonical (PRC2-independent) role for Jarid2 in coordinating this process.

RESULTS

Jarid2 Affects Nanog Expression in ESCs Maintained in Serum and Leukemia-Inhibitory Factor

Pluripotency factor expression by ESCs has been generally assumed to be unaffected by deletion of PRC2 (Azuara et al., 2008; Boyer et al., 2006; Lee et al., 2006; Pasini et al., 2007; Shen et al., 2008). Consistent with this, Jarid2-null ESCs (Landeira et al., 2010) expressed substantial levels of each of the core pluripotency factors Oct4, Sox2, and Nanog as indicated by western blotting analysis (Figure 1A, clones E4 and E8). Surprisingly, however, we detected an inverse correlation between Jarid2 gene dose and Nanog expression [JM8(wild-type) < A08(+/−) < E4(+/−) and E8(−/−)], Figure 1A]. At the single-cell level, immuno-labeling for Nanog followed by fluorescence microscopy (Figure 1B) or flow cytometry analysis (Figure 1C) revealed that the increased expression of Nanog by Jarid2-null ESCs was due to a reduced proportion of Nanog-low cells. Specifically, Jarid2-null ESCs appeared homogenously Nanog-high (Figure 1B, lower panel) whereas Nanog-high and -low cells were easily discerned among parental wild-type JMB8 ESCs (upper panel). This difference was confirmed by flow cytometry (compare green and gray traces, Figure 1C) where mutant E8 cells showed a reduced proportion of Nanog-low cells. To verify that altered Nanog expression was due to Jarid2 depletion rather than simply clonal variation between different ESC lines, we examined the properties Jarid2 mutant cells that had been independently generated by others (Shen et al., 2009). These Jarid2-null(−/−) ESCs also expressed constitutively high levels of Nanog protein detected by western blotting (Figure 1D), fluorescence-activated cell sorting (FACS) analysis (Figure 1E, 82% Nanog-high), and immunofluorescence microscopy (Figure 1F) (lower-right panel), as compared to matched wild-type controls (Jarid2 fl/fl). In addition, we noticed that, among cells plated at low density, Jarid2-null ESCs failed to properly self-organize into coherent colonies appearing as either “spread” (such as E8) or “disorganized” overlapping layers of Nanog-high cells (white arrows). To quantify such defects, we assessed the nuclear shape and dimensions of four to eight cell colonies that arose after plating E8 and JM8 ESCs at limiting dilution (Figure S1A). This unbiased comparison confirmed a profound difference in the nuclear height/width ratios of cells occupying central and peripheral positions within Jarid2-null colonies (Figures S1B and S1C). Taken together, these data suggest that Jarid2 has a role in regulating both Nanog levels and cell-cell interactions in ESCs.

Prior studies of ESCs lacking PRC1 (Ring1B) or PRC2 (Eed) components have shown similar levels of Nanog and Oct4 as matched controls (Eskeland et al., 2010; Leeb et al., 2010) suggesting that the effect of Jarid2 depletion on Nanog expression might not be mediated by PRC2. Furthermore, as Jarid2 does not directly bind to the Nanog promoter in mouse ESCs (Pasini et al., 2010), we investigated whether other repressors might be responsible for regulating Nanog expression differentially between Jarid2-null and wild-type ESCs. Chromatin immunoprecipitation (ChiP) analysis revealed that, although both cell lines expressed similar levels of Tcf3 (Figure S1D), wild-type ESCs showed a significant enrichment for Tcf3 bound to a consensus site in the Nanog promoter, as compared to Jarid2-null samples (Figure S1E). This result was consistent with Tcf3 mediating the repression of Nanog in ESCs (Cole et al., 2008) and suggests that Jarid2 may modulate Tcf3 activity.

Jarid2 Regulates Non-canonical Wnt Signaling in Undifferentiated ESCs

Tcf3 is a critical component of canonical Wnt signaling pathways that is implicated in regulating the balance between stem cell...
self-renewal and differentiation. Mouse ESCs maintained in media supplemented with inhibitors of glycogen synthase kinase 3 and Fgf-MAPK (2) have elevated Nanog levels (Wray et al., 2010; Ying et al., 2008) and, like Jarid2-null ESCs, are less prone to differentiation. To discover genes downstream of Jarid2, we examined publicly available gene-expression microarray data for Jarid2−/− ESCs (Shen et al., 2009) and found that among the 413 genes misregulated relative to controls (fold change > 2, p value < 0.05) many have roles in cell adhesion, the cytoskeleton, and extracellular matrix or are implicated in development.
We examined published ChIP-sequencing data for Jarid2 binding in undifferentiated ESCs (Pasini et al., 2010) to uncover which genes might be directly regulated by Jarid2. Among the 1,146 genic promoters enriched for Jarid2 binding, we noticed a strong preference for genes critical in Wnt signaling (29 genes) and Wnt-related pathways (Figure S2B). To explore this further, gene-expression profiling was performed to formally determine whether genes involved in Wnt signaling pathways were substantially deregulated in ESCs lacking Jarid2. For this, ESC clones that lacked Jarid2 (E8 and Jarid2−/− cells) were compared with wild-type ESCs (JM8 and Jarid2 fl/fl), and mRNA expression of Wnt signaling components was quantified by qPCR using commercially available PCR plates. Among the candidates showing significant de-regulation in ESCs lacking Jarid2 (p < 0.05) were Prickle1, Fzd2, Wisp1, Wnt9a, and Fosl1 (downregulated, Figure 2A, left) and Aes, Dkk1, Sfrp1, and Wnt11 (upregulated, Figure 2A, right). These candidates included genes with binding sites for Jarid2 within their promoters (Prickle, Fzd2, Fosl1, Wnt9a, Wnt11, Dkk1, and Sfrp1, Figure S2C) as reported previously (Peng et al., 2009). Careful pairwise comparison of gene-expression levels relative to matched parental controls verified a significant up- or down-regulation of these genes in Jarid2-null ESCs (Figure 2B, where black bars show wild-type and white bars indicate Jarid2 nulls). Differences between the two sources of Jarid2-deficient ESCs were apparent only for Dkk1, where expression appears particularly sensitive to cell density (data not shown). Fzd2 and Prickle1 are both well-established core components of the non-canonical Wnt, or PCP pathway in mammals, Wnt11 is a ligand associated with PCP (Gray et al., 2011), and Dkk1 and Sfrp1 are secreted Wnt inhibitors involved in PCP signaling in vertebrates (Caneparo et al., 2007; Satoh et al., 2008) (as depicted schematically in Figure 2C).

To check whether Jarid2-null ESCs have defects in planar cell polarity, we examined the distribution of Prickle1 and Vangl1 proteins (green) in wild-type and Jarid2-null ESCs using immunofluorescence with previously characterized antisera (Figure 2D). Both proteins were abundant within the cytoplasm and nucleus of wild-type ESCs (upper panels), whereas in Jarid2 nulls (E8−/−) Prickle1 and Vangl1 levels were reduced in nuclei and there was a paucity of both proteins in the cytoplasm. This difference was confirmed using quantitative analysis of labeling intensity (Figure S2D) that showed a significant decrease (5- to 10-fold) in Prickle1 and Vangl1 in the cytoplasm of Jarid2-null ESCs. E-cadherin labeling was also low in Jarid2-null ESCs and appeared patchy (Figure 2D, green lower right) as compared with contiguous labeling of cell boundaries in colonies of wild-type ESCs (Figure 2D, green upper right). These data highlight a deficiency of PCP proteins in Jarid2-null ESCs.

To extend this analysis, we generated three additional Jarid2-null ESC lines, using CRISPR/Cas9 to engineer mutations into the third exon of Jarid2 (Figures S2E–S2G). Clones were selected that showed frameshift mutation to both endogenous Jarid2 alleles (Figure S2G), and Western blots confirmed the absence of detectable Jarid2 proteins (Figure 2E). RT-PCR analysis showed that Prickle1, Fzd2, and Wnt9a expression was significantly reduced in each mutant clone relative to parental ESCs (Figure 2F), and flow cytometry analysis (Figure 2G) indicated a characteristic switch to constitutive Nanog-high expression (green versus gray trace) as illustrated for a Jarid2CRISPR−/− mutant cell. This extends previous observations made with established Jarid2-null lines, indicating that deregulated PCP and Nanog expression was likely to be a direct consequence of Jarid2 removal rather secondary effects or adaption to culture. Using a similar CRISPR/Cas9 approach, we also generated ESC lines lacking Prickle1, Fzd2, and Wnt9a (Figures S2E–S2G). Six clones were identified with frameshift mutations in one or both alleles of each of these genes, and each mutant cell line showed a switch to constitutive Nanog-high expression and defects in colony formation (“spread” or “layered” cells) that...
Figure 3. Sustained β-Catenin Activity and PCP Expression in ESCs Requires Jarid2 and Is Sensitive to Nanog Overexpression

(A) Western blot analysis of whole-cell extracts show similar levels of total β-catenin in wild-type (JM8+/+), Jarid2 heterozygous (A08+/–), and -null (E8–/–) ESCs. Lamin B provides a loading control.

(B) Western blots of active β-catenin detected in Jarid2-null (E8–/–, Jarid2–/–) versus parental ESC lines. Nanog is shown for comparison; Lamin B provides a loading control.

(C) β-catenin activity, evaluated by luciferase-based TOPFlash assays comparing matched pairs of wild-type (black bars) and Jarid2-null (white bars) ESCs: JM8:E8 (left), Jarid2 fl/fl: Jarid2–/– (middle), wt:Jarid2CRISPR#3 (right). Reduced β-catenin activity was evident in ESCs lacking Prickle1/Fzd2/Wnt9a (PCPCRISPR#5, gray bar). Mean ± SD of three experiments are shown; asterisks indicate statistical significance (p < 0.05; Student’s t test).

(D) RT-PCR analysis confirm a 7-fold increase in Nanog expression in ESCs engineered to overexpress Nanog (NanogOE, white bar), compared to wild-type (black bar).

(E) TOPFlash assays show reduced β-catenin activity in NanogOE ESCs (white bar) as compared with wild-type ESCs (wt, black bar). Mean ± SD of three experiments, where asterisks show statistical significance (p < 0.05; Student’s t test).

(F) RT-PCR analysis of Fzd2, Prickle1, and Wnt9a transcripts detected in wild-type (black bars) and NanogOE (white bars) ESCs. Mean ± SD of three experiments; asterisks show statistical significance (p < 0.05; Student’s t test).

(legend continued on next page)
resembled Jarid2-null ESCs. This is exemplified for PCP\textsuperscript{CRISPR}#5 (Figures 2H–2J), a clone with frameshift mutations in both endogenous Prickle1 and Fzd2 alleles and a single Wnt9a allele (Figure S2G). This cell line showed dramatically reduced expression of all three genes (Figure 2H), expressed Nanog constitutively (Figure 2I, green) (Figure 2J, green trace), and showed aberrant clonal morphology (Figure 2I, right, arrows). Taken together, these data showed a hitherto-unrecognized role for Jarid2 in regulating non-canonical Wnt signaling and Nanog expression in undifferentiated ESCs.

Although Jarid2 binds to the promoters of Prickle1, Fzd2, Wnt9a, Wnt11, Dkk1, and Sfrp1 (Pasini et al., 2010) (Figure S2C), ChIP analysis revealed similar H3K27me3 levels at these targets in Jarid2-wild-type (JM8), heterozygous (A08), and null ESC lines (E4, E8) (Figure S3A). This observation, together with the fact that Prickle1, Fzd2, and Wnt9a expression apparently requires Jarid2, suggests that Jarid2 may regulate PCP/Wnt signaling in ESCs in a way that is independent of its canonical role as a component of PRC2. A comparison of the replication timing of ESCs in a way that is independent of its canonical role as a Jarid2 (E8, E12, Jarid2\textsuperscript{+/-}, and Jarid2\textsuperscript{-/-}) compared to wild-type cells (JM8, Jarid2\textsuperscript{+/-}, and Jarid2\textsuperscript{-/-} (GFP+) ESCs with wild-type partners resulted in a switch from constitutive Nanog-high expression (Figure 4B, E8 \textsuperscript{-/-} left) to a profile that was indistinguishable from wild-type ESCs (Figure 4B, E8 \textsuperscript{-/-} right). Exposure of Jarid2-null ESCs to wild-type ESCs also restored \beta\textsuperscript{-}catenin activity in the mutant cells (Figure 4C) and improved their capacity to differentiate (Figures 4D and 4E). Following co-culture of E8 and wild-type ESCs increased overall E-cadherin expression in mixed colonies (Figure S4A, red), a close examination revealed that E-cadherin expression by mutant cells remained low in cultures at 16 hr (Figure S4B). These data showed that Jarid2-null ESCs have intrinsic defects in canonical and non-canonical Wnt signaling, likely to contribute to aberrant cell-cell interactions.

Remarkably, co-culture of Jarid2-null (GFP\textsuperscript{+}) ESCs with wild-type partners resulted in a switch from constitutive Nanog-high expression (Figure 4B, E8 \textsuperscript{-/-} left) to a profile that was indistinguishable from wild-type ESCs (Figure 4B, E8 \textsuperscript{-/-} right). Exposure of Jarid2-null ESCs to wild-type ESCs also restored \beta\textsuperscript{-}catenin activity in the mutant cells (Figure 4C) and improved their capacity to differentiate (Figures 4D and 4E). Following co-culture and leukemia-inhibitory factor (LIF) withdrawal, Jarid2-null E8-derived (GFP) cells expressing neural-associated markers such as Nestin and Mash1 (red) were readily identified within mixed cultures (47% of GFP\textsuperscript{+} cells expressed Nestin). Neural induction was less efficient than in control wild-type cultures (>80% Nestin positive by day 10, Figure S4C), and fewer cells were recovered overall (5%–10%). Co-culture of E8 cells with Jarid2-null ESCs, fibroblasts, T or B cells, or with conditioned media derived from wild-type ESCs did not rescue the capability of E8 cells to differentiate (data not shown) or enhance \beta\textsuperscript{-}catenin activity in these cells (as shown in Figure S4D). Because \beta\textsuperscript{-}catenin activity is reportedly required for ESC differentiation (Atlasi et al., 2013; Lyashenko et al., 2011), Jarid2-null ESCs may be unable to execute differentiation efficiently because of low \beta\textsuperscript{-}catenin activity. In this scenario, co-culture with wild-type ESCs

\textit{\textbf{\beta\textsuperscript{-}Cat}atenin Activity Is Reduced in ESCs that Lack Jarid2 or Overexpress Nanog}

We examined whether canonical Wnt signaling (\beta\textsuperscript{-}catenin-mediated) was also impaired in ESCs lacking Jarid2. As shown in Figure 3A, western blots revealed broadly similar amounts of \beta\textsuperscript{-}catenin in whole-cell extracts derived from Jarid2-null (E8), heterozygous (A08), and wild-type ESCs (JM8). However, using antibody specific for active forms of \beta\textsuperscript{-}catenin (Figure 3B), we observed reduced levels of active \beta\textsuperscript{-}catenin in ESCs lacking Jarid2 (E8, Jarid2\textsuperscript{2/-}) compared to wild-type cells (JM8, Jarid2\textsuperscript{+/-}). This reduction was confirmed in functional assays using TOPFlash (luciferase-based) reporters. \beta\textsuperscript{-}catenin activity was significantly reduced in both established (E8 and Jarid2\textsuperscript{2/-}) and newly generated (Jarid2\textsuperscript{CRISPR}#3) Jarid2-null ESCs (Figure 3C, white bars) compared with controls (black bars), and also in Prickle1/Fzd2/Wnt9a depleted ESCs (PCP\textsuperscript{CRISPR}#5, gray bar). Since each of these mutant ESCs also expressed Nanog constitutively, we asked whether Nanog expression per se was important for reduced \beta\textsuperscript{-}catenin activity. ESCs that stably express elevated levels of Nanog (Figure 3D, Nanog\textsuperscript{OE} white bars) were generated as described previously (Theunissen et al., 2011). These cells showed significant reductions in \beta\textsuperscript{-}catenin activity and reduced expression of Fzd2, Prickle1, and Wnt9a (Figures 3E and 3F), providing compelling evidence of a robust regulatory circuit between Nanog, PCP and \beta\textsuperscript{-}catenin activity that is sensitive to Jarid2 (illustrated in Figure 3G). Operationally, this balance between Nanog and Wnt signaling was also evident in ESCs expressing wild-type levels of Nanog (Chambers et al., 2007), where Nanog expression and \beta\textsuperscript{-}catenin activity were inversely correlated (Figure 3H).
restores β-catenin activity and may enable mutant ESCs to differentiate (Figure S4E shows a hypothetical scheme).

**Cytoplasmic Prickle1 Distribution Marks the Developing Epiblast In Vivo**

To investigate whether Jarid2 regulation of Nanog PCP/Wnt signaling is likely to be important in vivo as well as in vitro, we examined the distribution of Prickle1 in the pre-implantation mouse embryo. Depletion of PCP components, including Prickle1, has been reported to lead to defects in cell adhesion that affect the development of early mouse embryos (Larue et al., 1994; Na et al., 2007; Tao et al., 2009), and previous studies have shown that although Prickle1 is detected at E5.5 and earlier (Tao et al., 2009, 2012), canonical Wnt signaling is operational slightly later in development (Na et al., 2007). We examined mouse embryos from E3 to E4.5 for Prickle1 expression using DAPI as a counterstain (blue) to assess cell number. Immuno-labeling with antibodies to Nanog (red), Oct4 (data not shown), or Gata6 (red) was used to assess the stage and orientation of the developing inner cell mass (ICM) (Figures 5A and 5B). As illustrated in Figure 5A, Prickle1 (green) was abundantly detected in 32- and 64-cell embryos and showed a prominent cytoplasmic distribution in Nanog positive cells destined to form the epiblast (region outlined in white, Figure 5A). By E4.5, Nanog-high cells were condensed to the pole of the embryo where they expressed high levels of Prickle1 distributed equivalently between nuclei and cytoplasm. The surrounding Gata6-expressing cells (red) also expressed high levels of Prickle1 (green), but in this case expression was predominantly nuclear (Figure 5B, region highlighted in white). Most cells in the polar trophectoderm showed modest levels of mostly Prickle1. An exception was of a small group of two to six mural trophectoderm cells, in which Prickle1 expression was high and located in nuclei and particularly within nucleoli (summarized in the schematic diagram shown in Figure 5C). The selective distribution of cytoplasmic Prickle1 in cells of the epiblast versus primitive endoderm or trophectoderm was confirmed in optical sections of E4.5 embryos as shown in Figure 5A (green labeling and traces). E-cadherin (red, Figure 5D) was prominent throughout the blastocyst at these stages.

**Jarid2-Null ESCs Initiate Multiple ICMs upon Injection into Normal Blastocysts**

ESCs that lack Jarid2 expressed high levels of Nanog and low levels of Prickle1, Vangl1, and β-catenin activity and show severely compromised differentiation (Landeira et al., 2010). As these defects were partially compensated by in vitro co-culture with wild-type ESCs, we asked to what extent mutant cells would contribute to the developing embryo. Wild-type E3.5 blastocysts were injected with either 10–15 GFP^−Jarid2-null ESCs (E8) or an identical number of GFP-labeled wild-type ESCs (JM8) and then were cultured for 16–20 hr before being examined. Remarkably, we found that blastocysts injected with Jarid2-null ESCs routinely initiated the formation of more than a single ICM (16/39, illustrated in Figure 5E, lower panels), a feature that was not detected in any of the controls injected with wild-type ESCs (0/90, upper panels). Importantly, although these secondary ICMs appeared to be focused around small clusters of Jarid2-null ESCs (marked in green), in which higher levels of Nanog might be anticipated, Gata6 expression (red) was induced in the underlying wild-type cells (Figure 5F). This suggested that injection the mutant ESCs was sufficient to instruct neighboring wild-type blastomeres to upregulate Gata6 and thus contribute to the formation of multiple ICMs. To distinguish whether multiple ICM formation was due to defective PRC2 function, or defective Nanog, PCP, and Wnt circuitry, we injected E3.5 blastocysts with ESCs lacking Prickle1/Fzd2/Wnt9a, over-expressing Nanog, or that lacked Ed (Azuara et al., 2006) a core component of the PRC2 complex. As shown in Table 1, injection of wild-type (E14) and Ed-deficient (B1.3) ESCs generated only a single ICM in multiple experiments, whereas blastocysts injected with equivalent numbers of Jarid2-null (Jarid2^CRISPR#3), Prickle1/Fzd2/Wnt9a-depleted (PCPCRISPR#5), or Nanog^OE-overexpressing ESCs formed multiple ICMs in approximately 35%–48% of samples.

**DISCUSSION**

Our studies reveal several important and unanticipated results. First, we show that Jarid2, a Polycomb group member implicated in PRC2 recruitment, chromatin structure, and gene repression, acts as a positive regulator of PCP/Wnt signaling pathways in ESCs. Absence of Jarid2 or depletion of Prickle1/Fzd2/Wnt9a results in constitutive Nanog expression by ESC, which, in turn, results in reduced levels of active β-catenin. These observations identify an important Jarid2-sensitive core circuit in ESCs that regulates pluripotency and differentiation. In previous studies, Jarid2-null ESCs were shown to have a profoundly compromised ability to differentiate in culture (Landeira et al., 2010; Li et al., 2010; Pasini et al., 2010; Peng et al., 2009; Shen et al., 2010).
Here, we show that canonical and non-canonical Wnt signaling is reduced in Jarid2-null ESCs, and these cells are unable to establish coherent colonies with each other. Introduction of wild-type ESCs supports the formation of colonies and increases β-catenin activity within Jarid2-null ESCs, allowing them to properly execute differentiation programs in response to appropriate cues, although the nature of the support offered by wild-type ESCs is at present unclear.

These results are perhaps surprising in view of reports implicating Jarid2 in the regulation of Notch1 (Mysiwiec et al., 2011), or showing that Nanog represses the expression of Dkk1 (a secreted Wnt inhibitor) in ESCs to enhance β-catenin expression (Marucci et al., 2014). Current literature contains many conflicting reports on whether β-catenin is essential or dispensable for maintaining ESC pluripotency (Okumura et al., 2013; Soncin et al., 2009; ten Berge et al., 2011; Wray et al., 2011; Ying et al., 2008). This discrepancy probably reflects the fact that β-catenin is pleotropic and regulates both gene transcription and cell adhesion (reviewed in Brembeck et al., 2006 and Heuberger and Birchmeier, 2010). In addition, the requirement for and effects of β-catenin appear to be context dependent (Lyashenko et al., 2011; Okumura et al., 2013; Wray et al., 2011; Yi et al., 2008) and are reported to differ between naive and primed ESCs (Kurek et al., 2015; ten Berge et al., 2011). Our data show that Jarid2-null ESCs maintained in LIF and serum have low β-catenin activity despite unchanged levels of total protein. These cells can self-renew but have an impaired capacity to differentiate. Co-culture of mutant cells with restored β-catenin activity and allowed them to differentiate, consistent with several reports showing that nuclear β-catenin is required to execute differentiation in vitro (Aebischer et al., 2011; Lyashenko et al., 2011) and in vivo (Raegel et al., 1995; Huelskens et al., 2003).

Previous studies have shown that non-canonical Wnt signaling is present in mouse blastocyst before implantation (Tao et al., 2009, 2012). Early blastomeres express cytoplasmic Dishevelled (Dvl) proteins but may not activate canonical Wnt signaling as judged by sensitive BAT-gal Wnt reporters (Na et al., 2007). Overexpression of Dvl, a downstream target of Prickle1, Vangl, and Frizzled (Fz) (Gomez-Orte et al., 2013; Lapiebe et al., 2011), has been shown to dramatically alter the morphology and adhesion properties of 4-cell stage mouse embryos, resulting in a lack of coherence in the resulting blastocyst (Na et al., 2007). Likewise, deletion of Prickle1 in mouse embryos results in local disorganization of epiblast tissue (Tao et al., 2009) and early embryonic death (E5.5–E6.6), while lower levels of Wnt9a promotes cell proliferation (Xiang et al., 2008). Here, we show that PCP expression is reduced in Jarid2-null ESCs, and their introduction into E3.5 blastocysts induces the formation of multiple ICMs in around half of the embryos. Canonical Wnt signaling is thought to begin at implantation in the mouse (Na et al., 2007), and it is tempting to speculate that the group of cells in the mural trophectoderm identified by high levels of Prickle1 in nucleoli may be cells destined to mediate implantation into the uterus. Whatever the explanation, our demonstration that wild-type cells can rescue the differentiation of Jarid2-null ESCs in vitro may provide important insights for understanding the conflicting differentiation status of Jarid2 cells in vivo and in vitro. In homogenous clonally derived cultures, mutant cells are unable to establish bona fide cell-cell interactions or enhance β-catenin activity, whereas in heterogenous cultures (with wild-type ESC partners) or in vivo (with other blastocyst-derived cells) β-catenin activity and PCP/Wnt signaling may be partially compensated.

Our discovery that Jarid2 is a regulator of PCP/Wnt is also important for re-evaluating some of the emerging evidence that Jarid2 mutations (or SNPs) are risk factors for several human diseases. Genetic studies have for example linked Jarid2 with non-syndromic cleft lip (Scapoli et al., 2010). In mice, Jarid2 is highly expressed in epithelial cells and in the merging palatal shelves. In this context, as well as in congenital heart defects where Jarid2 mutations have also been reported (Volicik et al., 2004), the

### Table 1. Multiple ICMs Are Induced in E3.5 Mouse Blastocysts Injected with Jarid2-Null, Prickle1/Fzd2/Wnt9a-Depleted, or Nanog-Overexpressing ESCs

| ESC                        | Number of Blastocysts Injected/Analyzed | Number of Blastocysts with >1 ICM (%) |
|----------------------------|----------------------------------------|---------------------------------------|
| JM8+/−                      | 90                                     | 0                                     |
| E8−/−                       | 39                                     | 16 (41)                               |
| E14                        | 62                                     | 0                                     |
| Jarid2CRISPR#3              | 40                                     | 14 (35)                               |
| PCPRISPR#5                  | 23                                     | 11 (48)                               |
| Nanog-OE                    | 43                                     | 19 (44)                               |
| Eed-deficient B1.3           | 27                                     | 0                                     |

_a_ **Table 5.** ESCs Lacking Jarid2 Induce the Formation of Multiple ICMs upon Injection into E3.5 Mouse Blastocysts

(A) Confocal immunofluorescence images show mouse blastocysts at 32-cell (left panel), 64-cell (middle panel), or E4.5 (right panel) stages of development, immuno-labeled for Nanog (red), Prickle1 (green), and DAPI stain (blue). The dotted white line highlights the inner cell mass (32- and 64-cell stage), or epiblast (E4.5), indicated by high Nanog levels. Scale bar, 10 μm.

(B) Representative confocal images show Gata6 (red), Prickle1 (green) labeling, and DAPI staining (blue) of E4.5 blastocysts. The dotted white line marks the primitive endoderm as defined by high Gata6 expression.

(C) Schematic summary of Nanog, Gata6, and Prickle1 distribution in E4.5 blastocysts.

(D) Representative confocal image of a E4.5 blastocyst labeled for E-cadherin (red), Prickle1 (green), and DAPI (blue) stain. Scale bar, 10 μm.

(E) Representative confocal images of wild-type blastocysts that were labeled for Nanog (red) at E4.5, that had been previously injected (E3.5) with ten to 15 GFP labeled wild-type (JM8) ESCs (upper panels) or GFP-labeled Jarid2-null (E8) ESCs (lower panels). A single ICM was detected in all (90) samples injected with wild-type ESCs, whereas multiple ICMs were detected in ten of 24 and six of 15 blastocysts injected with Jarid2-null ESCs in two independent experiments. White dotted lines indicate the Nanog-high compartment corresponding to the developing epiblast. Scale bar, 10 μm.

(F) Confocal image of E4.5 blastocyst after injection of GFP-labeled E8 cells (green) (as in E) showing induction of Gata6 (red) in adjacent wild-type cells. Scale bar, 10 μm.

See also Figure S5.
potential for Jarid2 mutations to de-regulate PCP/Wnt signaling might be very informative for understanding the molecular basis of these malformations and could potentially offer different opportunities for intervention. In the case of cancer, Jarid2 mutations have been linked to metastases at diagnosis in soft-tissue sarcoma (Walters et al., 2014), to non-small cell lung carcinoma (Manceau et al., 2013), T-ALL, and to myeloproliferative disease (Saunthararajah and Maciejewski, 2012). Although it is possible that Jarid2 has an impact on these diseases based on its canonical role in PRC2-mediated chromatin modulation, it is also possible that Jarid2 is more directly involved in metastatic progression through its potential impact on cell sorting, cellular adhesion, and PCP/Wnt signaling. Thus, in addition to influencing PRC2 recruitment and H3K27 HMTase activity in ESCs, we have shown that Jarid2 is necessary to maintain a balance between Nanog expression and PCP/Wnt/β-catenin in ESCs that is essential to enable them to properly respond to differentiation cues. Regulation of this core circuit is also critical for normal pre-implantation development, since it appears to enable clusters of developing blastocysts to be discriminated and form a single inner cell mass. The discovery that Jarid2 regulates PCP/Wnt signaling in addition to its canonical role in PRC2 highlights an important intersection between cell signaling and chromatin-based regulation, relevant for understanding the interplay between pluripotency and differentiation.

**EXPERIMENTAL PROCEDURES**

Detailed experimental procedures are available in the Supplemental Experimental Procedures.

**Mouse ESC Culture**

ESC lines were grown using standard conditions on 0.1% gelatin-coated dishes in the presence of LIF and 10% fetal calf serum. Neural differentiation was carried out as described previously (Conti et al., 2005).

**Wnt Signaling Pathway and Gene-Expression Analysis**

Analysis of Wnt signaling pathway genes was performed using SYBR Green PCR array RT² profiler (SA Bioscience). Gene-expression analysis by RT-qPCR using SYBR Green (Qiagen) was performed as previously described (Landeira et al., 2010).

**Western Blot, Immunofluorescence, and Flow Cytometry Analysis**

Western blots were carried out using the following antibodies: mouse antisera to total β-catenin (BD Biosciences) and active β-catenin (Millipore), rabbit antisera to Nanog (Cosmo Bio), Jarid2 (Abcam), and goat antisera to Oct4 (Santa Cruz Biotechnology), Sox2 (Santa Cruz), Tcf3 (Santa Cruz), and Lamin B (Santa Cruz). Immunofluorescence analyses of ESC colonies and mouse blastocysts were carried out using the following primary antibodies: mouse antibodies against Oct4 (BD), E-cadherin (BD), Mash1 (BD); rabbit antisera against Nanog (Cosmo Bio), Vang1 (Sigma), and Prickle1 (gift from A.G. Bassuk (Bassuk et al., 2009)); and goat antisera against Gat6 (R&D Systems) and Nstin (Santa Cruz). Flow cytometry analysis of Nanog expression was carried out as previously described (Festuccia and Chambers, 2011) with the following modifications: 2 × 10⁶ cells were stained using rabbit antisera against Nanog (2.5 μg/ml, Cosmo Bio) and anti-rabbit conjugated to Alexa 647 (6 μg/ml, Molecular Probes).

**Injections of ESCs into Mouse Blastocysts**

10 to 15 GFP-expressing ESCs were injected into the inner cell mass of C57BL/6 blastocysts at E3.5. Injected embryos were cultured for 16 hr in KSOM media after which they were fixed for immunofluorescence analysis.

CRISPR/Cas9-Mediated Genome Editing

Single-guide RNA sequences were cloned into human codon optimized SpCas9 and chimeric guide RNA expressing px330 plasmid (Cong et al., 2013) followed by co-transfection into ESCs. Individual ESC clones were picked and sequenced to identify mutations.

**TOPFlash Assays**

Cells (10⁶ per well) were transfected with 1 μg TOPFlash and 0.1 μg Renilla luciferase plasmids in 6-well plates using Lipofectamine 2000 reagent (Life Technologies) and analyzed using the Dual Luciferase Reporter Assay System (Promega). 2 × 10⁵ ESCs were crosslinked using 1% formaldehyde. Tcf5 ChIP was carried out as described previously (Cole et al., 2008). H3K27me3 ChIP was carried out as described previously (Landeira et al., 2010).

**Animal Procedures**

Animals used in the study were maintained and handled according to the guidelines of the Imperial College Animal Welfare Ethical Review Committee and the regulations set out by the British Home Office.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.060.

**AUTHOR CONTRIBUTIONS**

A.G.F and D.L. conceived the study. Crispr/Cas9 mutation and β-catenin activity were assessed by H.B. and A.R.M. respectively. I.C. evaluated replication timing, N.B., Z.W., K.E.B., and E.N. performed embryo staining and injection.

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