Direct interaction between Id1 and Zrf1 controls neural differentiation of embryonic stem cells

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Abstract

Id proteins are dominant-negative regulators within the HLH family of proteins. In embryonic stem cells (ESCs), Id1 and Id3 maintain the pluripotent state by preventing neural differentiation. The Id1-interacting protein Zrf1 plays a crucial role as a chromatin-bound factor in specification of the neural fate from ESCs. Here, we show that Id1 blocks Zrf1 recruitment to chromatin, thus preventing the activation of neural genes in ESCs. Upon differentiation, Id1 expression decreases thus inducing Zrf1 binding to neural genes. Importantly, depletion of Zrf1 rescues the expression of Polycomb targets involved in neural specification which are up-regulated in Id1 knock-out ESCs. We therefore identified Zrf1 as transcriptional regulator of neural fate downstream of Id1 in ESCs.

Keywords epigenetics; Id1; neural development; Polycomb

Subject Categories Neuroscience; Stem Cells

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Introduction

Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocysts, are self-renewing, pluripotent cells capable of generating the primitive endoderm and the epiblast, which in turn gives rise to all cells types of the embryo [1]. Several signaling pathways, such as LIF, Bmp4, and Wnt signaling, regulate ESC self-renewal and pluripotency. The so-called inhibitor of differentiation or inhibitor of DNA binding (Id) family of proteins is known to be downstream Bmp4 targets in ESCs [2]. Id factors (namely, Id 1/2/3/4) belong to the basic helix-loop-helix (bHLH) family of transcriptional factors [3]. Id proteins lack a DNA binding motif and act as dominant-negative inhibitors of bHLH factors and other interactors, including MyoD and the NeuroD family of transcription factors [3], by preventing DNA binding or sequestering their heterodimerization partners.

Id proteins are involved in numerous processes, such as cell growth, cancer and differentiation [4–7]. In ESCs, Id1 has been reported to regulate the expression of Nanog [8] and to block the epiblast factor Tcf15 [9]. Overexpression of Id1 maintains the undifferentiated state by sustaining pluripotency markers including Cdh1 [10] and preventing neural commitment [2]. Surprisingly, key transcriptional regulators responsible for blocking the neural fate mediated by Id factors in ESCs have not yet been identified.

In the embryo, Id1 and Id3 have overlapping patterns of expression [3]. Interestingly, Id1 and Id3 single knock-out mouse models do not show any significant alteration during embryonic development. On the other hand, the Id1 and Id3 double knock-out mouse is embryonic lethal [11]. Although this suggests that Id1 and Id3 exert overlapping functions in the embryo, it has been recently demonstrated that Id1 can play a specific role in ESCs. Indeed, depleting Id1 in ESCs by gene targeting [Id1 knock-out (Id1KO) ESCs] leads to down-regulation of the pluripotency markers and to the induction of differentiation markers, including Fgf5 and T [8].

Of the Id1-interacting proteins identified to date, we focused our attention on Zrf1 (also named Mida1 or Dnajc2) [12,13]. We have recently identified Zrf1 as epigenetic regulator required for neural differentiation of ESCs [14]. Zrf1 acts as chromatin-bound protein by recognizing mono-ubiquitinated histone H2A (H2Aub1), through an ubiquitin-binding domain (UBD) [15]. Mono-ubiquitination of histone H2A is a repressive mark deposited by Polycomb repressive complex 1 (PRC1). Polycomb complexes are epigenetic repressors required for proper ESC differentiation and embryo development [16]. In differentiation conditions, Zrf1 is specifically recruited on target genes involved in specification of neural lineages. Once recruited to chromatin, Zrf1 promotes PRC1 and H2A displacement, thus inducing de-repression of neural genes [14]. Therefore, Zrf1 is required for specification of neural progenitor cells (NPCs) and for maintenance of their identity [14]. Importantly, co-immunoprecipitation (co-IP) experiments demonstrated that the domain of Zrf1 interacting with Id1 overlaps with the Zrf1 UBD [15], suggesting that Id1 prevents recognition of H2Aub1 and thus the epigenetic regulation mediated by Zrf1.

In the present study, we report that the interaction of Id1 with Zrf1 prevents Zrf1 binding to chromatin. Overexpression of

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Zrf1 restores neural differentiation in Id1-overexpressing ESCs. Moreover, in Id1KO ESCs, Zrf1 rescues the expression of PRC1 target genes implicated in neurogenesis. Together, our data demonstrate that Zrf1 plays a crucial role in the control of neural genes regulated by Id1.

Results and Discussion

Id1 interacts with Zrf1 in ESC and prevents its recruitment to chromatin

Previous reports indicate that Id1 and Zrf1 interact in several cell types, including the murine erythroleukemia (MEL) cells [13] and the human teratocarcinoma NTERA2 clone D1 cells [15]. The direct interaction between Id1 and Zrf1 was verified by GST pull-down and protein co-IP experiments [13,15]. To determine whether Id1 and Zrf1 interact also in mouse ESCs, we performed co-IP experiments. Indeed, we observed that the Zrf1 antibody was able to co-immunoprecipitate Id1, and vice versa (Fig 1A). As a control, we used ESCs differentiated toward neural lineages with retinoic acid (RA) (Fig 1A), which induced a significant decrease of Id1 expression as compared to ESCs (Supplementary Fig S1A). Interestingly, the Zrf1 domain that interacts with Id1 overlaps with the domain that recognizes the H2Aub1 mark [15]. Given the role of Id1 as dominant-negative regulator [5], we checked whether Id1 could block Zrf1 recruitment to chromatin in differentiation conditions [14]. For this, we generated Id1-Flag ESC cells stably overexpressing Id1 (Supplementary Fig S1B). Id1 overexpression impaired Zrf1 binding to chromatin and thus the activation of neural genes, such as Olig2, Dlk1, and HoxB1/B4, upon RA treatment (Fig 1B and C). Moreover, depletion of Zrf1 in ESCs (Supplementary Fig S1B) resembled Id1 overexpression (Fig 1B and C).

These data together indicate that Id1 interacts with Zrf1 and negatively regulates Zrf1 recruitment to chromatin. Importantly, co-factors induced during differentiation might further contribute to modulating recruitment of Zrf1 to chromatin.

Zrf1 overexpression restores neural differentiation in Id1-overexpressing ESCs

We recently reported that Zrf1 is essential for the neural commitment of ESCs [14]. Therefore, we reasoned that sequestration of Zrf1 by Id1 could account for the block of neural differentiation observed in ESCs upon overexpression of Id1 [2,10]. To test this hypothesis, Zrf1 human transgene was ectopically expressed in Id1-Flag ESCs (Supplementary Fig S2A). Cells were differentiated into neural lineages using N2B27 medium for 5 days [17]. Of note, mRNA levels of Id1 were not affected by Zrf1 overexpression (Fig 2A). The expression of the human Zrf1 transgene significantly decreased between days 3 and 5 of differentiation, whereas endogenous (mouse) Zrf1 levels remained unchanged during differentiation (Fig 2A).

In line with previous reports [2,10], Id1-overexpressing ESCs resisted differentiation and maintained high expression levels of the pluripotent markers Cdh1 and Oct4 (Fig 2A). Moreover, Id1-Flag ESCs did not up-regulate neural markers such as Sox1,

Pax6 and Olig2 and exhibited increased expression of the epiblast/mesodermal marker T in the neural medium N2B27 (Fig 2A). Importantly, Zrf1 overexpression in Id1-Flag ESCs was able to rescue, at least partially, the expression of master neural genes, including Sox1, Pax6, and Olig2 (Fig 2A). On the other hand, Zrf1 overexpression in Id1-Flag ESCs did not affect the expression of Cdh1, Oct4, or T (Fig 2A), indicating that the regulation of expression of pluripotent and non-neural factors is independent of Zrf1. Chromatin immunoprecipitation (ChIP) experiments demonstrated that Zrf1 overexpression in Id1-Flag ESCs caused Zrf1 binding to the promoter of Olig2, Dlk1 and HoxB1 genes (Fig 2B) upon differentiation. Yet, in undifferentiated control ESCs, Zrf1 overexpression led to an increased occupancy to chromatin without affecting gene activation (Supplementary Fig S2A–C), suggesting that additional factors induced by differentiation conditions are necessary for transcriptional activation of Zrf1 target genes. These data thus further support that upon ectopic expression of Id1, the impairment of Zrf1 recruitment to chromatin (Fig 1B) is a consequence of the Id1–Zrf1 direct interaction rather than the differentiation block caused by Id1 overexpression [2,10].

In agreement with the restored expression of several neural markers, Zrf1 overexpression in Id1-Flag ESCs fully rescued the expression of the neural progenitor marker Nestin and of the marker of post-mitotic neurons, β 3 tubulin at day 5 of differentiation (Fig 2C). Taken together, our data support that the interaction of Id1 with Zrf1 accounts for the role of Id1 as a repressor of neural fate.

Zrf1 controls the expression of Polycomb target genes involved in neural differentiation regulated by Id1

We then investigated the role of Zrf1 in the absence of Id1, taking advantage of Id1KO ESCs. As previously reported [8], Id1KO ESCs exhibited several features of differentiated cells, including decreased proliferation, decreased expression of pluripotency markers (such as Nanog, Klf4, and Rex1) and an up-regulated expression of the epiblast marker Fgf5 (Supplementary Fig S3A and B). Gene expression profile indicated that more than 6,000 genes belonging to the three germ layers (Fig 3A) were misregulated in Id1KO ESCs as compared to wild-type ESCs (1.5-fold, $P < 0.05$) (Supplementary Table S1). Although Zrf1 knock-down (Fig 3B) did not affect proliferation and expression of pluripotent markers (Supplementary Fig S3A and B), it rescued the expression of more than 50% of misregulated genes in Id1KO ESCs (Supplementary Fig S3C). Since it was previously demonstrated that, during differentiation of ESCs, Zrf1 is able to specifically de-repress Polycomb target genes involved in neural specification [14], we focused our attention on Polycomb targets misregulated in Id1KO ESCs. The expression of about 50% of Polycomb target genes (previously identified by ChIP-sequencing analysis [18]) misregulated in Id1KO ESC was affected by Zrf1 depletion (Fig 3C) (Supplementary Table S2). As expected, neural tissues (such as brain and spinal cord) represented top categories of the GO analysis of this set of genes (Fig 3D). For further analysis, 302 genes (86 up- and 216 down-regulated) whose expression was significantly enriched in brain and spinal cord were selected (Supplementary Table S2). As reported in the

Figure 1. Id1 overexpression impairs Zrf1 recruitment to chromatin during neural differentiation of ESCs.

A ESCs were grown in either self-renewal conditions (serum plus LIF) or differentiation conditions (knock-out serum replacement, KSR, plus retinoic acid, RA). Extracts were subjected to immunoprecipitation with an antibody against either Zrf1 or Id1. Levels of co-immunoprecipitation of Id1 with Zrf1 (left), or of Zrf1 with Id1 (right), were assessed by Western blot.

B Zrf1 ChIPs were performed in ESCs transfected with an empty vector (Ctrl) or in Id1-Flag-overexpressing ESCs. Zrf1-depleted ESCs were used as a control. Values are reported as percentage of input. Standard deviation (SD) is representative of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ (paired t -test) represent comparison with control ESCs at the corresponding time point.

C mRNA fold-change of Zrf1 targets under differentiation conditions (24 h RA). SD is representative of three independent experiments. * $P < 0.05$; ** $P < 0.01$ (paired t -test).

heatmap analysis, Zrf1 depletion rescued the expression of the majority of the 302 genes (Fig 3E), thus suggesting a key role for Zrf1 in the regulation of neural genes in Id1-dependent

manner. Given the role of Polycomb complexes in gene repression [16] and that of Zrf1 in the activation of Polycomb-repressed genes during neural specification [14], we validated by

Figure 2. Zrf1 overexpression rescues the expression of neural genes in Id1-overexpressing ESCs.

A mRNA fold-changes of Id1, endogenous (mouse) and transgenic (human) Zrf1, pluripotent markers Cdh1 and Oct4, the neural markers Pax6, Sox1, Olig2, Hoxb1 and the epiblast/mesodermal marker T in control ESCs, Id1-Flag ESCs, or Id1-Flag ESCs transiently overexpressing Zrf1. Self-renewing conditions (day 0), and days 3 and 5 of neural differentiation were analyzed. SD is representative of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ (paired *t*-test) represent comparison with control ESCs at the corresponding time point.

B ChIP analysis for Zrf1 in control ESCs, Id1-Flag ESCs, and Id1-Flag ESCs transiently overexpressing Zrf1, grown in self-renewing conditions (day 0) and during neural differentiation (day 5). SD is representative of three independent experiments. Dashed line represents IgG background. ** $P < 0.01$ (paired *t*-test).

C Immunostaining of the neural progenitor marker Nestin and of the post-mitotic neuronal marker β3 tubulin in control ESCs, Id1-Flag ESCs, and Id1-Flag ESCs transiently overexpressing Zrf1 at day 5 of neural differentiation.

Figure 3.

Figure 3. Zrf1 depletion restores expression of neural genes misregulated in Id1KO ESCs.

- A Gene ontology (GO) analysis of tissue expression of up- and down-regulated genes in Id1KO ESCs as compared to wild-type ESCs.
 B Western blot analysis of Id1 and Zrf1 expression in wild-type and Id1KO ESCs upon Zrf1 depletion.
 C Overlap between misregulated genes in Id1KO ESCs, genes significantly affected by Zrf1 depletion in Id1KO ESCs (identified by gene expression analysis), and Polycomb-bound genes in ESC (identified by ChIP-seq analysis).
 D GO analysis of tissue expression of 642 genes identified as Polycomb targets misregulated in Id1KO ESCs, which are significantly affected by Zrf1 depletion.
 E Heatmap analysis of Zrf1-regulated Polycomb genes whose expression is significantly enriched in brain and spinal cord.
 F mRNA fold-changes of Otx2, Pax6, Sox4, and Sox11 in control ESCs, Id1KO ESCs, and Zrf1-depleted Id1KO ESCs. SD is representative of three independent experiments. $**P < 0.01$ (paired t-test) represents comparison with Ctrl ESCs at the corresponding time point.
 G Zrf1 ChIPs at Otx2, Pax6, Sox4, and Sox11 promoters in control ESCs, Id1KO ESCs, and Zrf1-depleted Id1KO ESCs. SD is representative of three independent experiments. $**P < 0.01$ (paired t-test).

qPCR the expression of master genes and key transcription factors implicated in neural development (such as Pax6, Otx2, Sox4 and Sox11) [19–21] that were up-regulated in Id1KO ESCs (Fig 3F). Moreover, ChIP analysis indicated that Zrf1 was recruited to Otx2, Sox4 and Sox11 promoters in Id1KO ESCs (Fig 3G). In contrast, no binding was observed on Pax6 promoter (Fig 3G).

Taken together, our data indicate that Id1 prevents Zrf1 binding to neural genes, and therefore their transcriptional activation in

self-renewing conditions. During differentiation, Id1 expression diminishes, allowing Zrf1 occupancy at neural genes, and thus their activation. Consistently, Id1 overexpression blocks Zrf1 binding to chromatin and impairs neural differentiation. Finally, premature activation of neural genes in Id1KO ESCs is prevented by depletion of Zrf1 (Fig 4). Given the crucial role of Id1 and Zrf1 in senescence and cancer [5–7,22–24], our data suggest that modulation of the Id1–Zrf1 interaction could play a role in therapy for cancer and diseases.

Figure 4. Zrf1 acts as transcriptional regulator of neural genes downstream of Id1.

In wild-type ESCs, Id1 binds to Zrf1 and to other interactors (indicated as X). In self-renewing conditions, Id1 impairs Zrf1 binding to chromatin. Upon differentiation, Id1 expression decreases and Zrf1 is recruited to neural genes promoting their transcriptional activation by displacing Polycomb repressive complexes (PRC). Id1 overexpression blocks Zrf1 binding to chromatin, thus impairing the expression of neural genes during differentiation. In Id1KO ESCs, Zrf1 is bound on Polycomb target genes involved in neural differentiation, and promotes their expression. Correct expression of neural genes in Id1KO ESC is restored upon depletion of Zrf1.

Materials and Methods

Plasmids

Zrf1 depletion was obtained by lentiviral infection of Zrf1 shRNAs (pLKO Sigma), as previously reported [14]. Mouse Id1 cDNA was cloned into pCBA-3×Flag vector [25] to obtain the Id1-Flag vector. For overexpression of Zrf1, a plasmid encoding human coding sequence was used (hZrf1ve), as previously reported [14].

Cell culture and differentiation

E14Tg2A ESCs (ATCC) were cultured in GMEM medium (Gibco) supplemented with 15% FBS (Hyclone), NEAA, 0.1 mM β -mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin/streptomycin. Cells were differentiated with all-*trans* retinoic acid (RA) at the concentration of 10^{-6} M in 10% KSR (Invitrogen) or with N2B27 medium in the absence of LIF [17]. Stable Id1-Flag ESCs were generated by transfecting the Id1-Flag vector with Lipofectamine 2000 and then selecting the cells for 7 days with G418. For generation of transiently Zrf1-overexpressing cells, hZrf1ve was transfected in stable Id1-Flag ESCs grown in self-renewing conditions (serum plus LIF). Twenty-four hours post-transfection of Zrf1 transgene, neural differentiation was induced by using N2B27 medium. Id1KO ESCs and parental control cells were provided by Dr. Benezra [8].

RNA and gene expression profile

RNA was extracted with an RNA extraction kit (Qiagen). cDNA was generated from 1 μ g of RNA using the qScript kit (Quanta Bioscience). Primers used for RT-qPCR assays are listed in Supplementary Table S3. *P*-value of RT-qPCR experiments was calculated by using Student's paired *t*-test with a two-tailed distribution. For global gene expression analysis, RNA from four independent experiments was isolated and hybridized on Agilent-028005 SurePrint G3 Mouse GE 8 × 60K Microarray. Raw data were corrected for background noise using the normexp method [26]. A cut-off of $\pm$ 1.5-fold with *P* < 0.05 was used to determine genes that were differentially expressed (Supplementary Table S1). Microarray data have been deposited in GEO database (GEO GSE58627).

Immunostaining

Antibodies for Nestin (MAB353-Millipore) and β 3 tubulin (T8660-Sigma) were used for immunostaining of neural progenitor cells and post-mitotic neurons.

Immunoprecipitation

Co-immunoprecipitation (co-IP) experiments were performed as previously described [15] by using Zrf1 antibody [15] and Id1 antibody (c-20 Santa Cruz). ChIP experiments were performed as previously described [18]. Primers used in ChIP-qPCR experiments are listed in Supplementary Table S3.

Western blot

Protein extraction was performed as previously described [25]. For protein level analysis, antibodies against Zrf1 [15], Id1 (c-20 Santa

Cruz), Flag (clone M2, F3165 Sigma), and Gapdh (sc47724 Santa Cruz) were used.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

LA, AG, and JMC performed and analyzed the experiments. LA and LDC conceived the project and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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