FGF/ERK autocrine signaling is enhanced by NANOG in a subpopulation of pluripotent stem cells to execute autoregulation and induce heterogeneity

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SUMMARY:

FGF/ERK autocrine signaling induces *Nanog* heterogeneity, allelic switching, and differentiation in pluripotent cells, which are abolished in the naive-state (2i) by inhibition of MEK1/2 and GSK3β. Surprisingly *Nanog* autorepression is functional in 2i. The mechanisms activating FGF signaling and its downstream effectors leading to heterogeneity and allelic switching are unknown. We utilized genome-edited fluorescent reporter cell lines of both *Nanog* promotor and protein to elucidate, a NANOG induced FGF autocrine signaling mediated autoregulatory mechanism. We show that *Nanog* autoregulation is a cell non-autonomous process initiated by NANOG, but executed by FGF/ERK. FGFR2 differentially activates autoregulation in *Nanog*-high cells by activating ERK1/2. pERK1/2 recruits NONO (P54nrb) to repress *Nanog* transcription. Collectively, we show that *Nanog*-induced FGF autocrine signaling mediated autoregulation is the basis of *Nanog* heterogeneity and allelic switching. Our data introduces an unexpected reciprocal regulatory mechanism between NANOG and FGF/ERK signaling feeding into each other's heterogeneity.

INTRODUCTION

Embryonic stem (ES) cells are characterized by long-term self-renewal and the potential to differentiate to all cell types of the germ layers. ES cells cultured in presence of serum and LIF (metastable state) manifest transcriptional and functional heterogeneity. The heterogeneous

expression of transcription factors like *Nanog*, *Rex1*, *Stella*, *Esrrb*, *Klf4*, and *Tbx3* determine differential fate choice (Chambers et al., 2007; Festuccia et al., 2012; Filipczyk et al., 2013; Hayashi et al., 2008; Kalmar et al., 2009; Niwa et al., 2009; Torres-Padilla and Chambers, 2014; Toyooka et al., 2008; van den Berg et al., 2008). The molecular heterogeneity arising from the modulation of transcription factors by non-genetic factors underpins the functional heterogeneity of the subpopulations of ES cells. The core pluripotency factor, *Nanog* was identified as a factor conferring LIF independent self-renewal to ES cells by inhibiting differentiation (Chambers et al., 2003; Mitsui et al., 2003). *Nanog* imparts functional heterogeneity to ES cells through its heterogeneous expression leading to differential cell fate choices (Chambers et al., 2007; Kalmar et al., 2009). Unlike other core pluripotency factors, *Nanog* switches between mono-allelic and bi-allelic expression during embryonic development and in alternate pluripotency states (Filipczyk et al., 2013; Miyanari and Torres-Padilla, 2012). The expression of *Nanog* is restricted in ES cells to ensure their potential to differentiate by negative feedback autorepression and other repressive mechanisms (Fidalgo et al., 2012; Fidalgo et al., 2011; Mora-Castilla et al., 2010; Navarro et al., 2012; Pereira et al., 2006).

FGF/ERK signaling represses *Nanog* to induce heterogeneity and monoallelic expression, which are abolished in ESCs cultured in 2i (Filipczyk et al., 2013; Miyanari and Torres-Padilla, 2012). Intriguingly, *Nanog* autorepression is functional in 2i, suggesting MEK1/2 and GSK3β are dispensable for *Nanog* autoregulation (Navarro et al., 2012). Hence, the mechanisms regulating the three paradigms of *Nanog* regulation- heterogeneity, allelic expression, and autorepression are not entirely connected through FGF signaling. Although FGF/ERK represses *Nanog* expression (Hamazaki et al., 2006; Kim et al., 2014), the mechanistic delineation of this process is limited, and it is unclear how the NANOG protein levels are sensed by cells to trigger autorepression. Here, we utilized genetic approaches in *Nanog* fluorescent reporter cell lines and biochemical experiments to unravel a detailed mechanism of *Nanog* autoregulation. We show that MEK1/2 is essential for *Nanog* autoregulation. NANOG induces components of the FGF autocrine pathway in cells with higher *Nanog* expression, to trigger feedback repression by activation of ERK1/2. *Fgfr2* gene acts as a sensor of the NANOG protein in *Nanog* high cells. Further, we show that pERK1/2 interacts and recruits NONO to repress *Nanog* transcription. Surprisingly *Nanog* is essential only for triggering the autoregulation process but dispensable for its execution. Our results establish that the different facets of *Nanog* expression- heterogeneity, allelic switching, and autoregulation are regulated and interlinked by FGF autocrine signaling.

RESULTS

Residual MEK1/2 activity in the ground state prevents complete derepression of *Nanog*.

Nanog exhibits homogenous and biallelic expression in 2i. To uncouple the influence of MEK1/2 and GSK3β on *Nanog* expression, we analyzed the activity of endogenous *Nanog* gene activity reported by GFP in TNGA and TβC44Cre6 cell lines (Chambers et al., 2007) in different combinations of MEK1/2 and GSK3β inhibitors. *Nanog* promoter activity was derepressed in MEK1/2 inhibition (SLPD), GSK3β inhibition (SLCHIR), and 2i. Surprisingly *Nanog* promoter activity was higher in SLPD than in 2i (Figure1A, 1B). To analyze NANOG protein dynamics, we generated a NiRFP2A cell line with an iRFP670 coding sequence fused to the c-terminal of both alleles of *Nanog* (Figure S1A). Higher NANOGiRFP in SLPD (Figure1C, S1B), reaffirmed the highest induction of *Nanog* transcript and protein in SLPD. To dismiss the interference of any genetic modifications in the *Nanog* locus on its expression (Faddah et al., 2013); *Nanog* expression was analyzed in E14Tg2a cells. The *Nanog* transcript (Figure1D), transcriptional activity (Figure1E), and protein (Figure1F) were highest in SLPD. On the contrary, *Oct4* transcript and transcription activity were enhanced but protein changed very little in SLPD (Figure1D-F, S1C). NANOG was nearly 7-fold more in PD, which is twice that of 2i/SL2i (Figure1F). Although SLPD and 2i contain 1 μM PD, higher *Nanog* expression in SLPD indicates inefficient repression of *Nanog* in 2i/SL2i. We analyzed MEK1/2 substrate -pERK1/2 to investigate possible modulation of MEK1/2 activity by GSK3β (Yun et al., 2005). The pERK1/2 levels in ESCs changed dynamically throughout 24 hrs after the SL media was changed. The pERK1/2 remained undetectable up to 4 hrs in SLPD and 2i/SL2i. It gradually increased in 2i after 8 hrs but remained undetectable in SLPD (Figure 1G, S1D). We further analyzed pERK1/2 at different time points of treatment in PD, CHIR, and 2i/SL2i relative to SL after 24 hrs of media change (SL-24h). The pERK1/2 in SLCHIR and 2i significantly exceeded SL and SLPD respectively by 24 hrs (Figure 1H, S1E), suggesting a long-term CHIR treatment enhanced MEK1/2 activity in ESCs. Further, the PD and CHIR dose-responsive experiments confirmed that the pERK1/2 positively correlated with the CHIR concentrations (Figure 1I-J, S1F-I). Collectively, our data demonstrate that *Nanog* attains higher expression in MEK1/2 inhibition than in 2i. GSK3β activity negatively modulates MEK1/2 activity and its inhibition by CHIR increases pERK1/2

in 2i over time.

FGF autocrine signaling pathway components are essential for *Nanog* autoregulation.

The *Nanog* expression is higher in SLPD than 2i and *Nanog* autorepression is functional in 2i (Navarro et al., 2012). We asked if all repressive mechanisms including the *Nanog* autorepression are abolished in SLPD. To address this, we generated two independent NANOG restoration systems by integrating Flag-Avi-NANOG-ER^{T2} (NANOGER^{T2}) and a Doxycycline-inducible Flag-Avi-NANOG (FaNANOG) transgene in Tβc44Cre6 (Chambers et al., 2007) to derive the TNERT and TDiN cell lines respectively. TNERT and TDiN are similar to NERTc3 and 44iN (Festuccia et al., 2012; Navarro et al., 2012) where the NANOG function is reinstated by 4-Hydroxytamoxifen (OHT) or Doxycycline respectively, and endogenous *Nanog* gene activity is reported by GFP (Figure 2A, S2A-B). The repression of *Nanog*:GFP upon induction of transgenic NANOG by OHT/Dox is a functional readout of *Nanog* autoregulation. *Nanog*:GFP was repressed in OHT/Dox treated TNERT/TDiN in SL, SLCHIR, 2i, and SL2i, but not in SLPD (Figure 2A, S2C-D). To analyze the dynamics of endogenous NANOG in *Nanog* autoregulation, we generated NsGFPDiN cell line. The NANOG-GFP fusion protein is produced from one of the alleles of *Nanog* and a Doxycycline-inducible FaNANOG transgene is randomly inserted (Figure 2B). The NANOG-GFP was repressed in all treatments except in SLPD in Doxycycline treatment (Figure 2B, S2E). The data from distinct cell lines conclusively establish an essential role of MEK1/2 in *Nanog* autoregulation. We propose that the residual pERK1/2 resulting from residual MEK1/2 activity in 2i is sufficient for the functioning of *Nanog* autoregulation.

FGF autocrine signaling is the predominant inducer of MEK/ERK in pluripotent cells (Kunath et al., 2007; Lanner and Rossant, 2010), we investigated the role of FGF signaling components in autoregulation. NANOGER^{T2}/FaNANOG failed to repress *Nanog*:GFP in presence of FGFR inhibitor- SU5402, suggesting an essential role of FGFRs in *Nanog* autoregulation (Figure 2C, S2F). FGFR1, FGFR2, and FGF4 are major receptors and ligands of FGF signaling in early embryos respectively (Kang et al., 2017; Molotkov et al., 2017). FGFBP1 is a carrier protein expressed from early to late blastocyst (Figure 2D) (Park et al., 2015) that enhances FGF signaling (Tassi et al., 2001). We deleted *Fgfr1*, *Fgfr2*, *Fgf4*, and *Fgfbp1* in TNERT cells to analyze their role in autoregulation (Figure 2E, S2G-J). Except in

TNERTFgfr1-/-, *Nanog*:GFP was not repressed in TNERTFgfr2-/-, TNERTFgf4-/- and TNERTFgfbp1-/- cells upon OHT induction (Figure 2F). Our data suggest that FGF autocrine signaling and its components FGFR2, FGF4, and FGFBP1 are essential for *Nanog* autoregulation.

Nanog enhances the expression of FGF autocrine signaling pathway components.

We analyzed the expression of FGF autocrine signaling components during the time course of OHT induction. *Fgf4, Fgfr2, Fgfr1,* and *Fgfgbp1* transcripts were induced within 1-2 hrs (Figure 3A), *Esrrb* a direct target of NANOG was induced within 0.5 hrs. Increased pre-mRNA of *Fgf4, Fgfr2, Fgfbp1, and Fgfr1* indicated transcriptional activation of FGF signaling pathway components after OHT induction (Figure 3B). ChIP-seq data analysis identified NANOG occupancy on *Fgf4, Fgfbp1, Fgfr1,* and *Fgfr2,* which was further enhanced in *Oct4*+/- cells that have higher NANOG (Figure S3A) (Karwacki-Neisius et al., 2013). To analyze dosage-dependent occupancy of NANOG on these genes, we generated EDiN cell line by introducing a Doxycycline- inducible FaNANOG transgene in E14Tg2a. ChIP-PCR confirmed NANOG occupancy on *Fgf4, Fgfbp1, Fgfr1,* and *Fgfr2,* which was further enhanced in PD (Figure 3C) and EDiN+Dox (Figure 3D) which express higher NANOG. The data suggest a dose-dependent occupancy of NANOG on the FGF signaling component genes. FGFR1, FGFR2, and pERK1/2 were significantly increased upon OHT induction in TNERT (Figure 3E). The strength of FGF signaling depends on facilitation by carrier proteins (Tassi et al., 2001), the affinity of ligands (Ornitz et al., 1996; Zhang et al., 2006), and subsequent subcellular trafficking of the FGFRs after ligand-mediated activation (Auciello et al., 2013; Francavilla et al., 2013). The induction of NANOGER^{T2} enhanced FGFR2 on the cell surface (Figure 3F), unlike the FGFR1 (Figure S3B). The NANOGER^{T2} induction increased FGF4 and FGFBP1 secretion by TNERT (Figure 3G- H, S3C-D). Collectively the data shows that increased NANOG enhances FGFR2 on the cell surface, and secretion of FGF4 and FGFBP1 to intensify the FGF autocrine signaling. NANOG induces and enhances FGF autocrine signaling to execute *Nanog* autoregulation.

Nanog autoregulation is a cell non-autonomous process mediated by FGF autocrine/paracrine signaling.

Nanog autorepression is suggested to operate by a cell-autonomous process through intracellular proteins NANOG, ZFP281, and NURD complex (Fidalgo et al., 2012). However, Cell non-autonomous function of *Nanog* in the induction of primitive endoderm (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010) and the essential role of secreted proteins FGF4 & FGFBP1 in autoregulation from our experiments prompted us to investigate cell non-autonomous mechanisms. We assessed the ability of conditioned media from OHT induced TNERT cells, to repress *Nanog*:GFP in T*β*c44Cre6 lacking Nanog (Figure 3I). The conditioned media was sufficient to repress the *Nanog*:GFP (Figure 3J, S3E), suggesting that autoregulation operates via cell non-autonomous mechanisms and discounts the direct role of NANOG in autorepression as proposed earlier (Fidalgo et al., 2012). NANOG seems to be essential for triggering autoregulation through FGF signaling components but does not participate in repression. This led us to investigate the role of ZFP281 in *Nanog* autoregulation. The repression of *Nanog:*GFP upon OHT treatment, in TNERTZfp281-/- cell line lacking Zfp281 (S3F) suggests that ZFP281 is dispensable for *Nanog* autoregulation (Figure 3K).

To evaluate if FGF4 secretion was the causative factor of *Nanog* autoregulation in the conditioned media, we treated T*β*c44Cre6 with conditioned media from cells with loss or gain of FGF4. The conditioned media from an E14Tg2a cell line overexpressing FGF4 or supplementation of FGF4 (50ng/ml) could repress *Nanog*:GFP. Conversely, the conditioned media from OHT induced TNERTFgf4-/- or from TNERT+OHT-0 hr cells failed to repress *Nanog*:GFP, suggesting FGF4 is the key secreted factor essential for *Nanog* autoregulation (Figure 3L, S3G). The ELISA analysis confirmed the secretion and accumulation of FGF4 and FGFBP1 in the conditioned media (S3H-K). Collectively, our data establish that *Nanog* autoregulation is a cell non-autonomous process triggered by NANOG by augmenting FGF autocrine/paracrine signaling.

NANOG-induced FGFR2 triggers autoregulation predominately in the ES cell population with higher *Nanog* expression.

Nanog autoregulation was proposed to restrict NANOG levels within limits to retain the differentiation potential (Fidalgo et al., 2012; Navarro et al., 2012). *Nanog* autoregulation is expected to operate only in *Nanog*-high cells and not in *Nanog*-low cells. To evaluate this logic, we used TDiN cell lines with different induction levels of FaNANOG (Figure S4A).

The strength of *Nanog* autoregulation was found to be dependent on the FaNANOG levels and was completely abolished in TDiN clone with low FaNANOG (Figure S4B, 4C). To analyze autoregulation in *Nanog*-high and *Nanog*- low cells, we sorted the lowest and the highest 10% population of the TNERT expressing GFP and treated with OHT. The *Nanog:*GFP was repressed in *Nanog*-high cells but not in *Nanog*-low cells (Figure 4A). Our experiments demonstrate the logic of *Nanog* autoregulation and conclude that *Nanog* autoregulation predominately operates in *Nanog*-high and not in *Nanog*-low cell population.

FGF4 and FGFBP1 are secretory proteins, hence cannot distinguish between the *Nanog*- high and low cells in culture. Whereas FGFRs are essential for autoregulation and are retained on the cells, we asked if FGFRs functioned as a sensor for the NANOG levels to distinguish *Nanog*-high cells from low cells. We analyzed the correlation between the expression of FGFR1 and FGFR2 vis-à-vis NANOG in E14Tg2a by FACS. FGFR2 and FGFR1 showed a strong correlation with NANOG, which was further enhanced for FGR2-NANOG in SLPD (r=0.80) whereas decreased for FGFR1-NANOG (r=0.59) in SLPD (Figure 4B,) where NANOG levels are higher. These data suggested FGFR2 but not FGFR1 expression levels correlate and respond to NANOG concentration in the cells. FACS analysis showed high FGFR2 in the 10% NANOG high population and lower FGFR2 in the 10% NANOG low population (Figure 4C). The dose-dependent binding of NANOG to *Fgfr2* and a strong FGFR2-NANOG correlation suggest that the *Fgfr2* gene may function as a sensor for NANOG concentration in the cells. To test this possibility, we analyzed the NANOG binding sequences in the *Fgfr2* locus. Two NANOG binding regions (NBR) with multiple NANOG binding sequences were identified in the *Fgfr2* locus from the ChIP-seq (Karwacki-Neisius et al., 2013), NBR1 at 1.4 kb, and NBR2 at -0.2 kb relative to TSS of *Fgfr2*. NBR1 and NBR2 were deleted in TNERT (Figure 4D-E, S4D-E). Autoregulation was operational in TNERTNBR1-/- albeit at reduced strength, whereas it was abolished in TNERTNBR2-/- (Figure 4F), suggesting that NBR2 is essential for the binding of NANOG and activation of *Fgfr2* to trigger autoregulation. Together, our data suggest dose-responsive induction of *Fgf4*, *Fgfbp1*, and *Fgfr2* by NANOG. The *Nanog*-high cells secrete more FGF4 and FGFBP1, also express higher FGFR2 receptors. The FGF4 in presence of FGFBP1 binds to FGFR2 to enhance FGF signaling in *Nanog*-high cells to activate pERK1/2 and repress *Nanog*. The *Nanog*-low cells express relatively low FGFR2, resulting in weak FGF signaling and the absence of autoregulation (Figure 4G). We propose that FGFR2 distinguish the *Nanog*-high cells from the low cells to activate autoregulation predominately in *Nanog*- high cells.

ERK1/2 interacts and recruits NONO to repress *Nanog* transcription.

The FGF signaling cascade leads to phosphorylation of ERK1/2. pERK could activate downstream targets like *Tcf15* to repress *Nanog* (Davies et al., 2013) or ERK can recruit chromatin regulators like NONO to induce poised pol2 state in developmental bivalent genes (Ma et al., 2016). To examine these possibilities in autoregulation, we deleted *Tcf15* and *Nono* in TNERT (Figure 5A, S5A, B). Unlike in TNERTTcf15-/- cells, OHT treatment failed to repress *Nanog*:GFP in TNERTNono-/- cell indicating essential role of NONO in autoregulation (Figure 5B, S5C). pERK1/2 was substantially reduced in TNERTNono-/- despite OHT induction, unlike in TNERT (Figure 5C, S5D). Endogenous immunoprecipitation showed an interaction between NONO and ERK1/2 in the presence or absence of NANOG (Figure 5D). NONO colocalizes with ERK1/2 to bivalent developmental genes to maintain poised POL2 (Ma et al., 2016). Although *Nanog* is an actively transcribed gene lacking bivalent marks, the indispensability of NONO and pERK1/2 for autoregulation prompted us to investigate if they bind to *Nanog* and repress its transcription. The ChIP-seq data analysis (Ma et al., 2016; Tee et al., 2014) indicated NONO and ERK1/2 binding on the *Nanog* (S5E). We induced or repressed the pERK1/2 by treatment of E14Tg2a cells with FGF4 or PD respectively (Figure 5E) and analyzed the occupancy of NONO, pERK1/2, POL2, H3K4me3, and H3K27me3. The Transcription Start Site (TSS) and 5 kb upstream region (-5kb) are the two hubs of transcription factor binding and control of *Nanog* transcription (Chen et al., 2008; Loh et al., 2006). We performed ChiP-qPCR analysis with multiple primer sets spanning the -5.8 kb to +1.5 kb region relative to TSS (Figure 5F). pERK1/2 binding was detected in immediate downstream regions of the -5kb, and TSS. pERK1/2 binding was reduced significantly in PD and enhanced in FGF4 suggesting pERK1/2 binding to *Nanog* is dependent on FGF activity (Figure 5G). NONO binds to *Nanog* at the TSS and its downstream, which is enhanced in FGF4 and abolished in PD treatment suggesting pERK1/2 recruits NONO to *Nanog* (Figure 5H). POL2 occupancy seen in TSS and downstream region was reduced in FGF4 and enhanced in PD treatment. This suggested active transcription of *Nanog* in PD and repression in FGF4 (Figure 5I). This was further corroborated with enhanced enrichment of the transcription activating histone mark H3K4me3 in PD (Figure 5J) and enrichment of transcription repressive mark H3K27me3 at the -5kb region of the *Nanog* in FGF4 treatment (Figure 5K). pERK1/2 phosphorylates

NANOG, USP21 and affects NANOG stability and transactivation capability (Brumbaugh et al., 2014; Jin et al., 2016; Kim et al., 2014). In agreement with NANOG destabilization by pERK1/2 (Brumbaugh et al., 2014; Kim et al., 2014; Pokrass et al., 2020), The half-life of NANOG was significantly compromised in FGF4 treated cells but enhanced in PD (Figure 5L, S5F), suggesting that the FGF/ERK represses *Nanog* transcription and also affects NANOG stability. Collectively, these data suggest that FGF signaling activates pERK1/2 and its binding onto *Nanog* in a concentration-dependent manner. The recruitment of NONO to *Nanog* is dependent on the pERK1/2, NONO recruitment to *Nanog* is negligible in absence of pERK1/2. In the presence of pERK1/2-NONO, the POL2 loading on *Nanog* and its transcription activity is reduced resulting in repression of *Nanog* transcription. In the absence of active FGF signaling, pERK1/2-NONO occupancy on the *Nanog* is decreased permitting increased POL2 loading and transcription activation of the *Nanog*. The destabilization of NANOG by pERK1/2 further diminishes its ability to activate its promoter (Figure 5M).

FGF autocrine signaling regulates heterogeneity and monoallelic expression of Nanog.

The ESCs cultured in Serum+LIF on gelatin exhibit *Nanog* heterogeneity (Chambers et al., 2007; Filipczyk et al., 2013) which is lost when cultured on feeders or 2i (Faddah et al., 2013; Marks et al., 2012; Miyanari and Torres-Padilla, 2012). FGF signaling is substantially but discontinuously inhibited in 2i, leading to near homogeneity of *Nanog*. The loss of heterogeneity on feeders could be due to the induction of *Nanog* expression by the extracellular matrix (ECM)-integrin signaling (Lee et al., 2010). Alternately FGF4 might be titrated out by the FGFRs on the feeders or by ECM secreted by the feeders rendering it unavailable for inducing autoregulation resulting in loss of heterogeneity. To evaluate these possibilities, we cultured TNGA in presence of FGF4 and PD on gelatin or feeders. The *Nanog*:GFP was heterogeneous in SL on gelatin and nearly homogenous on feeders; it was homogenous in SLPD and 2i on gelatin and feeders. it was repressed further in presence of FGF4 vis-à-vis SL on gelatin. The *Nanog*:GFP was heterogeneous even in cells cultured on feeders in presence of FGF4, suggesting that heterogeneity could be induced even on feeders in presence of an excess of FGF4 (Figure 6A). This discounts any major role for ECM-integrin mediated activation of *Nanog* expression contributing to the loss of heterogeneity on feeders. To negate possibilities of variations due to targeting strategies in the reporter cell lines (Faddah et al., 2013), we analyzed *Nanog* heterogeneity in E14Tg2a cells. Consistent with

the observations in TNGA, the NANOG expression was homogenous in cells cultured in SLPD, 2i, and SLSU5402 on gelatin or feeders, and in SL on feeders (Figure S6A). The NANOG expression was heterogeneous in SL on gelatin and FGF4 was sufficient to induce heterogeneous NANOG expression in cells cultured on feeders (Figure 6B). Other growth factors like Insulin, EGF, and bFGF which can activate MEK1/2 also induced varying degrees of *Nanog* heterogeneity on feeders (S6A). *Nanog* expression was homogeneous in cells lacking FGF4 and FGFR2. Recombinant FGF4 substitution in media was sufficient to repress the *Nanog* and induce heterogeneity in FGF4-/- cells (Figure 6C). To quantify these observations, we generated OGNM cell line, where a randomly integrated *Oct4*-GFP transgene expression marks undifferentiated ES cells. The *Nanog* expression is reported by a T2AmCherry integrated in-frame with the last amino acid coding sequence of a *Nanog* allele (Figure 6D, S6B). The OGNM was cultured on gelatin or feeder or Feeder Secreted Matrix (FSM) and analyzed by FACS, to study the role of these substrates in *Nanog* heterogeneity. GFP expressing ES cells were gated and analyzed to avoid the feeders. Around 50% of cells cultured on gelatin expressed T2AmCherry, which increased to 64% on feeders and 55% on FSM. Since the feeder contains feeder cells and FSM, T2AmCherry expression was increased by 9% over the FSM (Figure 6E, S6D), suggesting that the diminished heterogeneity observed on the feeder is an additive impact of the FSM and feeder cells. FGF4 repressed *Nanog* and enhanced heterogeneity on feeders and FSM. The Nanog expression was homogenous in 2i and PD on all substrates. Repression of *Nanog* in FGFR inhibitor SU5402 was significantly lower than PD (Figure 6E, S6D). EGF, bFGF, and Insulin repressed *Nanog* expression (S6C-D) suggesting MEK1/2 may be predominately activated by FGF signaling, but other growth factors can also contribute to the MEK1/2 activity in ESCs. We propose that the feeder cells and the matrix secreted by the feeders titrate out the FGF4 secreted by the ES cells and interrupt autoregulation of *Nanog* resulting in loss of heterogeneity of *Nanog*.

Monoallelic expression of *Nanog* is suggested to be underlying mechanisms for *Nanog* heterogeneity in ES cells and the embryos (Filipczyk et al., 2013; Marks et al., 2012). To analyze the expression of both the alleles of *Nanog*, we integrated an iRFP coding sequence in one allele and a GFP coding sequence in another allele in-frame with the last coding sequence of the *Nanog* to generate NANOGiRFP and NANOGsfGFP fusion protein from the two alleles (NsGiR) (Figure 6F, S6E). Around 32% of the cells exhibited biallelic expression or no expression (32%) and the rest showed monoallelic expression (36%) in SL. CHIR reduced the monoallelic population (25%) and enhanced both the biallelic and non-expressing populations. The reduced MEK1/2 activity in 2i and PD leads to biallelic expression in 90%

of the cells. Around 75% of the cells show biallelic expression in SU5402 treatment, with a significant population either showing monoallelic or no expression of *Nanog*. This further strengthens the suggestion that other growth factor signaling pathways may be feeding to MEK1/2 activity in ESCs. FGF4 significantly repressed both biallelic as well as monoallelic expression of *Nanog* leading to loss of *Nanog* expression in 50% of the cells (Figure 6G). Together our data suggest that the FGF autocrine-driven *Nanog* autoregulation is interrupted by the FSM on feeders or by MEK1/2 inhibitor in 2i leading to loss of *Nanog* heterogeneity and monoallelic expression. This also establishes the primary role of feeder cells and FSM in the regulation of *Nanog* expression.

DISCUSSION

The inhibition of MEK1/2 and GSK3β in the ground state (2i) attenuates *Nanog* heterogeneity and monoallelic expression (Filipczyk et al., 2013; Nichols et al., 2009; Wray et al., 2010). Surprisingly, autorepression remains functional in 2i, discounting the role of MEK1/2 and GSK3β in *Nanog* autorepression (Navarro et al., 2012). We attribute the highest possible de-repression of *Nanog* locus to a consistent low MEK1/2 activity in SLPD, unlike in 2i. Wnt signaling can activate MEK1/2 at multiple levels (Yun et al., 2005), hence activation of MEK1/2 in presence of CHIR may be mediated by downstream components of Wnt signaling. We suggest that the inhibition of MEK1/2 prevents differentiation in 2i, but a time-dependent increase in MEK1/2 activity is significant and sufficient to facilitate *Nanog* autoregulation. Our findings open up the plausibility of other molecular processes regulated by MEK1/2 activity to be functional in 2i.

In this study, we show that FGF autocrine signaling and its components are essential for *Nanog* autoregulation. *Nanog* is vital for the secretion of FGF4 and endoderm differentiation by cell-autonomous mechanisms (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010). We show, NANOG binds to the promotors of *Fgf4*, *Fgfr1*, *Fgfr2*, and *Fgfbp1*; and induce their expression. As speculated earlier, NANOG targets-ESRRB and KLF4 may also induce some of the FGF signaling components, contributing to FGF signaling (Lakatos et al., 2014). Our data suggest that *Nanog* autoregulation is triggered above a threshold of NANOG, thereafter the intensity of repression is dependent on the levels of NANOG in the cells.

In a subpopulation of ES cells expressing high NANOG; NANOG activates the expression of *Fgf4*, *Fgfr2*, and *Fgfbp1*, leading to a state of high NANOG-high FGFR. The higher ERK1/2

activity induced by high FGFR in these cells leads to the destabilization of NANOG. The *Nanog* transcription is also decreased by transcriptional repression by pERK1/2-NONO, leading to a state of low/medium NANOG-high FGFR/ERK. In embryos, such cells may exit pluripotency to form primitive endoderm. In cell culture, such cells might continue to self-renew in presence of LIF. The reduced NANOG in these cells cannot anymore activate FGF signaling components leading to a low NANOG-low FGF/ERK state. The loss of FGF/ERK in such cells releases repression of *Nanog* and permits activation of *Nanog* by other transcription factors leading to cells with a high NANOG-low FGF/ERK state. This cycle of activation of FGF signaling by NANOG and repression of *Nanog* by FGF signaling might lead to heterogeneity of *Nanog* and heterogeneity of FGF/ERK (Figure 7A). ERK signaling heterogeneity is suggested to be a vital determinant of fate choice in ICM and ES cells (Bessonnard et al., 2014; Kang et al., 2017; Molotkov et al., 2017; Pokrass et al., 2020; Simon et al., 2020). The ERK heterogeneity has been suggested to originate due to differential local concentrations of FGF4 or heterogeneous expression of receptors FGFRs (FGFR1, FGFR2) or by negative feedback regulators (ETV5, DUSP1/6). Since FGFR1 is relatively uniformly expressed in the epiblast (Kang et al., 2017; Molotkov et al., 2017) and ESCs. Our findings may provide the basis of heterogeneous expression of FGFR2 and FGF4 in ESCs, which is dependent on NANOG concentration. The carrier protein FGFBP1 may locally enhance FGF signaling to induce ERK heterogeneity similar to heparan sulfate proteoglycans (Galanternik et al., 2015). We propose that reciprocal regulation of *Nanog* by FGF signaling and FGF signaling by NANOG as the basis for both NANOG and ERK heterogeneity.

Contradicting reports on *Nanog* heterogeneity (Chambers et al., 2007; Faddah et al., 2013; Filipczyk et al., 2013; Smith, 2013) attribute it to genetic manipulation strategies or culture on feeders or gelatin. Our study demonstrates that *Nanog* heterogeneity is induced by *Nanog* autoregulation triggered FGF autocrine signaling. The feeder cells and feeder secreted matrix interrupt this autoregulation to abolish heterogeneity (Figure 7B). *Nanog* autoregulation was speculated to regulate allelic switching and FGF4 signaling was considered dispensable for autorepression (Fidalgo et al., 2012; Navarro et al., 2012). Our study suggests that FGF autocrine signaling is essential for *Nanog* autoregulation and allelic switching (Figure 7C). We suggest that *Nanog* autoregulation is the molecular basis for *Nanog* heterogeneity.

Taken together, we demonstrate an essential role for FGF autocrine signaling in *Nanog* autoregulation, and different facets of *Nanog* regulation are intertwined and governed by FGF autocrine signaling.

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Author contributions

Conceptualization, H.T.K, and P.C.S Methodology, H.T.K and P.C.S; Investigation, H.T.K, R.S.R., V.V.V., and G.S.; Writing – Original Draft, H.T.K. and P.C.S.; Writing – Review & Editing, H.T.K., and P.C.S.; Funding Acquisition, P.C.S.; Resources, H.T.K, R.S.R., M.S., and D.J.; Visualization, H.T.K, and D.J.; Supervision, P.C.S.

Declaration of interest:

The authors declare no competing interests.

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Figure 1: Residual MEK1/2 activity in ground state prevents complete derepression of *Nanog*. (A) (left) FACS profiles of TNGA cultured in indicated conditions for 3 passages. The cells were cultured in Serum+LIF (SL) in presence of the 1 μM MEK1/2 inhibitor - PD0325901 (SLPD) or 3 μM GSK3β inhibitor -CHIR99021 (SLCHIR) or in serum-free media - N2B27 with PD0325901, CHIR99021, and LIF (2i). (right) *Nanog*:GFP population median of TNGA (n=4). (B) (left) FACS profiles of Tβc44Cre6 cultured in indicated conditions for 3 passages. TβC44Cre6 is a *Nanog* null cell line, where β–*geo* cassette is inserted into one allele and GFP into another allele of the *Nanog* gene. (right) *Nanog*:GFP population median of Tβc44Cre6 (n=3). (C) FACS profile of NANOG-iRFP protein in

NiRFP2A cells cultured in indicated conditions for 3 passages. (D) RT-qPCR of pluripotency factors in indicated conditions (SL2i= SL+ PD0325901+CHIR99021). (E) RT-qPCR analysis of pre- mRNA of *Nanog* and *Oct4*. (F) (left)Western blot of NANOG, OCT4, and SOX2. (right) Relative NANOG levels as estimated by densitometry (n=8). (G) Western blot of pERK1/2 and ERK1/2 at 0, 1, 4, 8, 12, 16, and 24 hrs after media change in indicated treatments. (H) Western blot of pERK1/2 and ERK1/2 in SLPD, SLCHIR, 2i, and SL2i after 8, 12, 16, and 24 hrs of culture relative to SL, where the cells in SL were harvested 24 hrs after the media change. (I) (left)Western blot of pERK1/2 and ERK1/2 in 1μM PD and increasing concentrations of CHIR in serum-free N2B27 media. (right) Relative pERK1/2 levels (n=3). (J) (left)Western blot of pERK1/2 and ERK1/2 in 3μM CHIR and increasing concentrations of PD in serum-free N2B27 media. (right) Relative pERK1/2 levels (n=6). All error bars in the figure represent s.e.m. See also Figure S1.

Figure 2: FGF autocrine signaling pathway components are essential for *Nanog* autoregulation. (A) (left) Schematic depiction of Tamoxifen (OHT) inducible TNERT cell line. (Middle) FACS profile of TNERT treated with OHT (red) or no OHT (blue). (Right) *Nanog*:GFP population median of TNERT (n=3). (B) (left) Schematic of NsGFPDiN cells reporting NANOG-GFP fusion protein and carrying a Doxycycline- inducible FaNANOG transgene. (Middle) FACS profiles of NsGFPDiN after 48 hrs with Doxycycline (red) or no Doxycycline (blue) treatment. (Right) *Nanog*:GFP population median of NsGFPDiN (n=3). (C) FACS profiles of TNERT, NsGFPDiN, and TDiN treated with 2μM SU5402, with OHT/Doxycycline (red) or no OHT/Doxycycline (blue). (D) Heat map representing transcript levels (FPKM) of *Fgfbp1* from 8-cell to blastocyst stage analyzed from the single-cell sequencing data. (E) Schematic depiction of TNERTFgfr1-/-, TNERTFgfr2-/, TNERTFgf4-/-, and TNERTFgfbp1-/- cell lines, which are derivatives of TNERT where *Fgfr1, Fgfr2, Fgf4,* and *Fgfbp1* are knocked out respectively. (F) FACS profiles of TNERT, TNERTFgfr1-/-, TNERTFgfr2-/-, TNERTFgf4-/-, and TNERTFgfbp1-/- cells, treated with OHT (red) or no OHT (blue). All error bars in the figure represent s.e.m. See also Figure S2.

Figure 3: *Nanog* enhances expression of FGF autocrine signaling pathway components. (A) RT-qPCR showing relative transcript levels after 0, 0.5, 1, 2, 4, 8, 12 and 18 hrs OHT treatment in TNERT (n=3). (B) RT-qPCR of relative levels of pre- mRNA at the above indicated time points after OHT treatment in TNERT (n=3). (C) ChIP analysis of NANOG on

Fgf4, *Fgfbp1*, *Fgfr1*, *Fgfr2*, and *Nanog* genes in E14Tg2a cells cultured in SL or SLPD for 48 hrs (n=4). (D) ChIP analysis of NANOG on promotors of above- indicated loci in EDiN cells cultured in Doxycycline (red) or no Doxycycline (blue) for 48 hrs (n=3). (E) Western blot of FGFR1, FGFR2, and pERK1/2 in TNERT after 18 hrs treatment with (red) or no OHT (blue) (n=3). (F) FACS analysis of FGFR2 on the cell surface of TNERT treated with (red) or no OHT (blue) (n=3). (G-H) ELISA-based relative quantification of FGF4 (G) and FGFBP1 (H) in conditioned media from TNERT treated with or no OHT (n=3). (I) Schematic of conditioned media experiment. (J) FACS analysis of Tβc44Cre6 cell line in conditioned media collected from TNERT treated with OHT after 0, 18, 24, and 48 hrs (K) (left) FACS analysis of TNERTZfp281-/- cells treated with (red) or with no OHT (blue) treatment. (right) *Nanog*:GFP population median of TNERTZfp281-/- (n=3). (L) FACS analysis of Tβc44Cre6 cell line in conditioned media from, TNERT+OHT 0 hrs, TNERTFGF4-/-, Tβc44Cre6 48 hrs, E14Tg2a- FGF4-OE (overexpression) 48 hrs, TNERT+OHT 48 hrs and 50ng/ml FGF4. All error bars in the figure represent s.e.m. See also Figure S3.

Figure 4: NANOG-induced FGFR2 triggers autoregulation predominately in the ES cell population with higher *Nanog* expression. (A) (top) FACS profile of TNERT, the position of the gates indicates the 10% low-*Nanog*:GFP (LN) and 10% high-*Nanog*:GFP (HN) population sorted for culture. (bottom left) FACS profiles of LN and HN after 18 hrs culture in SL. LN (dark green), HN (dark maroon) in SL, and LN (light green), HN (light maroon) in SL+ OHT. (bottom right) *Nanog*:GFP population median of TNERT (n=3). (B) FACS profile of E14Tg2a cultured in SL or SLPD for 48 hrs and co- immunostained with anti-NANOG and anti-FGFR1 or anti-FGFR2 antibodies. r-values represent the average of 3 independent experiments (n=3). (C) (left) FACS profile of E14Tg2a immunostained with anti-NANOG and anti-FGFR2 antibody, the gates mark the 10% low-NANOG (LN) and 10% high-NANOG (HN) population. (right) Histogram depicting the FGFR2 expression profiles in the gated LN and HN cell population (n=3). (D-E) Schematic representation of TNERTNBR1-/- (D) and TNERTNBR2-/- (E) cells, in which NANOG binding sequences at +1.4 kb (NBR1) and -0.2 kb (NBR2) are deleted respectively. (F) (Top) FACS profiles of TNERT, TNERTNBR1-/-, and TNERTNBR2-/- with (red) or no OHT treatment (blue). (bottom) *Nanog*:GFP population median of TNERT, TNERTNBR1-/- and TNERTNBR2-/- with or no OHT treatment. (G) A cartoon depicting *Nanog* autoregulation in *Nanog*-high cells. The *Nanog*-high cells secrete more FGF4 and FGFBP1. They contain higher levels of FGFR2 on

the surface and hence more sensitive to the FGF ligand triggering a stronger FGF signaling. The increased pERK1/2 in these cells recruit NONO to the *Nanog* locus and represses *Nanog* transcription. The *Nanog*-low cells secrete very little FGF4 and FGFBP1 and present fewer FGFR2 on their surface and less sensitive to FGF signaling. The pERK1/2 levels in *Nanog*-low cells are insufficient to execute *Nanog* autoregulation. All error bars in the figure represent s.e.m. See also Figure S4.

Figure 5: ERK1/2 interacts and recruits NONO to repress *Nanog* transcription. (A) Schematic of TNERTNono-/-cell line; a derivative of TNERT in which *Nono* is knocked out. (B) FACS profile of TNERTNono-/- treated with or no OHT (n=3). (C) Western blot of pERK1/2 and ERK1/2 in TNERT and TNERTNono-/- cells treated with or no OHT (n=3). (D) Immunoprecipitation analysis showing interactions between ERK1/2 and NONO in the presence or absence of *Nanog* induction by Doxycycline in TDiN cells. (E) (left) Western blot of pERK1/2 and ERK1/2 in E14Tg2a cells treated with PD or FGF4. (right) Relative levels of pERK1/2 in E14Tg2a cells treated with PD or FGF4 (n=4). (F) Schematic representation of *Nanog* locus comprising the -6.0 to +2kb region. The vertical bars represent relative positions of primer pairs used for ChiP-qPCR analysis. S1-S6 are located upstream of the TSS, S7 primer pair is located around TSS, S8 and S9 are located downstream in the first intron. (G-K) ChIP-qPCR analysis of pERK1/2 (G), NONO (H), Pol2 (I), H3K4me3 (J) and H3K27me3 (K) on *Nanog* 5' region in E14Tg2a cells (blue), treated with FGF4 (green) and with PD (pink) (n=3). (L) Cycloheximide chase assay of NANOG in SL, SLPD, and SLFGF4 in E14Tg2a cells. (M) A cartoon illustrating the repression of *Nanog* by FGF signaling and derepression of *Nanog* in absence of Fgf signaling. The FGF4 activates the Fgf signaling cascade, results in phosphorylation of ERK1/2. pERK1/2 interacts and recruits NONO to *Nanog* promoter and represses transcription of *Nanog*. pERK1/2 also affects the stability of the NANOG. In absence of FGF4, the pERK1/2 levels decrease resulting in enhanced stability of NANOG and transcription of *Nanog* locus by NANOG and other pluripotency factors resulting in derepression of *Nanog* locus. All error bars represent s.e.m. See also Figure S5.

Figure 6: FGF autocrine signaling regulates *Nanog* heterogeneity and monoallelic expression. (A) *Nanog*:GFP fluorescence in TNGA cultured on gelatin or feeders in SL, SLPD, SLFGF4, and 2i (n=3). Scale bars represent 75μM. (B) Immunofluorescence of

NANOG and OCT4 in E14Tg2a cells cultured on gelatin or feeders in SL, SLPD, and SLFGF4. Areas are marked to highlight the loss of NANOG. Scale bars represent 75μM. (C) Immunofluorescence of NANOG and OCT4 in E14Tg2a, *Fgfr2*-/- ESCs, *Fgf4*-/- ESCs, and *Fgf4*-/- ESCs rescued with Ef1α–*Fgf4* transgene. The marked area highlights the loss of NANOG. Scale bars represent 75μM. (D) Schematic of OGNM cell line with *Oct4*-GFP transgenic reporter and T2A-mCherry reporting one of the *Nanog* alleles. (E) FACS profiles of OGNM cultured in SL, SLFGF4, SLSU5402, 2i, and SLPD on gelatin (top), on feeders (middle), and FSM (bottom). (F) Schematic of NsGiR cell line, with allele-specific NANOG protein reporters. One allele of *Nanog* reports as NANOGiRFP fusion protein and the other as NANOGsfGFP fusion protein. (G) FACS profiles of NsGiR cell line cultured in SL, SLCHIR, 2i, SLSU5402, SLFGF4, and SLPD. See also Figure S6.

Figure 7: A cartoon illustrating the role of FGF autocrine signaling in *Nanog* autoregulation, heterogeneity, and monoallelic expression. (a) A subpopulation of ES cells with high-*Nanog* induce expression of FGF signaling components- *Fgf4*, *Fgfbp1*, and *Fgfr2*. FGF4 and FGFBP1 are secreted out of the cells. The FGFR2 is retained on the cells leading to a high NANOG-high FGFR state. Higher FGFR activates FGF signaling in these cells resulting in enhanced pERK1/2. The pERK1/2 leads to destabilization of NANOG and recruits NONO to *Nanog* locus to repress its transcription resulting in a low/medium NANOG-high FGF/pERK state. The repression of *Nanog* and reduced NANOG levels diminishes activation of FGF signaling components, leading to a low NANOG-low FGFR state, with low pERK1/2. The diminished pERK1/2 releases the repression on the *Nanog* and enables stabilization of NANOG. This permits transcriptional activation of *Nanog* locus by pluripotency factors, increasing the expression of *Nanog* resulting in a low/medium NANOG-low FGF/pERK state. Unrestricted induction of *Nanog* in low pERK1/2 state results in enhanced activation of *Nanog* resulting in a high-*Nanog* expressing cells. (b) A cartoon showing heterogeneous expression of *Nanog* in ESCs cultured on gelatin. FGF4 and FGFBP1 are free to activate FGF autocrine/paracrine signaling to induce *Nanog* autoregulation resulting in *Nanog* heterogeneity. In feeders or FSM, FGF4 is either titrated out by FGFRs on feeder cells and extracellular matrix rendering it unavailable for triggering FGF autocrine signaling to induce *Nanog* autoregulation. In the absence of *Nanog* autoregulation, most of the cells continuously express *Nanog* resulting in loss of heterogeneity. (c) Low FGF autocrine signaling prevents pERK1/2 and repression of *Nanog* locus resulting in biallelic expression. An increase in FGF

autocrine signaling activates ERK1/2 and leads to monoallelic expression. A further increase in FGF signaling results in complete repression of the *Nanog* locus.

Methods:

Cell Culture

The cell lines used in this study are listed in the resource table and their origin is depicted in supplemental flowchart 1. All the cells used in this study are derivatives of E14Tg2a ES cells (ATCC CRL1821). The cells were cultured as described earlier (Festuccia et al., 2012). 4-Hydroxytamoxifen (4-OHT) (Cat# H7904), Doxycycline (Cat# D9891), and Cycloheximide (Cat# 239764) were used at a concentration of 1 μg/ml, 1 μg/ml, and 100 μg/ml respectively. The TNERT and its derivative cell lines were treated with 4-OHT for 18 hrs except when indicated. TDiN, NsGFPDiN, and EDiN were treated with Doxycycline for 48 hrs unless indicated. CHIR99021 (CHIR) (Cat# SML1046), PD0325901 (PD) (Cat# PZ0162), and SU5402 (Cat# SML0443) were used at 3 μM, 1 μM, and 2 μM, respectively, except when indicated. FGF4 (Cat# 7460-F4-025), FGFBP1 (Cat# 1593-FB-025), Insulin (Cat# RP-10908), EGF (Cat# 01-107) and bFGF (Cat# RP-8628) were used at 50ng/ml, 50ng/ml, 100ng/ml, 25ng/ml and 10ng/ml concentrations respectively. The cells were cultured in Serum+ LIF (SL), SL+ PD (SLPD), SL+CHIR (SLCHIR), SL+SU5402 (SLSU5402), SL + PD +CHIR (SL2i) and N2B27+LIF+PD+CHIR (2i) for at least 2 passages before treating with either 4-OHT or Doxycycline.

The cells were cultured on cell culture dishes coated with 0.1% gelatin for all experiments. In experiments involving ESCs cultured on feeders, the feeder cells were seeded onto the culture dishes at least 48 hrs before seeding ESCs. To prepare feeder secrete matrix (FSM), feeder cells were seeded onto a culture dish and cultured for 4-5 days. The cells were lysed with 0.01% SDS for 30 mins followed by two washes with PBS for 30 mins each. Finally, the FSM was equilibrated with media for 30 mins. The conditioned media from the cells was collected after the specific treatments or indicated time points. The conditioned media was passed through a 0.22 μM filter and added to T*β*c44Cre6 or TNERT cells. The cells were cultured in the conditioned media for 24 hrs before FACS analysis.

Generation of Knock-out cell lines using paired CRISPR constructs: pU6-iRFP (pU6-

Cas9-T2A-iRFP-2A-PuroR) construct was engineered by replacing mCherry coding sequence with iRFP670-2A-PuroR cassette in pU6-(BbsI)-CBh-Cas9-T2A-mCherry plasmid (Addgene 64324) by Gibson assembly. For generating knock-out of a gene, two sgRNAs were designed with the expected cutting sites at least 30 bps apart to achieve deletion of at least 30 bps or more. For genotyping of the deletions, a set of genotyping primers was designed outside the deletion region flanking the sgRNA pair. The sgRNAs were designed using the UCSC genome browser and Deskgen or Benchling. The sequences of the sgRNAs and the genotyping primers are detailed in Supplemental data table 1. All sgRNAs were cloned into pU6-Cas9-T2A-iRFP-2A- PuroR plasmids. To generate a paired sgRNA construct, the U6-SgRNA cassette from one plasmid containing the sgRNA was amplified and Gibson assembled into the XbaI site of the plasmid containing the other sgRNA pair of the pair. Around 1 µg of paired sgRNA CRISPR plasmid was nucleofected in 1 million cells. The transfected cells were sorted by FACS for iRFP expression and cultured to obtain clones. The clones were genotyped by PCR using respective primer sets to identify the heterozygous and homozygous clones. The sequence of the derivation of cell lines is described in supplemental Figure 7.

Generation of Knock-in cell lines: A sgRNA encompassing the stop codon of *Nanog* was cloned into pU6-iRFP and co-transfected with the targeting vectors. Nanog-2A- mCherry targeting vector (Addgene 59995) was used for generating the OGNM cell line. The 2A-mCherry cassette was replaced with sfGFP or iRFP sequences by Gibson assembly to generate Nanog sfGFP fusion or Nanog iRFP670 fusion targeting vector respectively. Around 3 µg plasmid (targeting vector and CRISPR plasmid) were nucleofected in 3 million E14Tg2a cells. The cells were selected against G418. The derivation of cell lines is described in Supplemental Figure 7.

Real-time PCR analysis:

The RNA was extracted with TRIZOL reagent and quantified using Nanodrop2000 spectrophotometer (Thermo Fisher Scientific). One microgram of total RNA was reversed transcribed into cDNA by using superscript III. All real-time PCR was carried out with Power SYBR Green PCR master mix on the ABI prism 7900 HT sequence detection system (ABI) as per the manufacturer’s instructions. GAPDH was used as an internal control or normalizer. The data was analyzed by SDS 2.2 software provided with the instrument. The

primers used for real-time PCR are given in the supplemental data table 1.

Western blot analysis: The cells were harvested by using RIPA buffer with 25mM Tris HCl (pH 8.0), 150mM NaCl, 1%NP-40, 0.5% Sodium deoxycholate, 0.1% SDS and Complete Protease Inhibitor Cocktail Tablets (Cat# 11873580001). The protein samples were resolved by 4-20% gradient SDS-PAGE and electroblotted on to polyvinylidene difluoride (PVDF) membrane. The blot was blocked with 3% Blotto (Cat# SC-2324) for an hour and incubated overnight with a primary antibody at 4°C. Blots were washed thrice with TBST and hybridized with secondary antibody and the blots were visualized using enhanced chemiluminescence (ECL)detection kit. Western blot quantifications were performed using Image lab (Bio-rad).

Chromatin Immunoprecipitation (ChIP):

Cells were fixed by adding 270 µL of 37% formaldehyde into 10 ml of media and incubated for 10 minutes at 37^{0}C to crosslink the chromatin. Cells were washed twice with cold PBS containing protease inhibitors. Cells were scraped and harvested by centrifugation. The cell pellet was dissolved in 200 µL of SDS Lysis Buffer (1% SDS, 10 mM EDTA, and 50 mM Tris, pH 8.0) containing protease inhibitors (per 10^{6} cells) and incubated on ice for 10 min. The 25 cycles of sonication were used to shear DNA between 200 to1000 base pairs. The sample was centrifuged at 13,000 rpm for 10 min (at 4°C). The supernatant was diluted by adding 1800 µl ChIP Dilution Buffer (1.1% Triton X- 100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl with protease inhibitors). The 1% input was aliquoted from the supernatant. To reduce nonspecific background, diluted cell supernatant was preabsorbed for one hour at 4°C with protein A/G magnetic beads (Cat# 88803). The supernatant fraction was incubated overnight at 4°C with appropriate antibody and protein A/G magnetic beads were blocked with 4% BSA, 2µg salmon sperm DNA. The next day, pre-blocked beads were mixed with the sample and incubated for 1 hour to capture the antibodies. The supernatant was discarded and washed in the given order with 1 mL of each of the buffers - Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), LiCl Wash Buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8.0.), and TE buffer. DNA was eluted with elution buffer (1%SDS, 0.1M NaHCO3). The sample input and the ChIP chromatin were

reverse crosslinked with 20 µL of 5 M NaCl by heating at 65°C for 4 hours. Followed by one hour at 45°C with 10 µL of 0.5 M EDTA, 20 µL 1 M Tris- HCl, pH 6.5, and 2 µL of 10 mg/mL Proteinase K. Finally, DNA was eluted in 50 µL water using a minEleute PCR purification kit (Cat# 28006). Then 1µL of sample and input was used for qPCR analysis. The primers used for qPCR analysis were listed in supplemental data table 1.

Co-Immunoprecipitation in ES cells: 10-12 million ES cells were harvested by trypsinization, washed twice with cold PBS, and resuspended in 800 µl of CoIP Lysis Buffer (50 mM Tris-HCl, pH 67.5; 350 mM NaCl, 0.7% NP40, EDTA 0.1mM, 20% (v/v) glycerol, and protease inhibitor cocktail). The cell lysate was mixed with protein A/G magnetic beads (Cat# 88803) for 1 hour at 4°C for pre-clearing the background. Then 5% input was aliquoted and the remaining supernatant was incubated overnight with appropriate primary antibody. The protein A/G magnetic beads were blocked overnight at 4°C with 200 µl of CoIP Lysis buffer containing 4% BSA. The next day, the beads were transferred to the primary antibody incubated tubes and incubated for one hour at 4°C. The bead was washed three times with ice-cold TBS150 (50mM Tris, 150mM NaCl) and the protein was eluted with 2X sample buffer (125 mM Tris-HCl, pH 6.8**,** 4% SDS**,** 20% (v/v) glycerol, 0.004% bromophenol blue), by boiling for 5 min. The western was done for sample and input and the interaction was analyzed.

Immunocytochemistry: The cells were cultured in 24 well dishes and fixed at 70% confluency in 3.7% formaldehyde diluted in PBS for 15 mins at RT. After 3 washes with PBS, the cells were permeabilized and blocked with PBS containing 0.5% BSA and 0.3% Triton-X100 for 1 hour at room temperature. The cells were hybridized with primary antibody (1:100 dilution) in PBS containing 0.5% BSA at 4°C overnight in a humidified chamber. The cells were washed three times with PBS and hybridized to appropriate secondary antibody at 1:1000 dilution room temperature for 1hour. The nuclei were stained with DAPI in 1X PBS for 20 min at room temperature. The cells were washed thrice with PBS. The cells were layered with 100 µl of the mixture of PBS and Glycerol (1:1) and the images were acquired on the ZEISS Axio observer microscope and analyzed using ImageJ software.

ELISA Assay:

The condition media from the cell lines was collected at the respective time points. 100 µl of

the media was coated per well of 96 well of ELISA plate by incubating overnight at 4^oC. The wells were washed thrice with PBS containing 0.05% Tween-20 and blocked with PBS containing 2% BSA for one hour at room temperature. The wells were washed once with PBS and incubated with the appropriate primary antibody (1:100) for one hour. Washed thrice with PBST, appropriate HRP-labeled secondary antibody was hybridized for one hour at room temperature. The wells were washed thrice with PBST and incubated in substrate solution OPD (o-phenylenediamine dihydrochloride) (Cat# P8287) 3mg/ml with 6 µl/ml H_2O_2) for 30 min in dark. The reaction was stopped by using 2N H_2SO_4. The absorbance was measured at 492 nm in Power wave XS2 (Bio Tek instruments)

FACS analysis:

Reporter cells: Cells were trypsinized and collected by spinning at 800 rpm for 5 min. The media was removed and cells were resuspended in 300 µl of PBS containing 2% FBS at 10^6 cells/ml. The samples were analyzed in the Gallios flow cytometer (Beckman Coulter) or Fortessa flow cytometer (BD Biosciences). Sorting was performed on a MoFlo-XDP cell sorter (Beckman Coulter).

Immunostained cells: Cells were harvested by treatment with 0.5 mM EDTA and resuspended into single cells. The cells were fixed in PBS with 4% paraformaldehyde (PFA) for 20 min at room temperature. Cells were washed twice with cold PBS and incubated with methanol for 30 min for permeabilization. In the case of experiments involving analysis of FGFRs on the cell surface, the permeabilization step was excluded. Then cells were blocked with PBS containing 0.5% BSA for 60 min at room temperature. The cells were washed and hybridized to the appropriate primary antibody at 4^oC overnight. The cells were washed thrice with PBS and hybridized to the appropriate secondary antibody in PBS containing 0.5% BSA at1:1000 dilution for one hour at room temperature. The cells were washed thrice with PBS and the fluorescence profiles were acquired in the Gallios FACS analyzer (Beckman Coulter). All the FACS data were analyzed using FlowJo software (BD Biosciences).

Statistical analysis and reproducibility:

Statistical analysis was done by using a two-tailed paired or unpaired student t-test. The representation of data is in the form of means+/-SEM. The mean was calculated for more than

three independent experiments *P* value<0.05 is considered as statistically significant. * represents P<0.05, ** represents P<0.001, *** represents P<0.0001, and **** represents P<0.00001.

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Figure 1

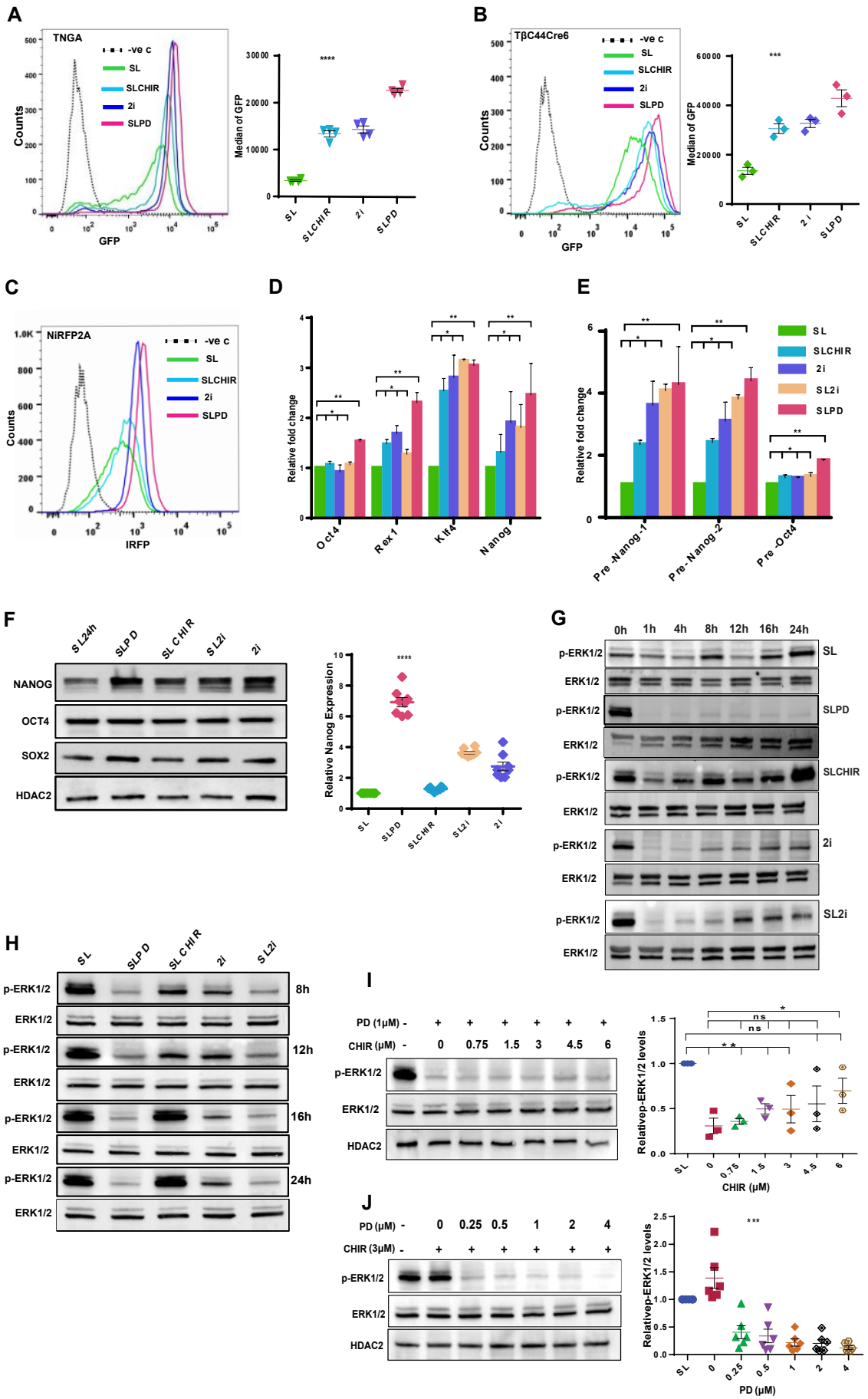

A
TNGA
-ve c
SL
SLCHIR
2i
SLPD
Counts
GFP
Median of GFP
B
TβC44Cre6
C
NiRFP2A
IRFP
D
Relative fold change
Oct4
Rex1
Klf4
Nanog
E
SL2i
Pre-Nanog-1
Pre-Nanog-2
Pre-Oct4
F
SL24h
SL CHIR
NANOG
OCT4
SOX2
HDAC2
Relative Nanog Expression
G
0h
1h
4h
8h
12h
16h
24h
p-ERK1/2
ERK1/2
H
8h
12h
16h
24h
I
PD (1μM)
CHIR (μM)
Relativep-ERK1/2 levels
J
PD (μM)
CHIR (3μM)

Figure 2

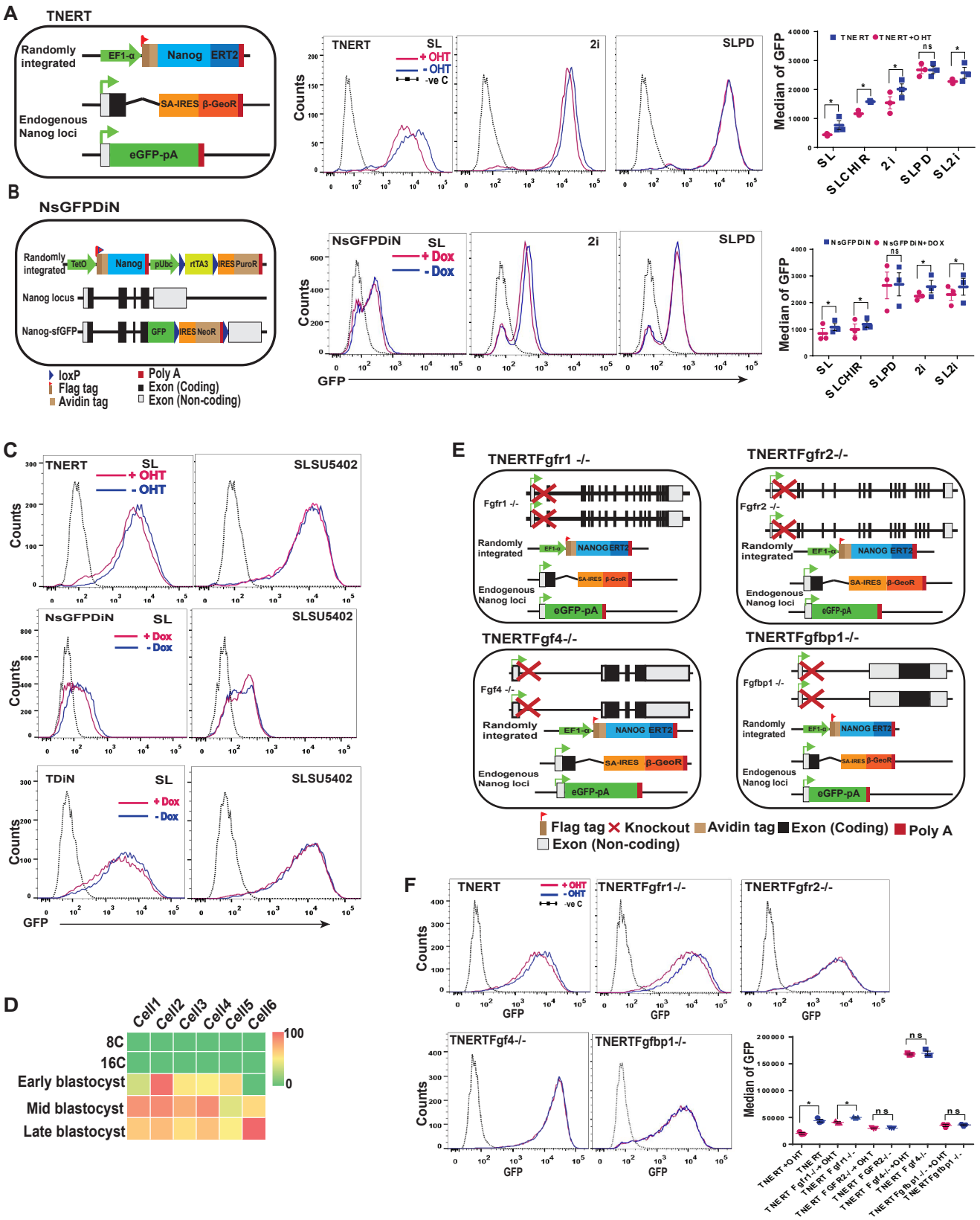

A
TNERT
Randomly integrated
EF1-α
Nanog
ERT2
Endogenous Nanog loci
SA-IRES
β-GeoR
eGFP-pA
TNERT
SL
+ OHT
- OHT
-ve C
2i
SLPD
Counts
Median of GFP
TNERT
TNERT +OHT
SL
SLCHIR
2i
SLPD
SL2i
B
NsGFPDiN
Randomly integrated
TetO
Nanog
pUbc
rtTA3
IRES PuroR
Nanog locus
Nanog-sfGFP
GFP
IRES NeoR
loxP
Flag tag
Avidin tag
Poly A
Exon (Coding)
Exon (Non-coding)
NsGFPDiN
SL
+ Dox
- Dox
2i
SLPD
GFP
NsGFPDiN
NsGFPDiN+DOX
C
TNERT
SL
+ OHT
- OHT
SLSU5402
NsGFPDiN
SL
+ Dox
- Dox
SLSU5402
TDiN
SL
+ Dox
- Dox
SLSU5402
GFP
D
Cell1
Cell2
Cell3
Cell4
Cell5
Cell6
8C
16C
Early blastocyst
Mid blastocyst
Late blastocyst
100
0
E
TNERTFgfr1 -/-
Fgfr1 -/-
Randomly integrated
NANOG
ERT2
Endogenous Nanog loci
eGFP-pA
TNERTFgfr2-/-
Fgfr2 -/-
EF1-β
TNERTFgf4-/-
Fgf4 -/-
EF1-α
TNERTFgfbp1-/-
Fgfbp1 -/-
Flag tag
Knockout
Avidin tag
Exon (Coding)
Poly A
Exon (Non-coding)
F
TNERT
+ OHT
- OHT
-ve C
TNERTFgfr1-/-
TNERTFgfr2-/-
TNERTFgf4-/-
TNERTFgfbp1-/-
Counts
GFP
Median of GFP
TNERT +OHT
TNERT
TNERT Fgfr1-/- +OHT
TNERT Fgfr1-/-
TNERT FGFR2-/- +OHT
TNERT FGFR2-/-
TNERT Fgf4-/- +OHT
TNERT Fgf4-/-
TNERT Fgfbp1-/- +OHT
TNERT Fgfbp1-/-

Figure 3

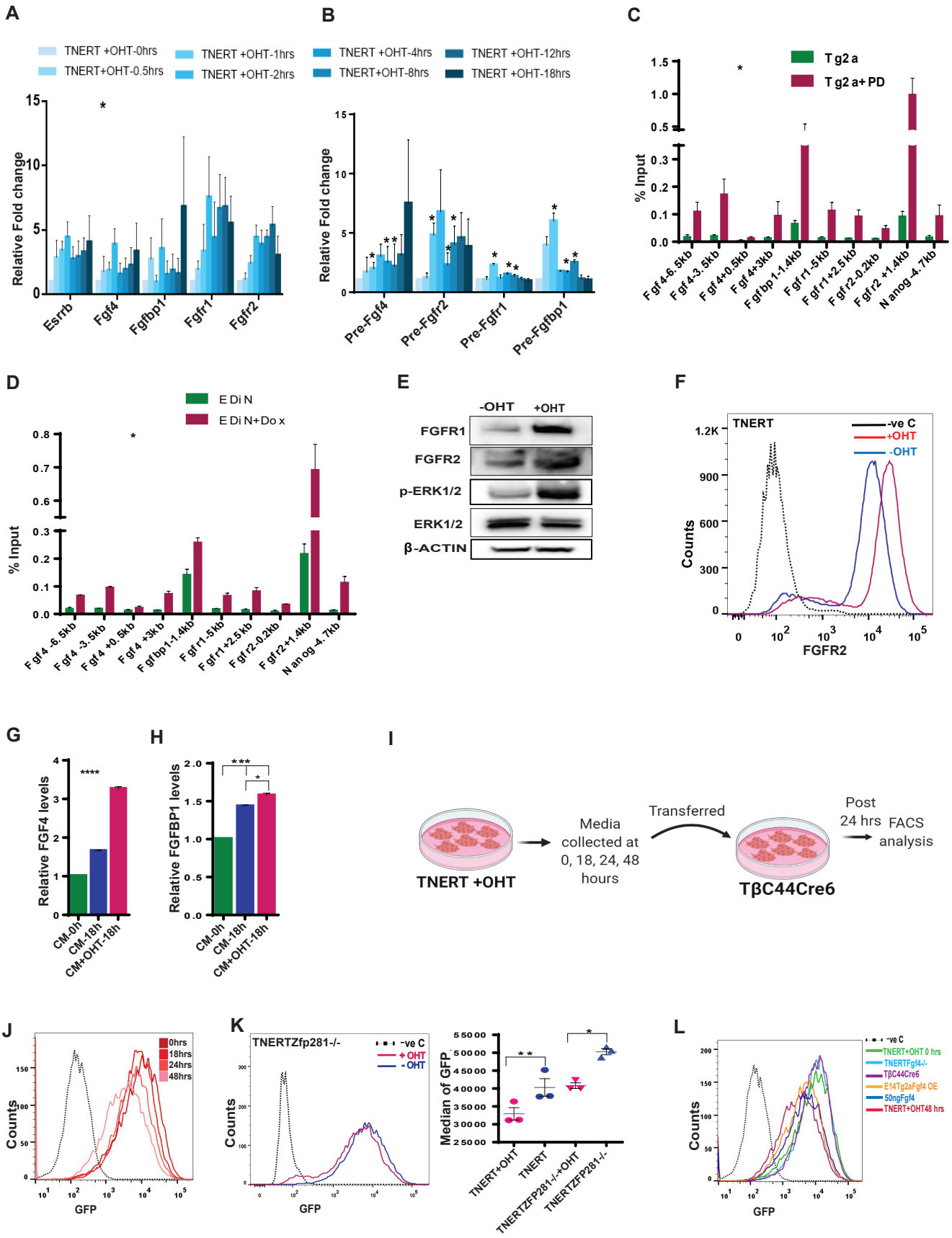

A
TNERT +OHT-0hrs
TNERT+OHT-0.5hrs
TNERT +OHT-1hrs
TNERT +OHT-2hrs
Relative Fold change
Esrrb
Fgf4
Fgfbp1
Fgfr1
Fgfr2
B
TNERT +OHT-4hrs
TNERT+OHT-8hrs
TNERT +OHT-12hrs
TNERT +OHT-18hrs
Relative Fold change
Pre-Fgf4
Pre-Fgfr2
Pre-Fgfr1
Pre-Fgfbp1
C
Tg2a
Tg2a+PD
% Input
Fgf4-6.5kb
Fgf4-3.5kb
Fgf4+0.5kb
Fgf4+3kb
Fgfbp1-1.4kb
Fgfr1-5kb
Fgfr1+2.5kb
Fgfr2-0.2kb
Fgfr2+1.4kb
Nanog-4.7kb
D
EDiN
EDiN+Dox
% Input
Fgf4-6.5kb
Fgf4-3.5kb
Fgf4+0.5kb
Fgf4+3kb
Fgfbp1-1.4kb
Fgfr1-5kb
Fgfr1+2.5kb
Fgfr2-0.2kb
Fgfr2+1.4kb
Nanog-4.7kb
E
-OHT
+OHT
FGFR1
FGFR2
p-ERK1/2
ERK1/2
β-ACTIN
F
TNERT
-ve C
+OHT
-OHT
Counts
FGFR2
G
Relative FGF4 levels
CM-0h
CM-18h
CM+OHT-18h
H
Relative FGFBP1 levels
CM-0h
CM-18h
CM+OHT-18h
I
TNERT +OHT
Media collected at 0, 18, 24, 48 hours
Transferred
TβC44Cre6
Post 24 hrs
FACS analysis
J
0hrs
18hrs
24hrs
48hrs
Counts
GFP
K
TNERTZfp281-/-
-ve C
+OHT
-OHT
Counts
GFP
Median of GFP
TNERT+OHT
TNERT
TNERTZFP281-/-+OHT
TNERTZFP281-/-
L
-ve C
TNERT+OHT 0 hrs
TNERTFgf4-/-
TβC44Cre6
E14Tg2aFgf4 OE
50ngFgf4
TNERT+OHT48 hrs
Counts
GFP

Figure 4

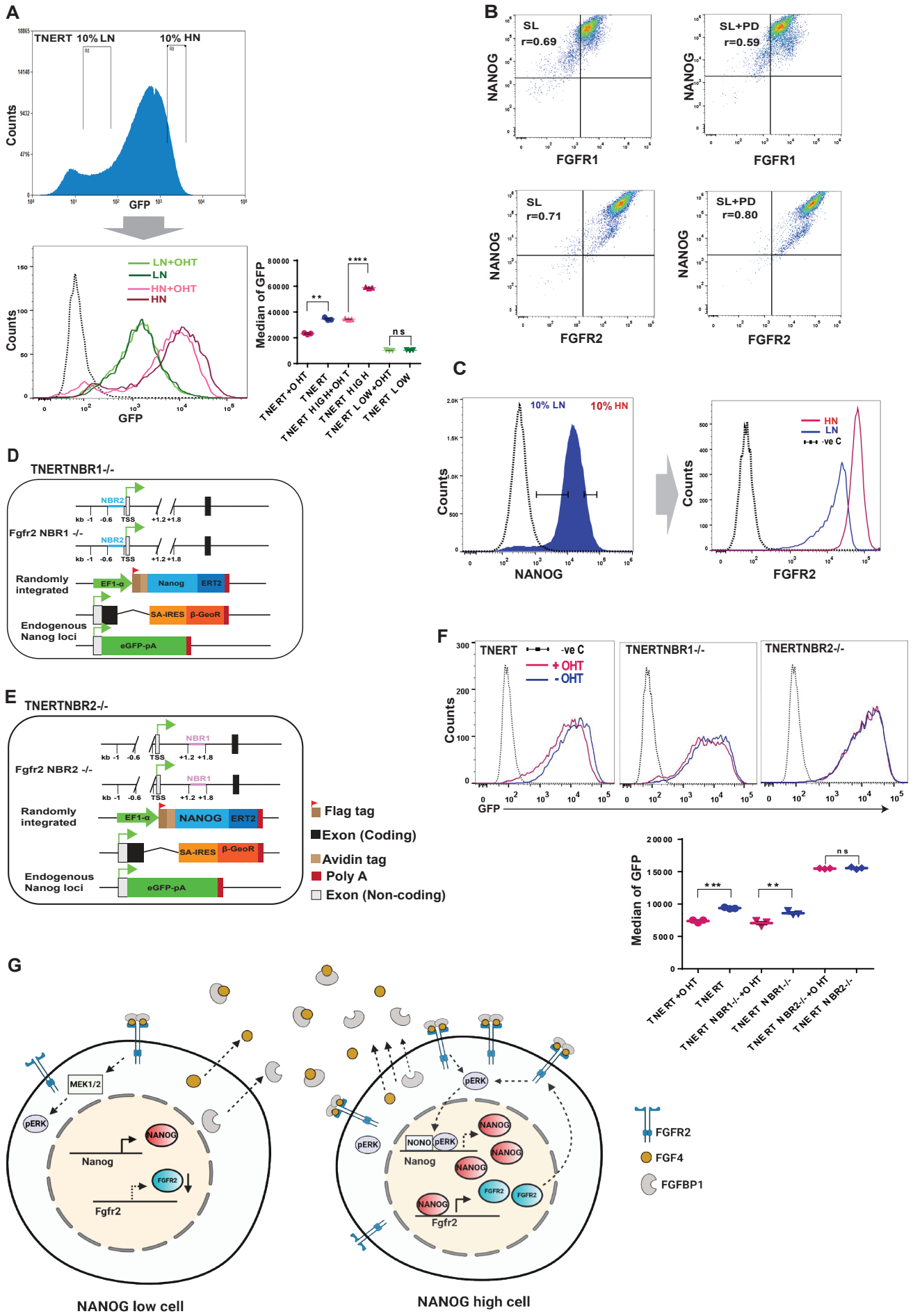
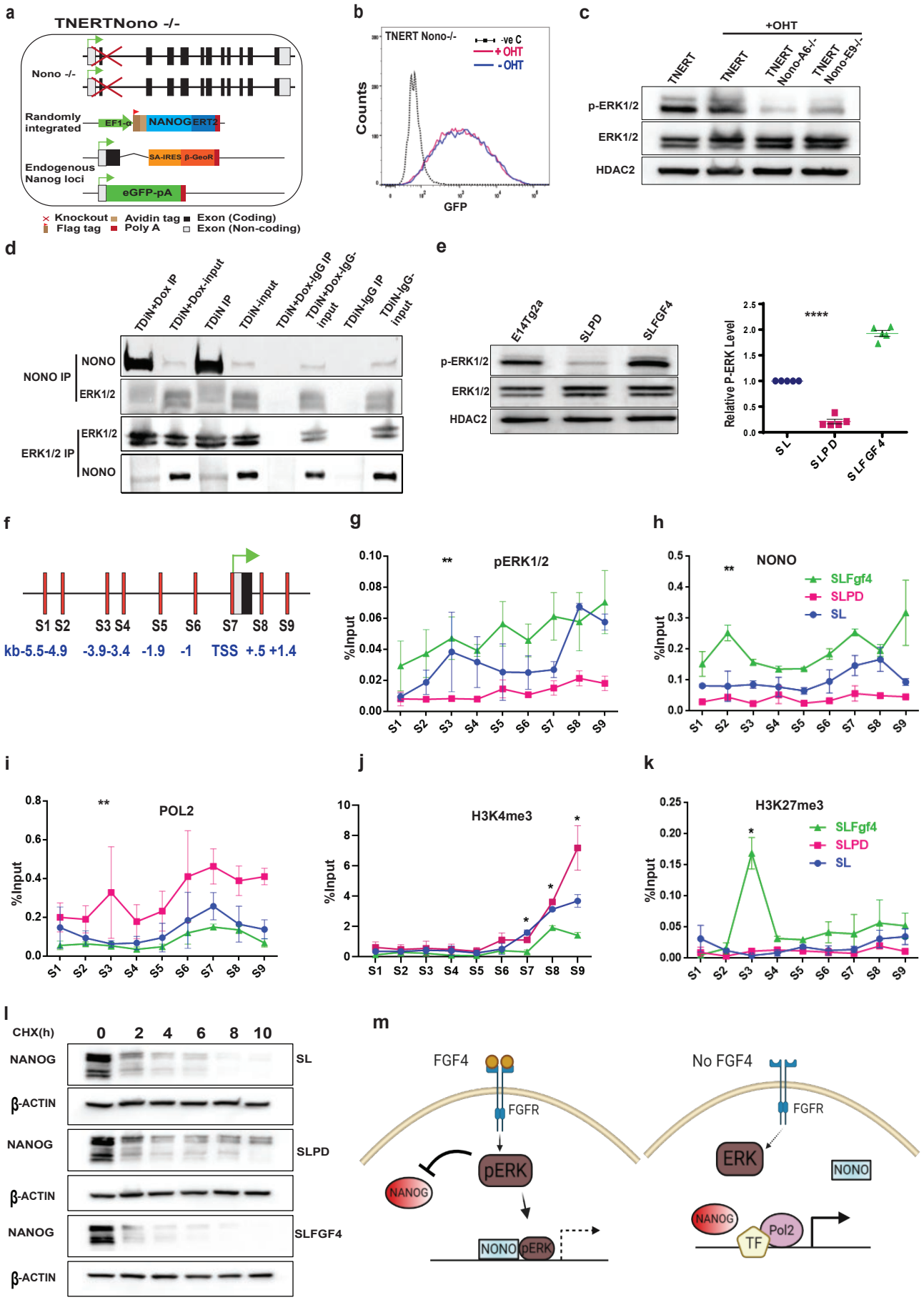

A
TNERT 10% LN
10% HN
Counts
GFP
LN+OHT
LN
HN+OHT
HN
Median of GFP
TNERT+OHT
TNERT
TNERT HIGH+OHT
TNERT HIGH
TNERT LOW+OHT
TNERT LOW
B
SL
r=0.69
SL+PD
r=0.59
SL
r=0.71
SL+PD
r=0.80
NANOG
FGFR1
FGFR2
C
10% LN
10% HN
HN
LN
-ve C
D
TNERTNBR1-/-
NBR2
Fgfr2 NBR1 -/-
Randomly integrated
EF1-α
Nanog
ERT2
SA-IRES
β-GeoR
Endogenous Nanog loci
eGFP-pA
E
TNERTNBR2-/-
NBR1
Fgfr2 NBR2 -/-
NANOG
Flag tag
Exon (Coding)
Avidin tag
Poly A
Exon (Non-coding)
F
TNERT
TNERTNBR1-/-
TNERTNBR2-/-
+OHT
-OHT
TNERT+OHT
TNERT
TNERT NBR1-/-+OHT
TNERT NBR1-/-
TNERT NBR2-/-+OHT
TNERT NBR2-/-
G
MEK1/2
pERK
NANOG
Nanog
FGFR2
Fgfr2
NONO
FGFBP1
NANOG low cell
NANOG high cell

Figure 5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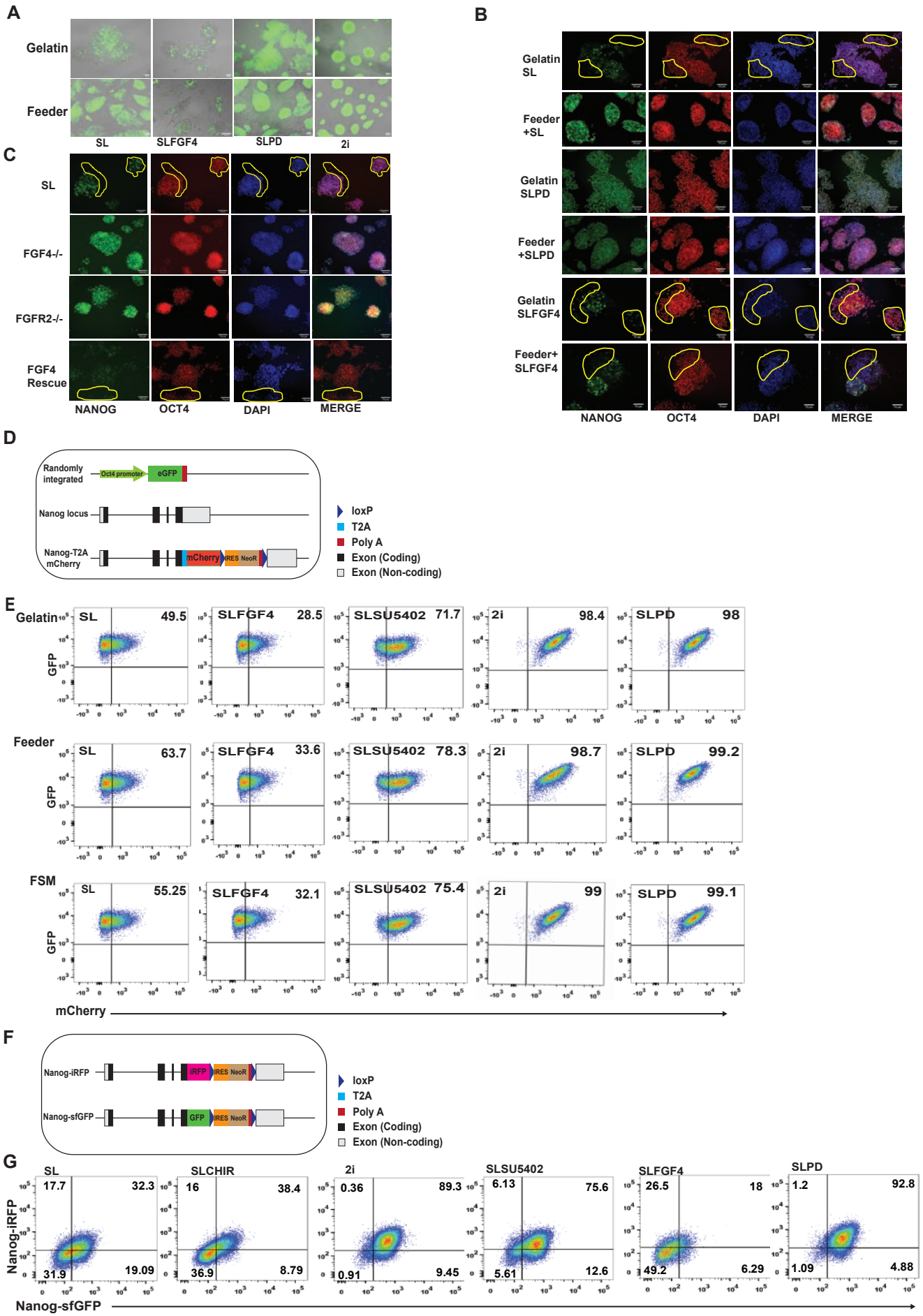
TNERTNono -/-
Nono -/-
Randomly integrated
EF1-α
NANOG
ERT2
Endogenous Nanog loci
SA-IRES
β-GeoR
eGFP-pA
Knockout
Avidin tag
Exon (Coding)
Flag tag
Poly A
Exon (Non-coding)
b
TNERT Nono-/-
-ve C
+ OHT
- OHT
Counts
GFP
c
+OHT
TNERT
TNERT
TNERT Nono-A6-/-
TNERT Nono-E9-/-
p-ERK1/2
ERK1/2
HDAC2
d
TDiN+Dox IP
TDiN+Dox-input
TDiN IP
TDiN-input
TDiN+Dox-IgG IP
TDiN+Dox-IgG-input
TDiN-IgG IP
TDiN-IgG-input
NONO IP
NONO
ERK1/2
ERK1/2 IP
ERK1/2
NONO
e
E14Tg2a
SLPD
SLFGF4
p-ERK1/2
ERK1/2
HDAC2
Relative P-ERK Level
****
SL
SLPD
SLFGF4
f
S1 S2 S3 S4 S5 S6 S7 S8 S9
kb-5.5-4.9 -3.9-3.4 -1.9 -1 TSS +.5 +1.4
g
pERK1/2
%Input
**
h
NONO
SLFgf4
SLPD
SL
i
POL2
j
H3K4me3
k
H3K27me3
l
CHX(h)
0 2 4 6 8 10
NANOG
β-ACTIN
SL
SLPD
SLFGF4
m
FGF4
FGFR
pERK
NANOG
NONO
pERK
No FGF4
FGFR
ERK
NONO
NANOG
TF
Pol2

Figure 6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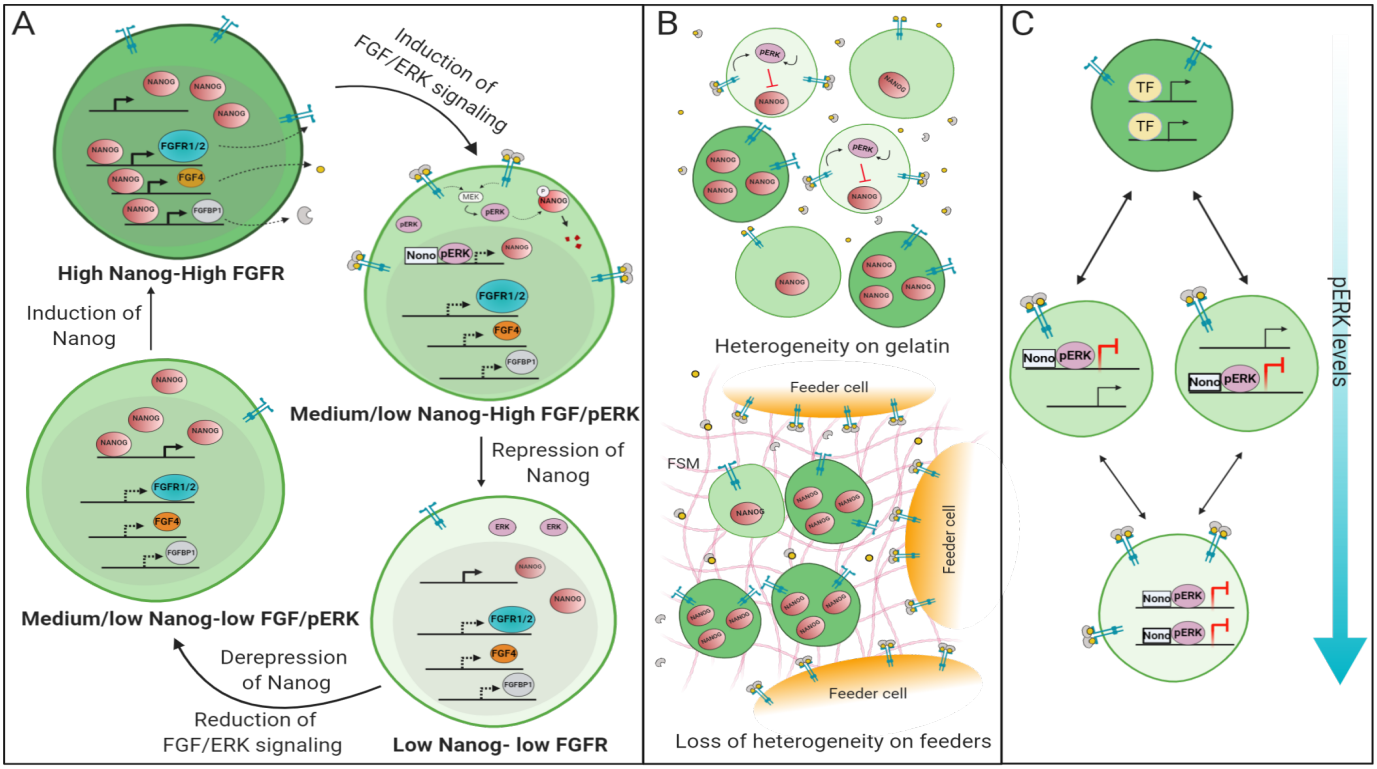
Feeder
SL
SLFGF4
SLPD
2i
B
Gelatin SL
Feeder +SL
Gelatin SLPD
Feeder +SLPD
Gelatin SLFGF4
Feeder+ SLFGF4
NANOG
OCT4
DAPI
MERGE
C
SL
FGF4-/-
FGFR2-/-
FGF4 Rescue
NANOG
OCT4
DAPI
MERGE
D
Randomly integrated
Oct4 promoter
eGFP
Nanog locus
Nanog-T2A mCherry
mCherry
IRES NeoR
loxP
T2A
Poly A
Exon (Coding)
Exon (Non-coding)
E
Gelatin
SL 49.5
SLFGF4 28.5
SLSU5402 71.7
2i 98.4
SLPD 98
Feeder
SL 63.7
SLFGF4 33.6
SLSU5402 78.3
2i 98.7
SLPD 99.2
FSM
SL 55.25
SLFGF4 32.1
SLSU5402 75.4
2i 99
SLPD 99.1
GFP
mCherry
F
Nanog-iRFP
iRFP
IRES NeoR
Nanog-sfGFP
GFP
loxP
T2A
Poly A
Exon (Coding)
Exon (Non-coding)
G
SL
17.7
32.3
31.9
19.09
SLCHIR
16
38.4
36.9
8.79
2i
0.36
89.3
0.91
9.45
SLSU5402
6.13
75.6
5.61
12.6
SLFGF4
26.5
18
49.2
6.29
SLPD
1.2
92.8
1.09
4.88
Nanog-iRFP
Nanog-sfGFP

Figure 7


A
Induction of FGF/ERK signaling
High Nanog-High FGFR
Induction of Nanog
Medium/low Nanog-High FGF/pERK
Repression of Nanog
Medium/low Nanog-low FGF/pERK
Derepression of Nanog
Reduction of FGF/ERK signaling
Low Nanog- low FGFR
B
Heterogeneity on gelatin
Feeder cell
FSM
Feeder cell
Feeder cell
Loss of heterogeneity on feeders
C
TF
TF
pERK levels