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Idax and Rinf facilitate expression of Tet enzymes to promote neural and suppress trophectodermal programs during differentiation of embryonic stem cells

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Abstract

The Inhibitor of disheveled and axin (Idax) and its ortholog the Retinoid inducible nuclear factor (Rinf) are DNA binding proteins with nuclear and cytoplasmic functions. Rinf is expressed in embryonic stem cells (ESCs) where it regulates transcription of the Tet-eleven translocation (Tet) enzymes, promoting neural and suppressing mesendoderm/trophectoderm differentiation. Here, we find that Idax, which is not expressed in ESCs, is induced upon differentiation. Like Rinf, Idax facilitates neural and silences trophectodermal programs. Individual or combined loss of Idax and Rinf led to downregulation of neural and upregulation of trophectoderm markers during differentiation of ESCs to embryoid bodies as well as during directed differentiation of ESCs to neural progenitors cells (NPCs) and trophoblast-like cells. These defects reminisce those of Tet-deficient ESCs. Consistently, Tet genes are direct targets of Idax and Rinf, and loss of Idax and Rinf led to downregulation of Tet enzymes during ESCs differentiation to NPCs and trophoblast-like cells. While Idax and Rinf single and double knockout (DKO) mice were viable and overtly normal, DKO embryos had reduced expression of several NPC markers in embryonic forebrains and deregulated expression of selected trophoblast markers in placentas. NPCs derived from DKO forebrains had reduced self-renewal while DKO placentas had increased junctional zone and reduced labyrinth layers. Together, our findings establish Idax and Rinf as regulators of Tet enzymes for proper differentiation of ESCs.

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Keywords

Idax; Rinf; Tet enzymes; ESC; differentiation; NPC; Trophoblast

1. INTRODUCTION

Embryonic stem cell (ESC) differentiation programs are tightly regulated by epigenetic regulation of gene expression. Epigenetic mechanisms involving DNA methylation and histone modifications ensure proper activation and silencing of master regulators of lineage specification upon differentiation (Jaenisch and Young, 2008). The Ten eleven translocation (Tet) family of dioxygenases (Tet1/2/3) promotes DNA demethylation by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and other derivatives (Pastor et al., 2013; Tahiliani et al., 2009). They can also partner with chromatin regulatory complexes such as PRC2 and Sin3a to promote gene silencing in a catalytic independent manner (Chrysanthou et al., 2022; Williams et al., 2011; Wu et al., 2011). Tet enzymes play critical roles in regulation of pluripotency and lineage specific genes in ESCs and during differentiation. Tet1 and Tet2 are expressed in ESCs and Tet3 is induced upon differentiation. Loss of Tet enzymes leads to aberrant skewed differentiation towards mesendoderm and trophectoderm lineages at the expense of neuroectoderm (Dawlaty et al., 2014; Koh et al., 2011). Consistently, proper expression of neuroectodermal genes and silencing of mesendodermal genes are mediated by the dual functions of Tet enzymes in DNA demethylation and in recruitment of repressive complexes to the chromatin (Chrysanthou et al., 2022; Pastor et al., 2013; Wu et al., 2011). Therefore, proper expression of Tet enzymes and their recruitment to their target genes are essential for gene regulation during differentiation.

Tet1 and Tet3 contain a CXXC DNA binding domain that binds to CpGs and facilitates their targeting to the chromatin (Liu et al., 2013; Pastor et al., 2013). The CXXC domain of Tet2 is believed to have undergone an evolutionary gene inversion giving rise to an independent gene *CXXC4* or *Idax* (Inhibitor of disheveled and axin) which is closely similar to a related gene *CXXC5* or *Rinf* (Retinoid inducible nuclear factor) (Ko et al., 2013; Liu et al., 2013). Rinf and Idax are small 30 kDa proteins that have very similar gene structures and domains. They are found both in the cytoplasm and in the nucleus of various cell types. In the cytoplasm, they negatively regulate Wnt signaling by binding to Disheveled (Dvl) and have biological roles in hematopoiesis, neurogenesis, wound healing and cancer (Kim et al., 2010; Kim et al., 2016; Lee et al., 2015; Pendino et al., 2009). In the nucleus, Rinf and Idax have been implicated in facilitating transcription in various cell types (Kim et al., 2014; Kim et al., 2016; Li et al., 2014; Ma et al., 2017) as well as negatively regulating Parp-mediated degradation of Tet2 (Ko et al., 2013).

Previously, we have shown that Rinf, but not Idax, is expressed in ESCs where it regulates transcription of pluripotency network genes and Tet enzymes, and is important for proper differentiation of ESCs (Ravichandran et al., 2019). In ESCs, Rinf is mainly present in the nucleus where its occupancy overlaps with those of Tet1, Tet2 and several key pluripotency factors. Rinf interacts with Tet1 and Tet2 as well as with the pluripotency

factors Nanog and Oct4 to facilitate their recruitment to promoters and enhancers of pluripotency and Tet genes, positively regulating their transcription. Consistently, Rinf-deficient ESCs have reduced expression of pluripotency genes and Tet enzymes, leading to restricted neuroectoderm and enhanced mesendoderm/trophectoderm differentiation. Here, we find that Idax, along with Rinf, is induced upon ESC differentiation. Given its close gene structure and domain similarity to Rinf, we sought to investigate if Idax and Rinf have analogous or compensatory regulatory functions during ESC differentiation. Using Rinf and Idax single and double knockout (DKO) ESCs and mice, we show that Idax, like Rinf, activates neural and silences trophectodermal programs. We found that individual or combined loss of Idax and Rinf led to downregulation of Tet enzymes during differentiation of ESCs to neural progenitor cells (NPCs) and to trophoblast-like cells. This resulted in restricting differentiation to neural cell types and enhancing differentiation to trophoblast-like cells. While Idax and Rinf single and DKO mice were viable and grossly normal, DKO embryos exhibited subtle defects in NPCs and abnormal placental morphologies. Together, our findings establish Idax, alongside Rinf, as regulator of Tet enzymes for enhancing neural differentiation and suppressing trophectodermal programs during differentiation of ESCs.

2. MATERIALS & METHODS

2.1. Generation of Idax and Rinf single and double knockout (DKO) ESCs and mice

Rinf$^{-/-}$ ESCs (V6.5 mixed 129/B6 background, male) were previously generated in our lab (Ravichandran et al., 2019). *Idax*$^{-/-}$ and DKO ESCs were generated by CRISPR/Cas9-mediated gene editing in wild type or *Rinf*$^{-/-}$ V6.5 mouse ESC (mixed 129/B6 background, male). Two pX330 vectors expressing Cas9 and gRNAs flanking exon 3 of Idax were used for gene editing following previous protocols (Wang et al., 2013). Targeted clones were screened by PCR using primers listed in Table S1. Loss of Rinf and Idax was confirmed at mRNA level by RT-qPCR in ESCs differentiated to EBs. Rinf and Idax targeted clones were injected into wild type blastocysts at the Einstein Transgenics Core to generate chimeric mice. Chimeras were bred to wild type B6 mice to generate heterozygote mice which were subsequently intercrossed to generate homozygous knockout mice. *Rinf*$^{-/-}$ and *Idax*$^{-/-}$ were intercrossed to generate double heterozygous (DHET) mice, which were further intercrossed to generate DKO embryos and mice. Mice were genotyped using DNA isolated from snippet of tails and primers described in Table S1. Mice were maintained on a mixed 129/B6 background on a 12/12 hour light/dark cycle. All mouse studies were performed in accordance with our IACUC approved protocols overseen by the Institute for Animal Studies of Albert Einstein College of Medicine.

2.2. Culture of mouse ESCs and differentiation to EBs

Three independent ESC lines of each genotype were cultured on irradiated feeders in standard ESC media with serum/LIF. For RNA and DNA extraction, ESCs were pre-plated on 0.2% gelatin-coated plates to remove feeders and then cultured on gelatin-coated plates overnight before harvesting for RNA and DNA extraction. To differentiate ESCs to embryoid bodies (EBs), ESCs were pre-plated, cultured in media without LIF in hanging drops for 3 days and then on non-adherent plastic surface for 3 days. EBs were collected on day 6 for analyses. Stable DKO ESCs were generated by transfection with PiggyBac-

hygro vectors expressing wild type and mutant Idax or Rinf transgenes (listed in Table S2) using Xfect mESC transfection reagent (Clontech) followed by 10-day selection with hygromycin (125ug/mL). Protein expression in stable lines were confirmed by western blot using primary antibodies (V5, CST 13202S, 1:1000; HA, Biolegend 901601, 1:1000; Actin, AC-15, Abcam, 1:40,000) and secondary antibodies (HRP-anti mouse, CalBiochem 401253, 1:5000; HRP-anti rabbit, CalBiochem 401393, 1:5000) following our previously used protocols (Chrysanthou et al., 2022; Ravichandran et al., 2019).

2.3. In vitro differentiation to NPCs and to trophoblast-like cells

ESCs were differentiated to NPCs following our previously used methods (Ravichandran et al., 2019). Briefly, ESCs were differentiated to EBs, transferred to tissue culture plates to attach and cultured in ITSFn media for 8 days. NPCs were propagated on poly-D-ornithine and laminin coated plates using N2 media containing Laminin (1μg/ml), bFGF (5 ng/ml) and EGF (20 ng/ml). ESCs were differentiated to trophoblast-like cells following previously used methods (Chrysanthou et al., 2018). Briefly, ESCs were cultured for 3 days on gelatin in TSC media, which was composed of 70% pre-conditioned media on MEFs, 30% TS base media (20% FBS, 1mM sodium pyruvate, 50uM β-mercaptoethanol, 1x PenStrep in RPMI 1640), 1 ug/ml heparin, 25 ng/ml FGF. Three independent ESC lines of each genotype were used in both differentiation assays.

2.4. Gene expression profiling by RNA-seq and ChIP-seq data analysis

Total RNA was extracted from day 6 EBs of each genotype (one line of each genotype) using Omega E.Z.N.A Total RNA kit and subjected to mRNA-seq at Novogene using their Illumina Novoseq 6000 platform, which generated ~30 million reads per sample. Adaptors were trimmed with trim galore (v 0.6.5) and clean reads were mapped to mouse genome (mm10) by STAR software (v 2.7.3a) (Dobin et al., 2013) with default parameters. Mapped reads were assigned to genes using featureCounts (Liao et al., 2014) with --largestOverlap parameter. Differential expression analysis was performed on raw counts by edgeR software(Robinson et al., 2010). A p-adjusted value of < 0.05, and a log2 fold-change of > 1 between wild type and $Idax^{-/-}$ or $Rinf^{-/-}$ or DKO samples was used as criteria to identify differentially expressed genes (DEGs). DEGs were subjected to GO and KEGG pathway analysis using DAVID (https://david.ncifcrf.gov/tools.jsp). RNA-seq data sets have been deposited in the Gene Expression Omnibus (GEO) database (Accession GSE191241). Previously published Myc-tagged Idax ChIP-seq in HEK293 cells (Ko et al., 2013), Rinf ChIP-seq in mouse ESCs (Ravichandran et al., 2019), Flag-Tet1 CUT&Tag in mouse ESC (Chrysanthou et al., 2022), Flag-Tet2 ChIP-seq in mouse ESCs (Rasmussen et al., 2019), and V5-Tet1 ChIP-seq in human ESCs (Dixon et al., 2021) were visualized by Integrative Genomics Viewer (IGV) v 2.5.0 to assess enrichment of Idax, Rinf and Tet enzymes at genes. Empty vector (EV) track for IDAX and Rinf knockout (KO) track for Rinf ChIP-seq were used as negative controls. For Myc-Idax, V5-Tet1 and Flag-Tet2 ChIP-seq, raw data was downloaded from GEO, trimmed using trim galore and mapped to human or mouse genome using Bowtie2 (v 2.3.5.1). Samtools (v 1.9) was used to sort, index and balance mapped reads to sample with lowest counts. Bam files were removed of duplicates with Picard tools (v 2.3.0). For visualization on IGV, balanced bam files

were converted to bigwig files using deepTools (v 3.1.0) function bamCoverage with option --binSize 10.

2.5. RT-qPCR

1.5μg RNA extracted from feeder-free ESCs (three independent lines of each genotype), day-6 EBs, NPCs, trophoblast-like cells or embryo tissues (Omega E.Z.N.A Total RNA kit) was subjected to cDNA synthesis (Superscript III First-Strand synthesis system, Invitrogen). Real time quantitative PCR using SYBR green master mix (Applied Biosystems) was performed in QuantStudio 6 Flex Real-Time PCR system using primers listed in Table S1. Comparative ct method was used to calculate relative gene expression level and data was normalized to *Gapdh*. One-way-ANOVA test and GraphPad Prism 8 software was used for calculating statistical significance.

2.6. Teratoma formation assay

Cultured mouse ESCs (1.5×10^6) were subcutaneously injected into SCID mice (Taconic) following standard procedures (Dawlaty et al., 2014). After six weeks mice were euthanized to harvest tumors, which were fixed in formalin for 48hrs and then imbedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for histological analysis.

2.7. Analyses of E12.5 placentas and NPCs

Adult double heterozygous mice were time-mated and pregnant mice were sacrificed at E12.5. Embryos and placentas were harvested for genotyping and downstream analyses. To isolate NPCs, telencephalic neural tissue was isolated, digested in trypsin (0.05% trypsin with 0.02% EDTA and 0.2% BSA) at 37°C for 10min, quenched with trypsin inhibitor and centrifuged for 5min 300g. Cells were washed with PBS and then counted and 50,000 cells/ml were seeded on poly-D- ornithine and laminin-coated plates in N2 media (bFGF 5ng/ml, EGF 20 ng/ml, laminin 1 μg/ml, and heparin 2 μg/ml). For neurosphere assay, cells were seeded at a density of 5 cells/well in ultra-low attachment 6-well plates (Corning 3471) in N2 media (bFGF 5ng/ml, EGF 20 ng/ml, laminin 1 μg/ml, and heparin 2 μg/ml) for 6 days (37°C, 5% CO_2, humidified incubator). Both the number of spheres formed per well and the number of living cells (trypan blue negative) was counted. For secondary neurosphere formation, cells were reseeded (5 cells/well) as above. At least three biological replicates were used in all analyses. One-way-ANOVA test and GraphPad Prism 8 software was used for calculating statistical significance.

3. RESULTS

3.1. Idax and Rinf are induced upon ESC differentiation and their loss leads to down regulation of neural and up regulation of trophectodermal gene expression programs

Rinf, but not Idax, is expressed in mouse ESCs (Ravichandran et al., 2019). To examine if Idax is expressed upon differentiation, we differentiated wild type ESCs to embryoid bodies (EBs) and quantified mRNA expression of *Idax* and *Rinf* by RT-qPCR. We found a robust induction of both *Idax* and *Rinf* in day 6 EBs (Fig. 1A). To establish the molecular and biological significance of Rinf and Idax during ESC differentiation, we generated Idax and Rinf single and double knockout (DKO) ESCs. *Rinf*$^{-/-}$ ESCs were previously generated

by deleting exon 2 of *Rinf (Ravichandran et al., 2019). Idax*$^{-/-}$ ESCs were generated by deleting exon 3 of *Idax* using two gRNAs flanking it (Fig. S1A). *Idax*$^{-/-}$*;Rinf*$^{-/-}$ ESCs were generated by deleting exon 3 of *Idax* in *Rinf*$^{-/-}$ ESCs. The deleted exons encode a major portion of each protein and their loss completely abolished Idax and Rinf expression. We confirmed genotypes of properly targeted single and DKO ESC lines by PCR (Fig. S1B) and validated the complete loss of *Idax* and *Rinf* mRNA by RT-qPCR in EBs derived from these ESCs (Fig. S1C). We also noted that the loss of either Rinf or Idax did not lead to an induction of the other (Fig. S1C).

To examine how loss of Rinf and Idax affects gene expression programs of ESCs during differentiation, we compared the transcriptomes of single and double knockout EBs to wild type EBs by RNA-seq (Fig. 1B). We found 809 genes that were significantly deregulated (505 up and 304 down) in DKO EBs in contrast to 41 (32 up and 9 down) in *Idax*$^{-/-}$ and 58 genes (40 up and 18 down) in *Rinf*$^{-/-}$ EBs. Gene Ontology analysis revealed that the differentially expressed genes (DEGs) in DKO EBs that were downregulated were enriched in neurodevelopmental processes and those that were upregulated were enriched in vasculature formation (Fig.S1D). The downregulated DEGs included many regulators and markers of neurogenesis including *Sox2, Nestin,* and *Olig1*, which were expressed at comparably low levels in single and double knockout EBs (Fig. 1C). The neural marker *Pax6* was reduced only in DKO EBs, while the neuronal marker *Tuj1* and the astrocyte marker *Gfap* were not significantly changed (Fig. 1C). The upregulated DEGs included several regulators and markers of placenta development and trophoblast cells including *Tpbpa*, *Plac1, Plf, Eomes and Krt7*, which were expressed at significantly higher levels in *Rinf*$^{-/-}$ EBs and at much higher levels in DKO EBs (Fig. 1D). *Cdx2* was equally upregulated in both *Rinf*$^{-/-}$ and DKO EBs while *Tfeb* and *Pl1* were upregulated only in DKO EBs (Fig. 1D). This indicates that Rinf and Idax facilitate expression of key neural regulators and suppress expression of trophectodermal genes during ESC differentiation.

3.2. Individual or combined loss of Idax and Rinf in ESCs restricts neural and induces trophectoderm differentiation

To examine if loss of Idax and Rinf influences lineage specification along the neural and trophectoderm lineages, we differentiated *Idax*$^{-/-}$, *Rinf*$^{-/-}$ and DKO ESCs to neural progenitor cells (NPCs) and to trophoblast-like cells. We found that both Idax and Rinf were upregulated during differentiation of wild type ESCs to NPCs (Fig. 2A). Individual or combined loss of Idax and Rinf led to profoundly reduced expression of NPC markers *Nestin*, *Sox2 and Pax6*. We also found reduced expression of the oligodendrocyte marker *Olig1* and neuronal marker *Tuj1* in single and DKO NPCs and increased expression of the astrocyte marker *Gfap* in DKO NPCs. This suggests a deregulation of NPC programs in the absence of Idax and Rinf. Likewise, we found that both Idax and Rinf were upregulated during differentiation of wild type ESCs to trophoblast-like cells (Fig. 2B). Individual or combined loss of Idax and Rinf led to robust upregulation of trophectoderm markers *Eomes*, *Cdx2 and Tpbpa.* Several trophoblast markers such as *Krt7*, *Plac1* and *Tfeb* were upregulated in *Rinf*$^{-/-}$ and DKO trophoblast-like cells while *Plf* and *Pl1* were upregulated only in DKO trophoblast-like cells. Together, these findings suggest that Idax and Rinf

facilitate neural and inhibit trophoblast differentiation, and their individual or combined loss restricts ESC differentiation to NPCs and induces a trophoblast-like state.

To examine if loss of Idax and Rinf affects pluripotency of ESCs, we subjected *Idax*$^{-/-}$, *Rinf*$^{-/-}$ and DKO ESCs to a teratoma formation assay. While single and DKO ESCs were pluripotent and formed teratomas containing structures of all three germ layers, teratomas derived from DKO ESCs were hemorrhagic and contained trophoblast giant cells (Fig. S2A–B). This is indicative of aberrant and skewed differentiation towards the trophoblast lineage, consistent with the observations in EBs and trophoblast-like cells derived from single and DKO ESCs.

Next, we tested if the DNA binding function of Idax and Rinf, which is mediated through their CXXC domains, is involved in proper ESC differentiation. We overexpressed wild type or DNA binding mutant forms of Idax and Rinf in DKO ESCs followed by differentiation to EBs (Fig. 2C and Fig S2C). These mutants have defined amino acid substitutions or deletions in the CXXC domain that are previously shown to completely abolish binding of Idax and Rinf to DNA (Kim et al., 2014; Ko et al., 2013). We found that wild type Idax or Rinf transgenes, but not their DNA binding mutant forms, boosted neural and prevented aberrant trophoblast differentiation in DKO EBs. This is evident from the enhanced expression of neural markers *Nestin* and *Sox2* and diminished expression of trophoblast markers *Plac1* and *Tpbpa* (Fig. 2C). This suggests that the DNA binding function of Idax and Rinf contributes to proper regulation of ESC differentiation.

3.3. Deficiency of Rinf and Idax leads to deregulation of Tet enzymes during directed differentiation of ESCs to NPCs and trophoblast-like cells

The transcriptomic deregulation (i.e. downregulation of neural and upregulation of trophectodermal markers) and differentiation defects (i.e. restricted differentiation to NPC, induced differentiation to trophoblast, and formation of hemorrhagic teratomas) observed in Idax and Rinf deficient ESCs reminisce those in Tet deficient ESCs, where loss of Tet enzymes promotes mesendoderm/trophectoderm differentiation at the expense of neuroectoderm (Chrysanthou et al., 2022; Dawlaty et al., 2014; Dawlaty et al., 2013; Dawlaty et al., 2011; Koh et al., 2011). This promoted us to examine if individual or combined loss of Idax and Rinf deregulates Tet enzymes during differentiation. To this end, we quantified the expression of Tet1/2/3 during differentiation of *Idax*$^{-/-}$, *Rinf*$^{-/-}$ and DKO ESCs to NPCs, trophoblast-like cells and EBs. We found robust down regulation of all three *Tet* genes in single and DKO NPCs (Fig. 3A). Likewise, *Tet1* and *Tet2* were significantly down regulated while *Tet3* was upregulated in single and DKO trophoblast-like cells (Fig. 3B). During differentiation to EBs, we found *Tet1* and *Tet2* to be significantly reduced only in DKO EBs (Fig. S3A). Re-expression of wild type Idax or Rinf, but not their DNA binding mutant forms, boosted *Tet1/2* expression in DKO EBs (Fig. S3B) suggesting that Idax and Rinf DNA binding is important for regulation of Tet enzymes. Genome browser tracks of previously published Myc-tagged Idax ChIP-seq in HEK293 cells (Ko et al., 2013) and Rinf ChIP-seq in ESCs (Ravichandran et al., 2019) revealed prominent enrichment of Idax and Rinf at all three *Tet* genes as well as at selected regulators of neural and trophectoderm lineages (i.e. *Pax6*, *Eomes, Cdx2*) (Fig. 3C). Together, these findings

suggest that Idax and Rinf are enriched at Tet and lineage genes to facilitate their expression during differentiation (Fig 3D). Moreover, genome browser tracks of published Flag-Tet1 and Flag-Tet2 occupancy in mouse ESCs (Chrysanthou et al., 2022; Rasmussen et al., 2019) and V5-Tet1 occupancy in human ESCs (Dixon et al., 2021) revealed that Rinf and Idax peaks overlap with Tet1 and Tet2 peaks at several lineage genes (Fig. S3C). This suggests that Rinf and Idax may work together with Tet enzymes in regulating lineage genes during differentiation. For example, they may facilitate Tet recruitment to lineage genes, just as Rinf mediates Tet recruitment to pluripotency genes in ESCs (Ravichandran et al., 2019).

3.4. Individual or combined loss of Idax and Rinf in mice is compatible with development but leads to subtle defects in embryonic NPCs and formation of placental layers

To define the biological significance of Idax and Rinf in development, we generated *Idax*$^{-/-}$ and *Rinf*$^{-/-}$ mice, which were viable and fertile. We intercrossed them to generate double heterozygous mice, which were then bred to generate DKO mice. Single and DKO embryos and mice were produced at the expected mendelian frequency and were grossly normal (Fig. S4A–B). To examine if the restricted *in vitro* differentiation to NPCs observed in single and DKO ESCs has developmental implications, we analyzed forebrains and NPCs from single and DKO embryos (Fig. 4A). We found that the expression of NPC markers *Pax6, Sox2* and *Nestin* were significantly reduced in embryonic forebrains of DKO embryos (Fig. 4B) while expression of neural differentiation makers *Olig1*, *Tuj1* and *Gfap* were not changed (Fig. 4SC). Interestingly, NPCs derived from embryonic forebrains of DKO embryos expressed reduced levels *Pax6, Sox2 and Nestin* as well as neuronal marker *Tuj1* and oligodendrocyte marker *Olig1*. Astrocyte maker *Gfap* was upregulated in both single and DKO NPCs (Fig. 4C). We subjected the NPCs to neurosphere formation assay and found that *Rinf*$^{-/-}$ and DKO NPCs have reduced primary and secondary neurosphere forming capacity (Fig. 4D). These findings suggest that Idax and Rinf, though not required for embryonic neurogenesis, have subtle effects on proper expression of NPC markers and properties *in vivo*.

To examine if the induced *in vitro* differentiation to trophoblast-like cells observed in single and DKO ESCs has developmental implications, we analyzed placentas from single and DKO embryos (Fig. 4E). We found that the expression of trophoblast giant cell markers *Pl1* and *Plf* were significantly upregulated in DKO placentas (Fig. 4F). However, other trophectoderm markers that were induced in single and DKO EBs and *in vitro* derived trophoblast-like cells (i.e. *Tpbpa, Cdx2, Eomes, Tfeb and Krt7*) were not significantly changed (Fig. S4D). Histological analysis of single and DKO placentas revealed that while placentas of all genotypes contained all layers, there was a subtle but significant expansion of junctional zone and reduction of labyrinth layer in DKO placentas (Fig. 4G&H). These findings suggest that deficiency of Idax and Rinf, consistent with aberrantly activating a trophoblast-like transcriptional program in ESCs, have a subtle influence on placenta development.

4. DISCUSSION

Idax and Rinf have been linked to Tet enzymes (Ko et al., 2013), which has prompted investigations into their biological significance in development. In ESCs, Rinf ensures

proper neuroectoderm differentiation and prevents aberrant mesendoderm/trophectoderm differentiation by binding to gene regulatory regions of Tet1 and Tet2 and facilitating their transcription (Ravichandran et al., 2019). A standing question has been what role Idax plays in ESC biology, and whether Idax and Rinf, by virtue of their gene structure and protein sequence similarity, have redundant functions and compensate for each other. This study finds that Idax, which is not expressed in ESCs, is induced upon differentiation and, like Rinf, facilitates expression of Tet enzymes to enhance neural and prevent trophectoderm specification. It also reveals that the two proteins possess both redundant and distinct roles in differentiation and during development.

Our findings that deficiency of Idax or Rinf leads to downregulation of Tet enzymes is consistent with our previous findings in ESCs where Rinf facilitated transcription of Tet enzymes, and the differentiation defects of *Rinf*$^{-/-}$ ESCs (i.e. restricted neural and enhanced mesendoderm/ trophectoderm differentiation) were corrected by overexpression of Tet enzymes (Ravichandran et al., 2019). Since Tet enzymes are key regulators of the neural and mesendoderm/trophectoderm lineages in ESCs (Chrysanthou et al., 2022; Dawlaty et al., 2014; Koh et al., 2011), our data suggest that Idax and Rinf influence these lineages by promoting expression of Tet enzymes (Fig. 3D). As Idax and Rinf are also enriched at some lineage genes, it is plausible that they regulate the lineage genes directly too, just as Rinf directly regulates pluripotency genes in ESCs (Ravichandran et al., 2019). We note that trophectoderm genes were more robustly deregulated than neural genes in DKO EBs. This may suggest that Rinf and Idax are more important in the trophectoderm lineage regulation than they are in neural lineage regulation. This is also evident from the increased presence of trophoblast lineage cells (i.e. giant cells) in the teratomas derived from Rinf/Idax DKO ESCs. We also note that *Tet3* was upregulated in single and DKO trophoblast-like cells. This induction is likely triggered by reduction of Tet1 and Tet2 in these cells, but is not sufficient to restore the phenotypes of DKO trophoblast-like cells suggesting that Tet3 cannot compensate for Tet1 and Tet2 in this context.

Rinf and Idax have both nuclear functions in gene regulation and cytoplasmic roles in negative regulation of Wnt signaling (Kim et al., 2010). Our findings that wild type Idax or Rinf, but not their CXXC mutant forms, boosted expression of neural and trophectoderm genes and Tet enzymes in DKO EBs suggests that the DNA binding function is involved in regulation of Tet and lineage genes. While Rinf binds to gene regulatory regions of Tet enzymes and pluripotency genes in ESCs(Ravichandran et al., 2019), lack of ChIP-seq grade antibodies for Idax has hindered mapping its genomic occupancy during ESC differentiation. A previous study using Myc-tagged Idax in HEK293 cells reports a significant enrichment of Idax at gene promoters (Ko et al., 2013). We find that many of DEGs in DKO EBs, including the *Tet* genes and lineage genes, are directly bound by Idax at their promoters or in gene bodies. This supports that Idax, like Rinf, can bind to gene regulatory regions to affect transcription. Rinf is implicated in recruitment of transcription factors and Tet enzymes to gene regulatory regions in ESCs (Ma et al., 2017; Ravichandran et al., 2019) and we find that Idax and Rinf peaks overlap with Tet peaks at lineage genes. Thus, it will be interesting to map the genomic occupancy of Idax and Rinf during differentiation and assess how their loss affects recruitment of Tet enzymes or lineage-specific transcription factors. Likewise,

reciprocal genome-wide mapping of Rinf and Idax in *Idax*$^{-/-}$ and *Rinf*$^{-/-}$ cells can further elaborate if they have any overlapping targets.

RNA-seq analysis revealed more genes to be deregulated during differentiation of DKO ESCs than during differentiation of single knockout ESCs. This suggests that Rinf and Idax may compensate for each other at the global level. Thus, their combined loss may have synergistic effects on global gene expression programs leading to more robust gene expression changes. While neural programs were more downregulated in DKO EBs by RNA-seq, individual or combined loss of Rinf and Idax had similar effects during *in vitro* differentiation to NPCs with key neural transcription factors and markers being comparably downregulated in single and DKO NPCs when quantified by RT-qPCR. Likewise, while trophectodermal programs were more upregulated in DKO EBs by RNA-seq, key trophectoderm transcription factors and markers were upregulated in both single and DKO trophoblast-like cells when quantified by RT-qPCR, with the effect being more robust and likely additive in DKO trophoblast-like cells. One explanation for these differences could be that differentiation to EBs results in a mixed population of cells while differentiation to NPCs or trophoblast-like cells yields a more uniform cell population of each lineage. Another explanation could be that candidate-gene-specific assessment of expression by RT-qPCR is more accurate than the global assessment of transcriptional changes by RNA-seq where statistical cutoffs may have masked expression changes in single knockout cells.

The aberrant neural differentiation defects observed in single and DKO ESCs have *in vivo* implications in NPCs isolated from *Rinf*$^{-/-}$ and DKO mice. This supports a specific *in vivo* requirement for Rinf during neural differentiation. The aberrant trophectoderm differentiation defects observed in single and DKO ESCs only manifests in DKO placentas, albeit very subtly, affecting labyrinth and junctional zone layers. This suggests that Idax and Rinf may have compensatory roles in the placenta. However, given that both single and DKO mice are viable and overtly normal, it suggests that Rinf and Idax are not fully essential for regulation of neural and placental development and that parallel mechanisms in their absence can promote normal development along these lineages. This is also supported by the fact that not all neural and placental markers that are deregulated in *in vitro* systems are affected in embryonic forebrain and placenta. Although another explanation for this could be that the cellular composition of embryonic forebrain and placenta is very heterogenous and thus differences in expression of some makers belonging to a specific cell type are masked by their expression in other cell types.

Loss of Rinf and Idax reduces Tet expression but DKO mice, unlike Tet knockout mice, do not have overt developmental defects and embryonic lethality. This is because combined loss of Rinf and Idax only reduces the expression of Tet enzymes to about half the normal levels. Based on our prior work reducing Tet dosage to half (i.e. Tet triple heterozygous mice) (Dawlaty et al., 2014) is compatible with normal development. Our findings have implications beyond ESCs into various somatic cell types where Idax and Rinf are expressed. It will be of interest to examine how expression of Tet enzymes and 5hmC levels are affected in various cell types and organs of single and DKO mice and whether

that has any implications in cellular homeostasis or disease pathology, such as cancer, where Rinf and Idax are dysregulated (Knappskog et al., 2011; Pendino et al., 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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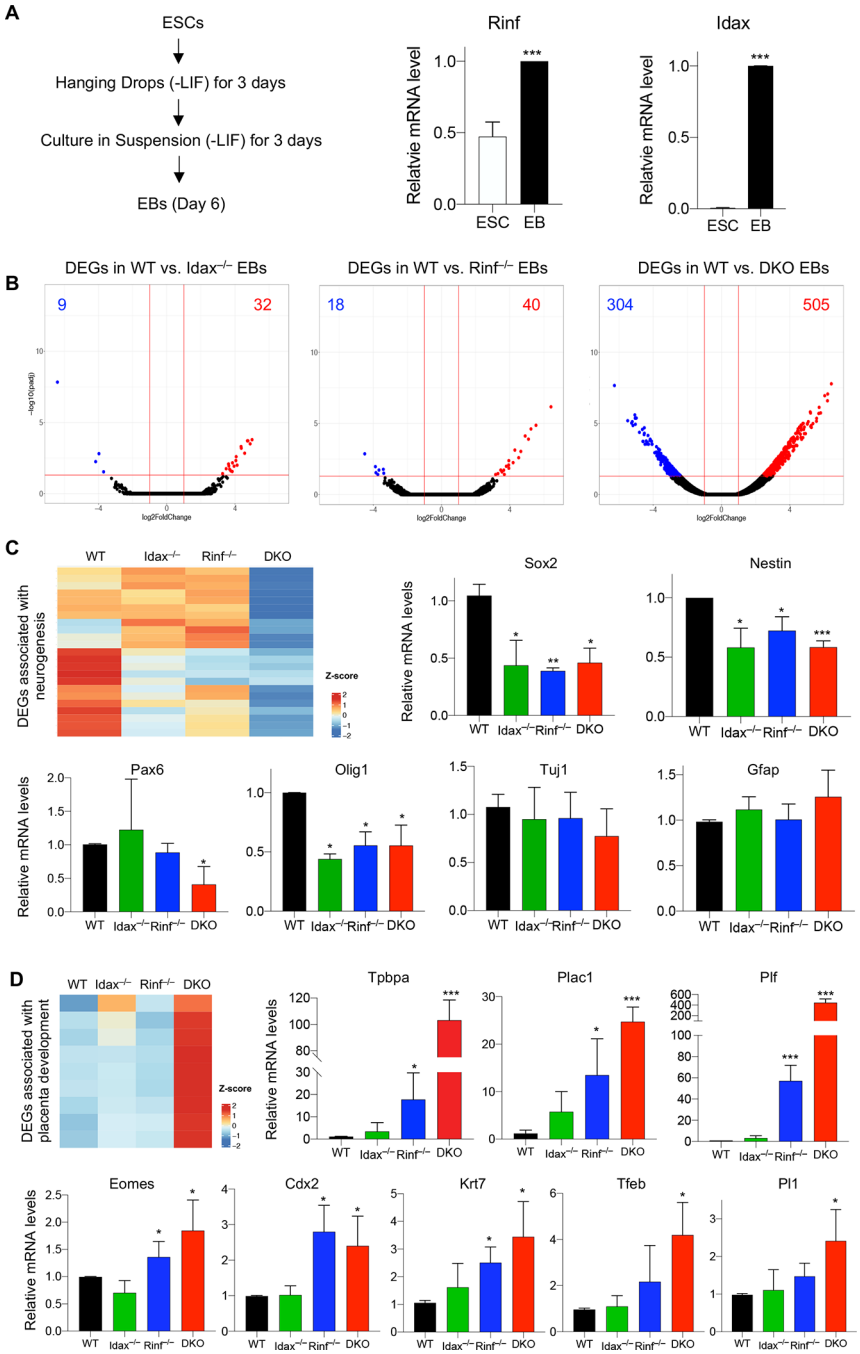

Figure 1: Loss of Rinf and Idax leads to downregulation of neurogenesis and upregulation of placental gene expression programs during differentiation of ESCs to EBs.

A. Schematic of differentiation of ESCs to embryoid bodies (EBs) (left) and mRNA levels of *Rinf* and *Idax* quantified by RT-qPCR in wild type ESCs and EBs (right).

B. Volcano plots of differentially expressed genes (DEGs) in EBs derived from wild type, *Idax*$^{-/-}$, *Rinf* $^{-/-}$ and DKO ESCs.

C. Heatmap of neurogenesis genes differentially expressed among EBs derived from wild type, *Idax*$^{-/-}$, *Rinf* $^{-/-}$ and DKO ESCs (left) and quantification of mRNA levels of neural markers in EBs of indicated genotypes by RT-qPCR (right).

D. Heatmap of trophectodermal and placental genes differentially expressed among EBs derived from wild type, *Idax*$^{-/-}$, *Rinf*$^{-/-}$ and DKO ESCs (left) and quantification of mRNA levels of trophoblast markers in EBs of indicated genotypes by RT-qPCR (right).
For all panels data are normalized to *Gapdh* and to wild type levels for each gene. Three independent lines of each genotype are used in all analyses. Data are presented as mean ± SD. Statistically significant (*p<0.05, **p<0.01, ***p<0.001) when compared to wild type.

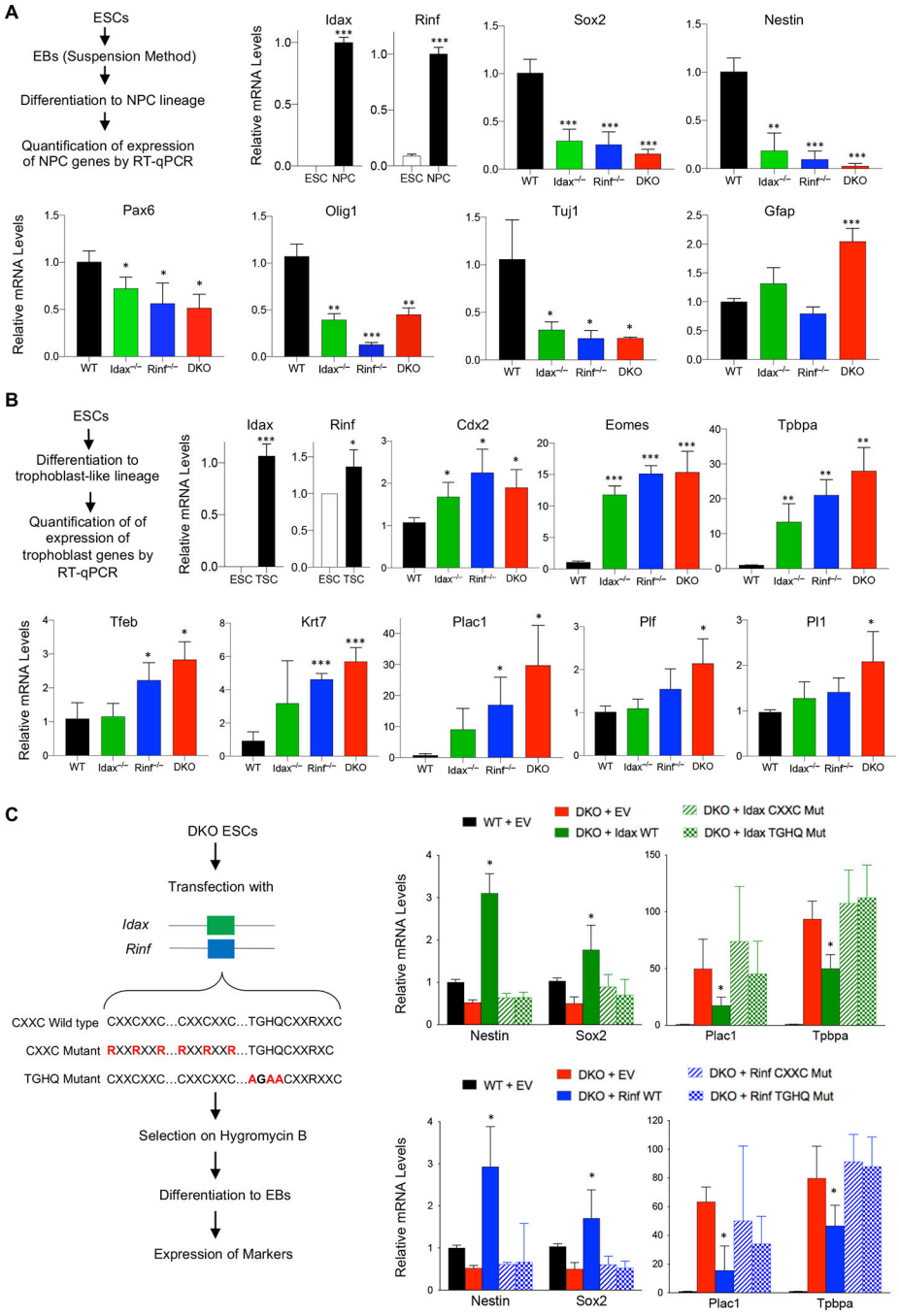

Figure 2: Deficiency of Rinf and Idax impairs ESC-to-NPC differentiation and induces ESC-to-Trophoblast-like cell differentiation.

A. Quantification of mRNA levels of *Rinf* and *Idax* and neural markers during differentiation of ESCs of indicated genotypes to neural progenitor cells (NPCs) by RT-qPCR.

B. Quantification of mRNA levels of *Rinf* and *Idax* and trophoblast markers during differentiation of ESCs of indicated genotypes to trophoblast-like cells by RT-qPCR.

C. Schematic of expression of Rinf and Idax wild type and mutant transgenes in DKO ESCs followed by differentiation to EBs (left). Quantification of mRNA levels of neural markers *Nestin* and *Sox2* and trophoblast markers *Plac1* and *Tpbpa* in EBs by RT-qPCR (right). For all panels data are normalized to *Gapdh* and to wild type levels for each gene. Three independent lines of each genotype are used in all analyses. Data are presented as mean ± SD. Statistically significant (*p<0.05, **p<0.01, ***p<0.001) when compared to wild type in panels A-B and to empty vector in panel C.

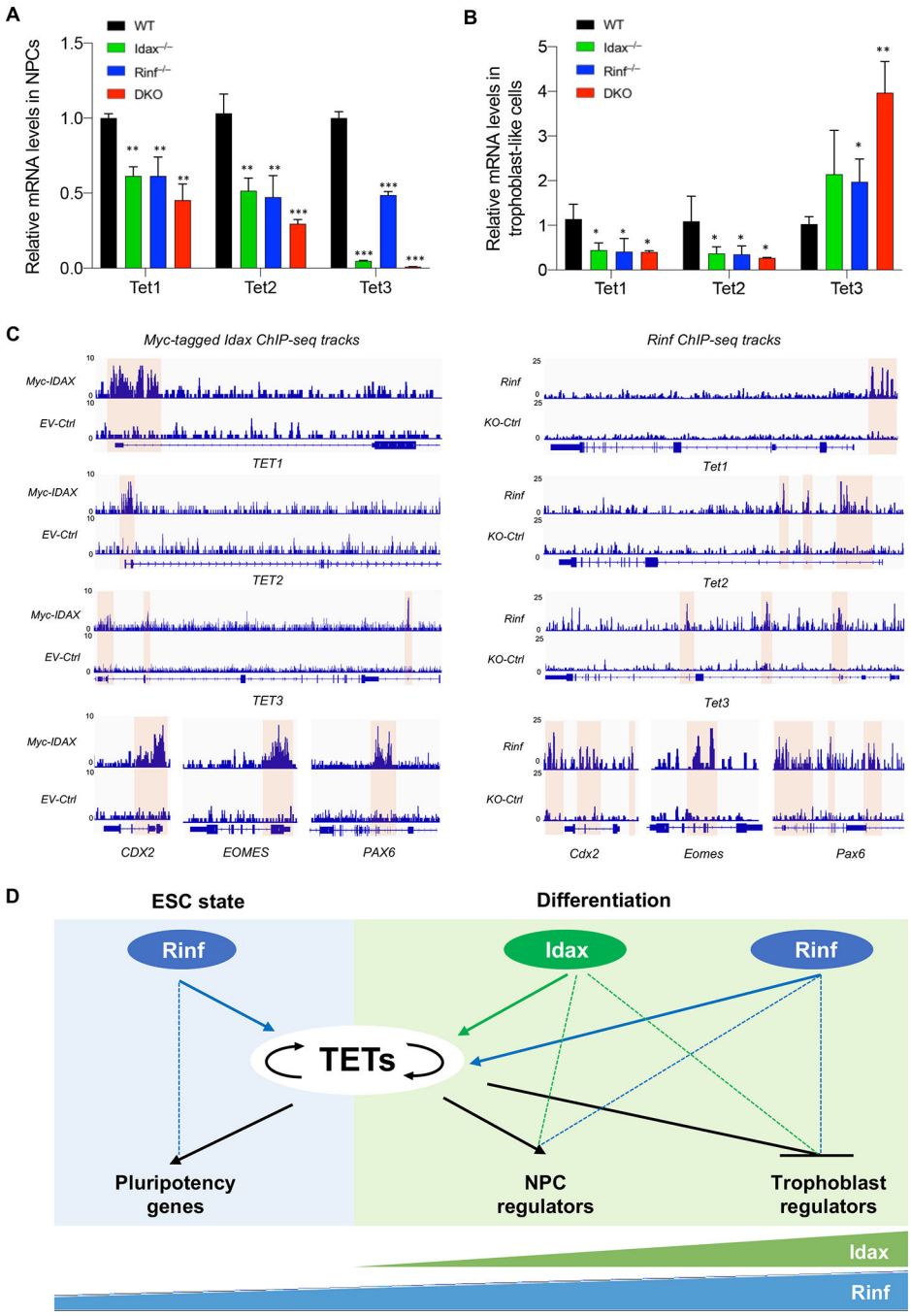

Figure 3: Loss of Rinf and Idax compromises proper expression of Tet enzymes during differentiation of ESCs.

A. Quantification of *Tet1/2/3* mRNA levels during differentiation of ESCs to NPCs by RT-qPCR and normalized to *Gapdh*. Three lines of each genotype were used. Note all three *Tet* genes are robustly downregulated in single and double knockout NPCs.

B. Quantification of *Tet1/2/3* mRNA levels during differentiation of ESCs to trophoblast-like cells by RT-qPCR and normalized to *Gapdh*. Three lines of each genotype were used. Note that *Tet1* and *Tet2* are robustly downregulated while *Tet3* is upregulated in single and double knockout trophoblast-like cells.

C. Genome browser tracks of published Myc-tagged Idax and endogenous Rinf ChIP-seq peaks in HEK293 cells and ESCs, respectively (see methods), confirming enrichment of Rinf and Idax at *Tet* genes and selected lineage markers. Empty vector (EV) track for IDAX and Rinf knockout (KO) track for Rinf ChIP-seq are used as negative controls (Ctrl).
D. Model for the roles of Idax and Rinf in regulation of ESC differentiation. In ESCs Rinf promotes expression of Tet enzymes and pluripotency genes as shown in our previous study (Ravichandran et al, 2019). Upon differentiation both Idax and Rinf are induced to promote Tet expression and subsequently enhance neural and inhibit trophectodermal programs.
For all panels data are normalized to *Gapdh* and to wild type levels for each gene. Three independent lines of each genotype are used in all analyses. Data are presented as mean ± SD. Statistically significant (*p<0.05, **p<0.01, ***p<0.001) when compared to wild type.

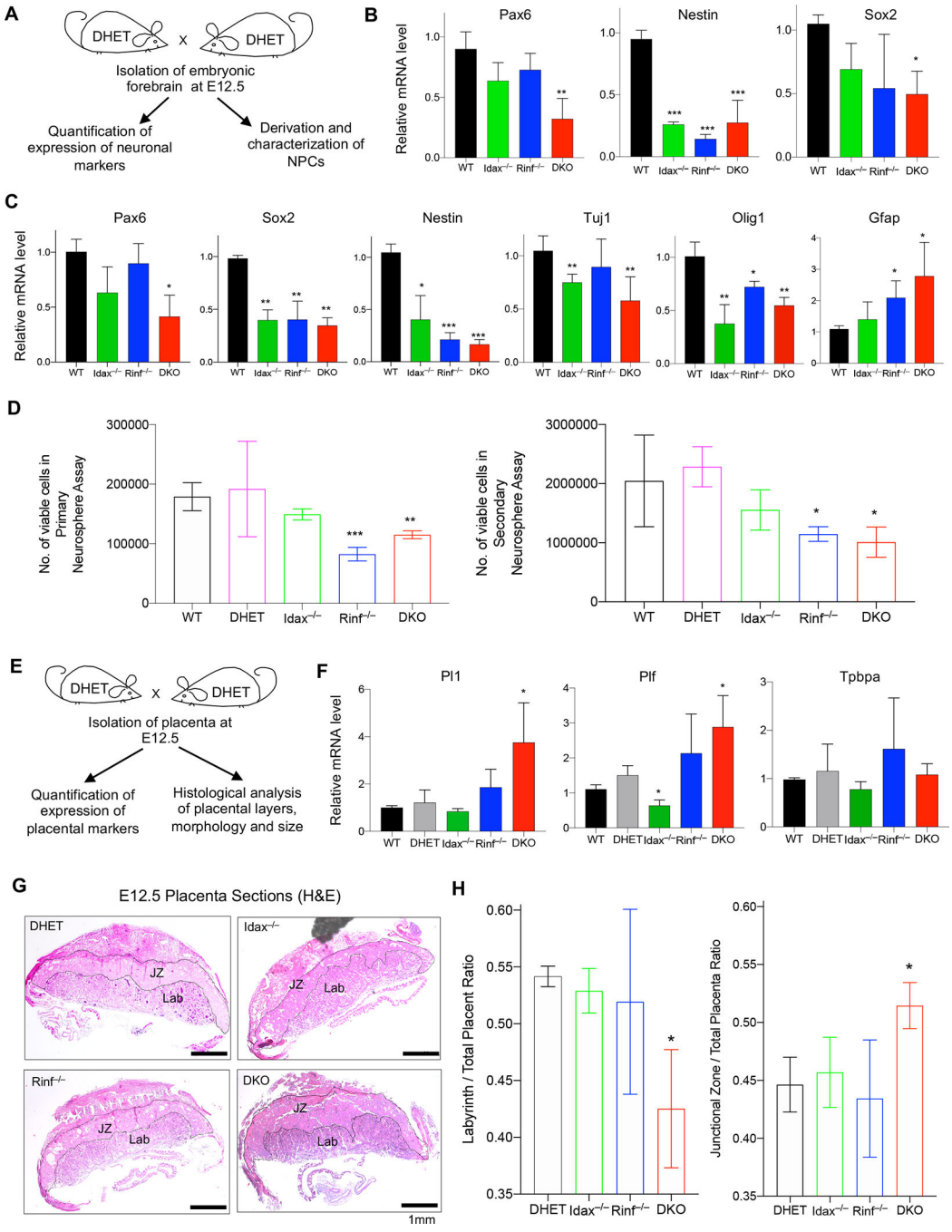

Figure 4: NPCs derived from DKO embryos have reduced neurosphere formation capacity and DKO placentas have increased junctional zone and reduced labyrinth layers.

A. Schematic of derivation of NPCs from E12.5 embryonic forebrain obtained from intercross of *Rinf/Idax* double heterozygous (DHET) mice.

B. Quantification of mRNA levels of selected neural markers in E12.5 forebrain by RTqPCR.

C. Quantification of mRNA levels of selected neural stem cell and neural differentiation markers in NPCs derived from E12.5 forebrains of indicated genotypes by RTqPCR.

D. Number of viable cells in primary and secondary neurosphere formation assays using isolated NPCs of indicated genotypes.
E. Schematic of characterization of E12.5 placentas obtained from intercross of *Rinf/Idax* double heterozygous (DHET) mice.
F. Quantification of trophoblast markers in E12.5 placentas of indicated genotypes by RTqPCR.
G. H&E staining of E12.5 placenta sections of indicated genotypes. The labyrinth (Lab) and junctional zone (JZ) are demarcated by a dotted line.
H. Labyrinth to total placental area and junctional zone to total placental area ratios are plotted. Four independent placentas of each genotype were quantified in this analysis.
For all panels RTqPCR data are normalized to *Gapdh* and to wild type levels for each gene. At least three independent samples of each genotype are used in all analyses. Data are presented as mean ± SD. Statistically significant (*p<0.05, **p<0.01, ***p<0.001) when compared to wild type (WT) or double heterozygote (DHET).