The Death-inducer Obliterator 1 (Dido1) Gene Regulates Embryonic Stem Cell Self-renewal

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The regulatory network of factors that center on master transcription factors such as Oct4, Nanog, and Sox2 help maintain embryonic stem (ES) cells and ensure their pluripotency. The target genes of these master transcription factors define the ES cell transcriptional landscape. In this study, we report our findings that Dido1, a target of canonical transcription factors such as Oct4, Sox2, and Nanog, plays an important role in regulating ES cell maintenance. We found that depletion of Dido1 in mouse ES cells led to differentiation, and ectopic expression of Dido1 inhibited differentiation induced by leukemia inhibitory factor withdrawal. We further demonstrated that whereas Nanog and Oct4 could occupy the Dido1 locus and promote its transcription, Dido1 could also target to the loci of pluripotency factors such as Nanog and Oct4 and positively regulate their expression. Through this feedback and feedforward loop, Dido1 is able to regulate self-renewal of mouse ES cells.

The abilities to divide indefinitely and differentiate into multiple lineages are two defining characteristics of embryonic stem (ES) cells (1, 2). The last decade has seen tremendous progress in our understanding of the factors and pathways that mediate ES cell self-renewal and pluripotency, in particular, the regulatory network that centers around master transcription factors such as Oct4, Nanog, and Sox2 (3–14). Through complex feedforward and feedback regulatory loops, these factors maintain the ES cell transcriptional landscape (6, 7, 11, 15). Oct4, Nanog, and Sox2 also participate directly or indirectly in recruiting chromatin remodeling proteins to regulate gene expression (16–20). For mouse ES cells, Oct4 is required for both pluripotency and self-renewal maintenance (5, 9) and is a key factor in reprogramming somatic cells (21–26). Although Nanog is not required for the pluripotency of ES cells (27), it is essential for mouse ES cells in the absence of the leukemia inhibitory factor (LIF)3 (4). Studies have shown that LIF promotes ES cell self-renewal and pluripotency through activating the master transcription factors (10, 28–30). When Nanog was ectopically expressed, mouse ES cells were able to compensate for the lack of LIF (4, 28).

The transcriptional network that encompasses the target genes of Nanog, Oct4, Sox2, and other stem cell transcription factors ensures the pluripotent state of stem cells and determines cell fate. Genome-wide transcriptome and occupancy studies have offered great insight into many of the targets of the master transcription factors that help mediate cell fate decisions, but the function of a large number of potential targets remain poorly understood. In this report, we describe findings that indicate Dido1, the shortest splicing variant of the death inducer obliterator (Dido) gene, as a novel regulator for mouse ES cell maintenance. The Dido gene encodes three splicing variants (Dido1, 2, and 3) and has been implicated in apoptosis and development (31–35). The longest and most widely expressed isoform Dido3 was shown to be dispensable for ES cell self-renewal and pluripotency (34); however, the role of isoform Dido1 in ES cell maintenance has not been explored. We showed here that ectopic expression of Dido1 isoform in mouse ES cells inhibited differentiation induced by LIF withdrawal, whereas knocking down Dido1 facilitated differentiation. Furthermore, Dido1 could target to the loci of key pluripotency factors such as Nanog and Oct4 and positively regulate their expression. Our data indicate that Dido1 helps maintain ES cells by forming feedforward and feedback regulatory loops with canonical ES cell factors, and they high-

3 The abbreviations used are: LIF, leukemia inhibitory factor; Dido, death inducer obliterator; qPCR, quantitative PCR; RA, retinoic acid.
light the importance of exploring the cross-talk between noncanonical pluripotency regulators and master transcription factors.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Expression Constructs**—Mouse AB2.2 ES cells were provided by the Darwin Core facility at Baylor College of Medicine and cultured in medium supplemented with 15% fetal bovine serum and 0.01% LIF. cDNAs encoding GFP, mouse Nanog, and human DIDO1 were cloned into murine stem cell virus retroviral vectors under the control of EF1a promoter and tagged with HA and FLAG. The murine stem cell virus vectors also contain a puromycin resistance marker for selection. Retroviral transduction was used to introduce the constructs into ES cells, followed by puromycin selection.

**Antibodies**—Immunoprecipitation and Western blotting experiments were carried out as described previously (16), using the following antibodies: anti-HA (ab9110; Abcam), antitubulin (ab52901; Abcam), anti-GAPDH (sc-25778; Santa Cruz Biotechnology), anti-Nanog (BL1662 for Western blotting and BL-2663 for ChIP; Bethyl Laboratories), anti-Oct4 (sc-8628 for Western blotting and sc-9081 for ChIP; Santa Cruz Biotechnology), anti-Sox2 (ab59776; Abcam), anti-FLAG (F7425; Sigma), anti-phospho-STAT3 (9131; Cell Signaling), anti-STAT3 (610189; BD Biosciences).

**RNAi Knockdown and RT-Quantitative PCR (RT-qPCR)**—The Stealth siRNA for Dido1 (Invitrogen) was transfected into ES cells in 6-well plates as described previously (36). At 2 days after transfection, ES cells were passaged and transfected with the same oligonucleotides again. Total RNA was extracted using RNeasy Mini Kit (Qiagen) 2 days after the second round of transfection. An equal amount of RNA was used for each reverse transcription reaction with iScript Select cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using an ABI PRISM 7300 Sequence Detection System and SYBR Green Master Mix. 18S was used as an internal control for qPCR. Primer sets for RT-qPCR can be found in [supplemental Table I](#).

The stealth siRNA sequences are: siDIDO1_1, 5'-GCAACAGAGACAUAGGCUAGCAGAAA; siDIDO1_2, 5'-CCAAAGGC-UAUCAAAACCACCCAGUAA; siDIDO1_3, 5'-GCCUUACCG-UUGAAGGACUUCAGAA; control siRNA sequence, 5'-UUCUCUCCACGCAGCAGAUAUU.

**Chromatin Immunoprecipitation (ChIP)**—ChIP experiments were performed as described previously (16), Primer sets can be found in [supplemental Table II](#).

**Self-renewal and Differentiation Assay by LIF Withdrawal and Retinoic Acid (RA) Treatment**—To determine self-renewal activity, mouse ES cells ectopically expressing different genes were cultured in ES medium without LIF and passaged every 4 days for ~21 days (~6 passages). For differentiation assays, ES cells were plated at clonal density in 6-well plates and then cultured without LIF. At different time points following LIF withdrawal, alkaline phosphatase staining was performed with the alkaline phosphatase staining detection kit (Millipore), and RNA was extracted for RT-qPCR analysis. RA was used at a final concentration of 1 μM.

**RESULTS**

**Dido1 Is Important for Maintaining ES Cells**—Human and mouse DIDO1 share 76% identity, and both contain a highly conserved pleckstrin homology (PH) domain, suggesting functional importance of Dido1 (Fig. 1A) (31, 32). Unlike isoforms Dido2 and 3, the shortest isoform Dido1 lacks the C-terminal transcription elongation factor S-II subunit M (TFSIIIM) domain and the open paralog and ortholog (SPOC) domain. We found the Dido1 isoform to have higher expression in mouse ES cells compared with mouse embryonic fibroblasts; conversely, the Dido3 isoform appeared to be expressed at a lower level in mouse ES cells compared with mouse embryonic fibroblasts (Fig. 1B), suggesting distinct roles for these two isoforms in mouse ES cells. In addition, when we examined Dido1 mRNA expression during differentiation, we found that the Dido1 level decreased ~2-fold during differentiation induced by either RA treatment or LIF withdrawal (Fig. 1C), suggesting that Dido1 may have an important function in mouse ES cells. We then ectopically expressed HA-tagged DIDO1 in mouse ES cells and examined these cells following LIF withdrawal using alkaline phosphatase staining as a self-renewal marker. HA-tagged Nanog and GFP were used as positive and negative controls, respectively (Fig. 1, D and E). As expected, overexpression of Nanog was able to suppress LIF withdrawal-induced differentiation compared with cells expressing GFP. Importantly, ES cells stably expressing DIDO1 also had more undifferentiated colonies and fewer completely differentiated colonies, suggesting that overexpression of DIDO1 was able to inhibit differentiation induced by LIF withdrawal (Fig. 1F). Next, we carried out RT-qPCR to examine a collection of lineage markers in these cells. Consistent with previous reports, LIF withdrawal resulted in de-repression of trophoderm (Cdx2 and Eomes), endoderm (Afp and Goosecoid), mesoderm (Brachyury and Mix1), and ectoderm (Nestin and Fgf5) markers in control cells (Fig. 1, G and 1H). This de-repression (with the exception of Fgf5) could be suppressed with Nanog overexpression. Similarly, activation of most of the lineage markers examined was significantly reduced in cells expressing exogenous DIDO1, indicating its role in maintaining the pluripotent state of mouse ES cells.

**Nanog and Oct4 Occupy Dido1 Promoter and Positively Regulate Its Expression**—We noticed up-regulated Dido1 gene expression in Nanog overexpression cells (Fig. 2A), which supports the idea that Dido1 may be targeted and regulated by Nanog and other master transcription factors. To test this possibility, we performed ChIP assays for endogenous Nanog and Oct4 using primers specific for the promoter region of Dido1 gene (Fig. 2B). We found that both Nanog and Oct4 were enriched on the Dido1 promoter compared with the control locus. The region with the highest enrichment was at ~7 kbp upstream of Dido1 transcriptional start site. The notion that Dido1 could be targeted by Nanog/Oct4 was further confirmed by our RT-PCR analysis of cells transiently transfected with siRNAs targeting Oct4 or Nanog. The knockdown efficiency for both genes was >80% (Fig. 2, C and D). Importantly, depletion of Oct4 and Nanog led to down-regulated Dido1 mRNA expression. Taken together, these data indicate that Nanog and
FIGURE 1. Ectopic expression of DIDO1 inhibits LIF withdrawal-induced differentiation of mouse ES cells. A, schematic represents mouse Dido1. NLS, nuclear localization signal. PHD, PH domain zinc finger motif. B, RT-qPCR was carried out in mouse ES cells and mouse embryonic fibroblasts (MEF) using two sets of primers specific for the Dido1 and Dido3 isoforms. PCR products were verified by sequencing. Error bars indicate S.D. (n = 3). C, mouse ES cell lines AB2.2 (left) and E14 (right) were either treated with RA or cultured without LIF, and then collected for RT-qPCR at the indicated time points with primers specific for the Dido1 gene. D, mouse ES cells stably expressing HA-tagged GFP (negative control), Nanog (positive control), and DIDO1 were analyzed by Western blotting using anti-HA antibodies. Tubulin was used as loading control. E, cells from D were cultured with or without LIF for 4 days before being examined for AP staining. F, results from E are quantified. For each cell line, three independent experiments were conducted (50 colonies for each experiment). G and H, cells from E were also examined by RT-qPCR for the indicated lineage markers. Error bars indicate S.D. (n = 3). **, p < 0.01 for all panels.
Oct4 positively regulate Dido1 gene expression and that Dido1 may be part of the Nanog/Oct4 pluripotency circuitry in ES cell maintenance.

Depletion of Dido1 in Mouse ES Cells Induces Differentiation—To test whether Dido1 was required for maintaining pluripotency and self-renewal of mouse ES cells, we generated cells transiently knocked down for Dido1 using three different siRNAs and cultured these cells in the presence of LIF. Compared with control knockdown cells, Dido1 depletion led to weaker AP staining and an increase in the number of differentiated colonies (Fig. 3, A and B). To further rule out off-target effects of siRNAs, we then performed genetic rescue experiments. To accomplish this, we introduced one of the Dido1 siRNA oligonucleotides into mouse ES cells stably expressing HA-tagged human DIDO1 that was resistant to the siRNA oligonucleotide. Cells expressing HA-tagged GFP were used as negative controls. Expression of the HA-tagged exogenous proteins was confirmed by Western blotting, and knockdown efficiency was measured by RT-qPCR (~70%) (Fig. 3, C and D). Compared with Dido1 knockdown cells that were rescued with control vectors, the Dido1 knockdown cells that also expressed human DIDO1 exhibited morphology and AP staining patterns...
Dido1 Participates in ES Cell Maintenance

A

NC  siDido1_1
siDido1_2  siDido1_3

B

Percentage of colonies

Differentiated
Partially differentiated
Undifferentiated

NC  siDido1_1  siDido1_2  siDido1_3

C

\(\alpha\)-HA
DIDO1
\(\alpha\)-Gapdh
GFP

D

E

GFP + ctr siRNA

GFP + siDido1_3

Human DIDO1 + siDido1_3

F

Differentiated
Partially differentiated
Undifferentiated

G

Relative expression

GFP+Ctrl siRNA
GFP+siDido1_3
hDIDO1+siDido1

Relative expression

Cdx2  Eomes  Brachyury  Mix1  Pdgfra  Goosecoid  Nestin  Cxcl12

Trophoderm  Mesoderm  Endoderm  Ectoderm
similar to GFP-expressing cells with control knockdown (Fig. 3E). Consistent with their morphological changes, de-repression of lineage markers was evident in Dido1 knockdown cells as assessed by RT-PCR (e.g. Cdx2 and Brachyury) (Fig. 3F). In DIDO1 rescue cells, trophodermectoderm, ectoderm, and mesoderm markers were partially repressed. These observations provide further evidence that Dido1 represents an additional player in the ES cell pluripotency circuitry and participates in ES cell maintenance.

**Dido1 Directly Regulates the Expression of Pluripotency Factors**—We speculated that Dido1 might function as part of the master transcription factor network by regulating the expression of pluripotency markers. To test this idea, we cultured mouse ES cells stably expressing DIDO1 or GFP in the presence of LIF and compared by RT-PCR the expression levels of pluripotency markers Nanog, Oct4, and Sox2. We found elevated expression of all three factors in DIDO1-overexpressing cells (Fig. 4A). Additionally, this increase in message expression was accompanied by a slight but reproducible increase in the protein levels of Nanog, Oct4, and Sox2 as well (Fig. 4B). This Dido1-dependent increase in protein amount was likely driven by increased transcription. In fact, similar patterns were also obtained in DIDO1 overexpression cells in the absence of LIF, although the induction of pluripotency markers was to a lesser degree compared with cells maintained in LIF (Fig. 4C). It is also interesting to note that when we examined by Western blotting Dido1 RNAi cells and those that were also rescued with DIDO1 expression, we found Dido1 KD cells to display reduced levels for Oct4, Nanog, and Sox2 (Fig. 4D).

To rule out the possibility that DIDO1-mediated inhibition of LIF withdrawal-induced differentiation and up-regulation of pluripotency factors was simply a result of activation of the LIF autocrine loop, we examined whether Dido1 expression led to STAT3 phosphorylation and activation, because activation of STAT3 is a central pathway in LIF-dependent maintenance of mouse ES cells (28, 37, 38). As shown in Fig. 4E, whereas addition of LIF led to clear accumulation of phosphorylated STAT3 regardless of Dido1 status, DIDO1 overexpression alone did not have an effect on STAT3 activation. These observations indicate that Dido1 likely directly regulates the pluripotency factors. Indeed, when we carried out ChIP experiments with DIDO1 overexpression cells, we found enrichment of both exogenous and endogenous Dido1 on the promoter regions of Nanog, Oct4, Sall4, and Sox2 (Fig. 4F). Taken together, our data support a feedback and feedforward loop between Dido1 and pluripotency factors, where Dido1 can occupy the loci of key pluripotency markers and positively regulate their expression.

**DISCUSSION**

Studies of the Dido gene and its variants support their important function in a number of cellular processes such as apopositis and chromosomal segregation, particularly in the hematopoietic system (31–33, 39–41). Homozygous mutant mice that had disrupted 5′ regions of the Dido gene were viable but exhibited defective hematopoiesis and reduced fertility (33). Furthermore, deletion of the 3′ region of Dido gene led to embryonic lethality, likely a result of delayed onset of differentiation (34). These observations point to the important function of Dido proteins during development and suggest possible roles for Dido1 in ES cell biology.

In this study, we provide evidence that Dido1 is a noncanonical pluripotency factor in mouse ES cells. Our siRNA and rescue experiments demonstrated that Dido1 expression rescued ES cell self-renewal, suggesting that Dido1 was responsible for the knockdown phenotype. LIF withdrawal induces major changes in mouse ES cells, such as chromatin modification and gene expression, ultimately leading to differentiation (28, 42). Up-regulating master regulators such as Nanog helps maintain self-renewal and pluripotency of ES cells in the absence of LIF (4, 28). Dido1 is transcriptionally regulated by Nanog, Oct4, and Sox2 and can also target to the loci of master transcription factors to enhance their expression. Dido1 contains a highly conserved PH domain finger. PH domains are capable of binding post-translationally modified histones (43–46). The Dido1 PH domain was reported to bind histone H3 (47), with a higher affinity for trimethylated lysine 4 (H3K4me3) (47, 48). Perhaps Dido1 could act as a transcription co-activator, and ectopically expressed Dido1 was able to promote expression of the pluripotency markers through its binding to active histone marks. Dido3 was reported to be dispensable for ES cell self-renewal and pluripotency (34). Loss of Dido3 expression by specific deletion of the C-terminal domains unique to Dido3 (Dido3ΔC) resulted in sustained Oct4 expression and compromised differentiation of embryonic stem cells. Interestingly, expressing a dominant negative form of Dido1 reduced Oct4 expression and relieved differentiation block in Dido3ΔC ES cells. Together with our findings, these results suggest that Dido1, but not Dido3, regulates ES cell self-renewal and raise the possibility that Dido1 may negatively regulate Dido3 activity.

It is interesting to note that homologs of Dido1 in yeast and other organisms (based on sequence homology) appear to be involved in DNA regulation and chromatin stability and assembly into higher complexes linked to active chromatin (39). These observations are also consistent with the positive role of Dido1 in regulating pluripotency marker expression. Previous genome-wide ChIP-seq analysis identified the Dido1 locus as a possible target for Nanog, Oct4, and other transcription factors (8). Furthermore, RNA sequencing studies of human ES cells identified Dido1 as one of the transcripts up-regulated in undifferentiated human ES cells (42). Taken together with our data, we speculate that Dido1 might function as part of a transcriptional complex that regulates pluripotency factors.
these observations support a feedback and feedforward loop between Dido1 and master transcription factors.

Both proteomic and genomic studies have helped illustrate the network of proteins that interact with Nanog- and Oct4-mediated signaling pathways and contributed to our understanding of their function (7, 8, 11, 12, 16, 49–52). Our studies with Dido1 add an additional player to the complex network of factors that intersect with Nanog/Oct4/Sox2. It will be interesting to determine whether additional players can be brought into the circuitry through their interaction with Dido1. Further studies should allow us to gain a more comprehensive understanding of the transcriptional circuitry at work in ES cells.

**REFERENCES**

1. Chambers, I., and Smith, A. (2004) Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene* 23, 7150–7160
2. Rossant, J. (2001) Stem cells from the Mammalian blastocyst. *Stem Cells* 19, 477–482
3. Avilion, A. A., Nicolis, S. K., Pevny, L. H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003) Multipotent cell lineages in early mouse development...
depend on SOX2 function. *Genes Dev.* **17**, 126–140

4. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–655

5. Nichols, J., Zevnik, B., Anastassiadi, K., Niwa, H., Klevे-Nebenius, D., Chambers, I., Schüler, H., and Smith, A. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* **95**, 379–391

6. Jaenisch, R., and Young, R. (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell** 132**, 567–582

7. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., and Young, R. A. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122**, 947–956

8. Kim, J., Chu, J., Shen, X., Wang, J., and Orkin, S. H. (2008) An extended transcriptional network for pluripotency of embryonic stem cells. *Cell** 132**, 1049–1061

9. Niwa, H., Miyazaki, J., and Smith, A. G. (2000) Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat. Genet.* **24**, 372–376

10. Mitsuji, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631–642

11. Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V. B., Wong, E., Orlov, Y. L., Zhang, W., Jiang, L., Roh, Y. H., Yeo, H. C., Yeo, Z. X., Narang, V., Govindarajan, K. R., Leong, B., Shahab, A., Ruan, Y., Bourque, G., Sung, W. K., Clarke, N. D., Wei, C. L., and Ng, H. H. (2008) Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. *Cell** 133**, 1106–1117

12. Silva, J., Nichols, J., Theunissen, T. W., Guo, G., van Oosten, A. L., Barrandon, O., Wray, J., Yamanaka, S., Chambers, I., and Smith, A. (2009) Nanog is the gateway to the pluripotent ground state. *Cell** 138**, 722–737

13. Niwa, H., Masui, S., Chambers, I., Smith, A. G., and Miyazaki, J. (2002) Phenotypic complementation establishes requirements for specific Pou domain and generic transactivation function of Oct-3/4 in embryonic stem cells. *Mol. Cell. Biol.* **22**, 1526–1536

14. Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A. A., Ko, M. S., and Niwa, H. (2007) Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat. Cell Biol.* **9**, 625–635

15. Sharov, A. A., Masui, S., Sharova, L. V., Piao, Y., Aiba, K., Matoba, R., Xin, L., Niwa, H., and Ko, M. S. (2008) Identification of Pou5f1, Sox2, and Nanog downstream target genes with statistical confidence by applying a novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. *BMC Genomics* **9**, 269

16. Liang, J., Wan, M., Zhang, Y., Gu, P., Xin, H., Jung, S. Y., Qin, J., Wong, J., Cooney, A. J., Liu, D., and Songyang, Z. (2008) Nanog and Oct4 associate via immunoprecipitation data. *Cell** 132**, 145–158

17. Gaspar-Maia, A., Alajem, A., Meshorer, E., and Ramalho-Santos, M. (2009) Chd1 regulates open chromatin in pluripotency and reprogramming. *Cell** 137**, 625–635

18. Gatchalian, J., Fütterer, A., Rothbart, S. B., Tong, Q., Rincon-Arano, H., Groudine, M., Strahl, B. D., Martínez-A, C., and Valencia, A. (2005) Death inducer obliterator protein 1 in the context of DNA regulation. Sequence analyses of distant homologues point to a novel functional role. *Genes Dev.* **19**, 115–126

19. Loh, Y. H., Zhang, W., Chen, R., Liu, D., Barton, M., and Songyang, Z., Thomson, J. A., and Zwaka, T. P. (2008) Ronin is essential for embryogenesis and the pluripotency of mouse embryonic stem cells. *Cell** 133**, 1162–1174

20. Burdon, T., Smith, A., and Savatier, P. (2002) Signalling, cell cycle and pluripotency in embryonic stem cells. *Trends Cell Biol.* **12**, 432–438

21. García-Domínguez, D., Leonard, E., Grandien, A., Martinez, P., Albar, J. P., Izpisúa-Belmonte, J. C., and Martinez-A, C. (1999) DIO-1 is a gene involved in onset of apoptosis in vitro, whose misexpression disrupts limb development. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7992–7997

22. García-Domínguez, D., Ramírez, D., González de Buitrago, G., and Martínez-A, C. (2003) Death inducer-obliterator 1 triggers apoptosis after nuclear translocation and caspase up-regulation. *Mol. Cell. Biol.* **23**, 3216–3225

23. Fütterer, A., Campanero, M. R., Leonard, E., Criado, L. M., Flores, J. M., Hernández, J. M., Fernández-A, C., and Valencia, A. (2007) Dio gene expression alterations are implicated in the induction of hematological myeloid neoplasms. *J. Clin. Invest.* **115**, 2351–2362

24. Fütterer, A., Raya, A., Llorente, M., Izpisúa-Belmonte, J. C., de la Pompa, J. L., Platt, K., and Martinez-A, C. (2012) Ablation of Dio3 compromises lineage commitment of stem cells in vitro and during early embryonic development. *Cell Death Differ.* **19**, 132–143

25. Gatchalian, I., Fütterer, A., Rothbart, S. B., Tong, Q., Ronin-Arono, H., Sánchez de Diego, A., Groudine, M., Strahl, B. D., Martinez-A, C., van Wey, K. H., and Kutateladze, T. G. (2013) Dio3 PHD modulates cell differentiation and division. *Cell Rep.* **4**, 148–158

26. Wan, M., Liang, J., Xiong, Y., Shi, F., Zhang, Y., Lu, W., He, Q., Yang, D., Chen, R., Liu, D., Barton, M., and Songyang, Z. (2013) The trithorax group protein ashl2 is essential for pluripotency and maintaining open chromatin in embryonic stem cells. *J. Biol. Chem.* **288**, 5039–5048

27. Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev.* **12**, 2048–2060

28. Mitsuji, K., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. (1999) STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J.* **18**, 4261–4269

29. Rojas, A. M., Sanchez-Pulido, L., Fütterer, A., van Wely, K. H., Martinez-A, C., and Valencia, A. (2005) Death inducer obliterator protein 1 in the context of DNA regulation. Sequence analyses of distant homologues point to a novel functional role. *FEBS J.* **272**, 3505–3511

30. Trachana, V., van Wely, K. H., Guererro, A. A., Fütterer, A., and Martínez-A, C. (2007) Dio3 disruption leads to centrosome amplification and mitotic checkpoint defects compromising chromosome stability. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2691–2696

31. Wang, Y., Chen, Y., Chen, Z., Wu, Q., Ke, W. J., and Wu, Q. L. (2008)
Gambogic acid induces death inducer-obliterator 1-mediated apoptosis in Jurkat T cells. *Acta Pharmacol. Sin.* **29**, 349–354

42. Lee, J. H., Hart, S. R., and Skalnik, D. G. (2004) Histone deacetylase activity is required for embryonic stem cell differentiation. *Genesis* **38**, 32–38

43. Li, H., Ilin, S., Wang, W., Duncan, E. M., Wysocka, J., Allis, C. D., and Patel, D. I. (2006) Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. *Nature* **442**, 91–95

44. Wysocka, J., Swigut, T., Xiao, H., Milne, T. A., Kwon, S. Y., Landry, J., Kauer, M., Tackett, A. J., Chait, B. T., Badenhorst, P., Wu, C., and Allis, C. D. (2006) A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **442**, 86–90

45. Peña, P. V., Davrazou, F., Shi, X., Walter, K. L., Verkhusha, V. V., Gozani, O., Zhao, R., and Kutateladze, T. G. (2006) Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* **442**, 100–103

46. Shi, X., Hong, T., Walter, K. L., Ewalt, M., Michishita, E., Hung, T., Carney, D., Peña, P., Lan, F., Kaadige, M. R., Lacoste, N., Cayrou, C., Davrazou, F., Saha, A., Cairns, B. R., Ayer, D. E., Kutateladze, T. G., Shi, Y., Côté, J., Chua, K. F., and Gozani, O. (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442**, 96–99

47. Prieto, I., Kouznetsova, A., Fütterer, A., Trachana, V., Leonardo, E., Alonso Guerrero, A., Cano Gamero, M., Pacios-Bras, C., Leh, H., Buckle, M., García-Gallo, M., Kremer, L., Serrano, A., Roncal, F., Albar, J. P., Barbero, J. L., Martínez-A, C., and van Wely, K. H. (2009) Synaptosomal complex assembly and H3K4me3 demethylation determine DIDO3 localization in meiosis. *Chromosoma* **118**, 617–632

48. Santiveri, C. M., García-Mayoral, M. F., Pérez-Cañadillas, J. M., and Jiménez, M. A. (2013) NMR structure note: PHD domain from death inducer obliterator protein and its interaction with H3K4me3. *J. Biomol. NMR* **56**, 183–190

49. Wang, J., Rao, S., Chu, J., Shen, X., Levasseur, D. N., Theunissen, T. W., and Orkin, S. H. (2006) A protein interaction network for pluripotency of embryonic stem cells. *Nature* **444**, 364–368

50. Kim, J., Cantor, A. B., Orkin, S. H., and Wang, J. (2009) Use of *in vivo* biotinylation to study protein-protein and protein-DNA interactions in mouse embryonic stem cells. *Nat. Protoc.* **4**, 506–517

51. Pardo, M., Lang, B., Yu, L., Prosser, H., Bradley, A., Babu, M. M., and Choudhary, J. (2010) An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell* **6**, 382–395

52. van den Berg, D. L., Snoek, T., Mullin, N. P., Yates, A., Bezstarosti, K., Demmers, J., Chambers, I., and Poot, R. A. (2010) An Oct4-centered protein interaction network in embryonic stem cells. *Cell Stem Cell* **6**, 369–381