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

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Background: *Dido1* plays important roles in development.
Results: *Dido1* inhibition led to ES cell differentiation, and its expression promoted ES cell self-renewal.
Conclusion: Dido1 participates in ES cell maintenance and forms feedback and feedforward loops with canonical ES cell factors such as Nanog and Oct4.
Significance: Dido1 represents a new factor in the ES cell regulatory circuitry for maintaining self-renewal of ES cells.

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-

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[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), anti-tubulin (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′-GCACAAGAGACUAGCGUCAGAGAAA; siDIDO1_2, 5′-CCAAGGCUAUCAAACCCACCAGUAA; siDIDO1_3, 5′-GCCUUACGUUGAAGGAACUUCAGAA; control siRNA sequence, 5′-UUCCUCUCCACGCGCAGUACAUUUA.

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. 1*A*) (31, 32). Unlike isoforms *Dido2* and *3*, the shortest isoform *Dido1* lacks the C-terminal transcription elongation factor S-II subunit M (TFSIIM) domain and the spen 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. 1*B*), 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. 1*C*), 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. 1*F*). 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 trophectoderm (*Cdx2* and *Eomes*), endoderm (*Afp* and *Goosecoid*), mesoderm (*Brachyury* and *Mixl1*), and ectoderm (*Nestin* and *Fgf5*) markers in control cells (Fig. 1, *G* and 1*H*). 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. 2*A*), 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. 2*B*). 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 kb 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

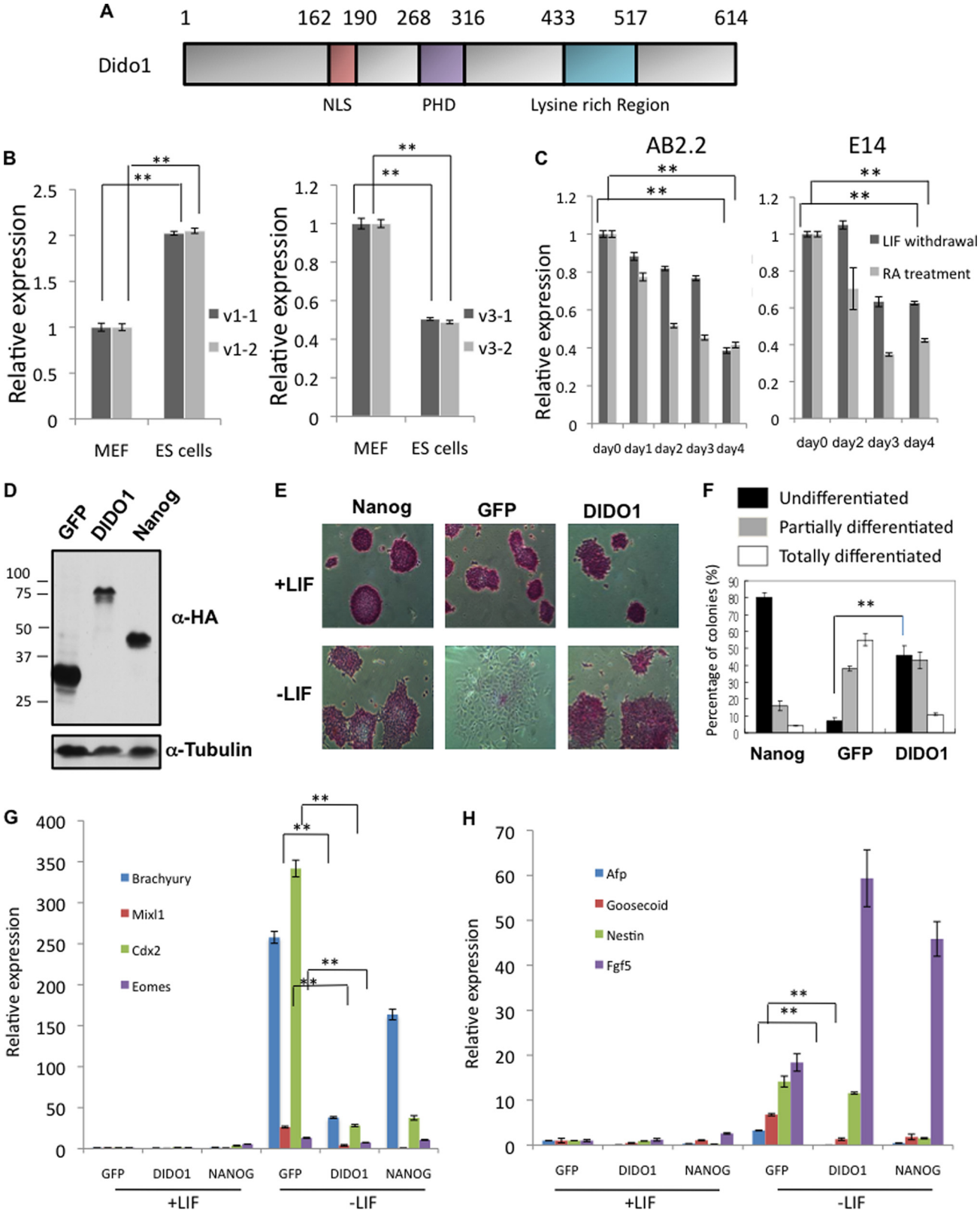


FIGURE 1. **Ectopic expression of DIDO1 inhibits LIF withdrawal-induced differentiation of mouse ES cells.** *A*, schematic represents mouse Dido1. *NLS*, nuclear localization signal. *PHD*, PH domain zinc finger motif. *B*, RT-qPCR was carried out in mouse ES cells and mouse embryonic fibroblasts (*MEF*) 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*.

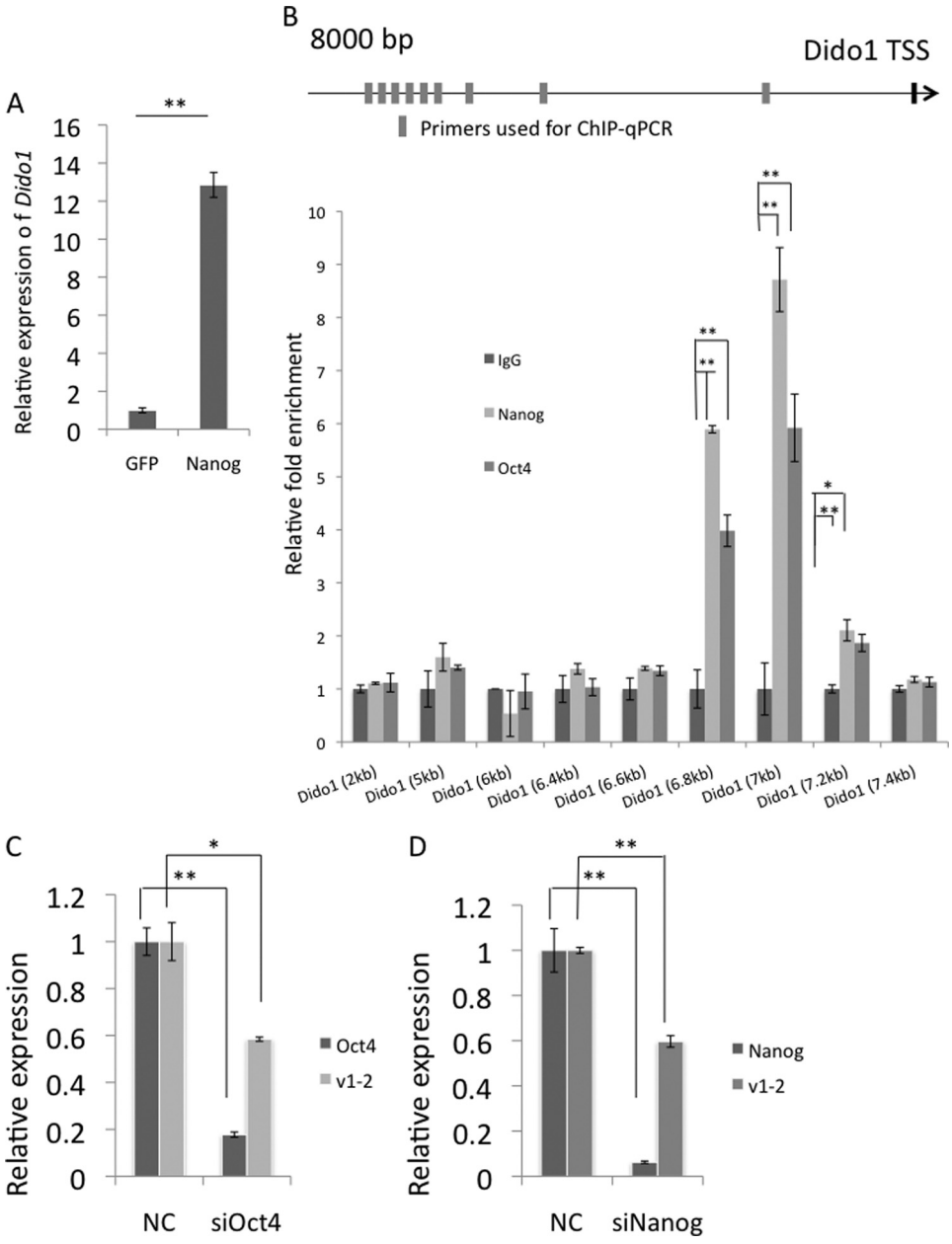
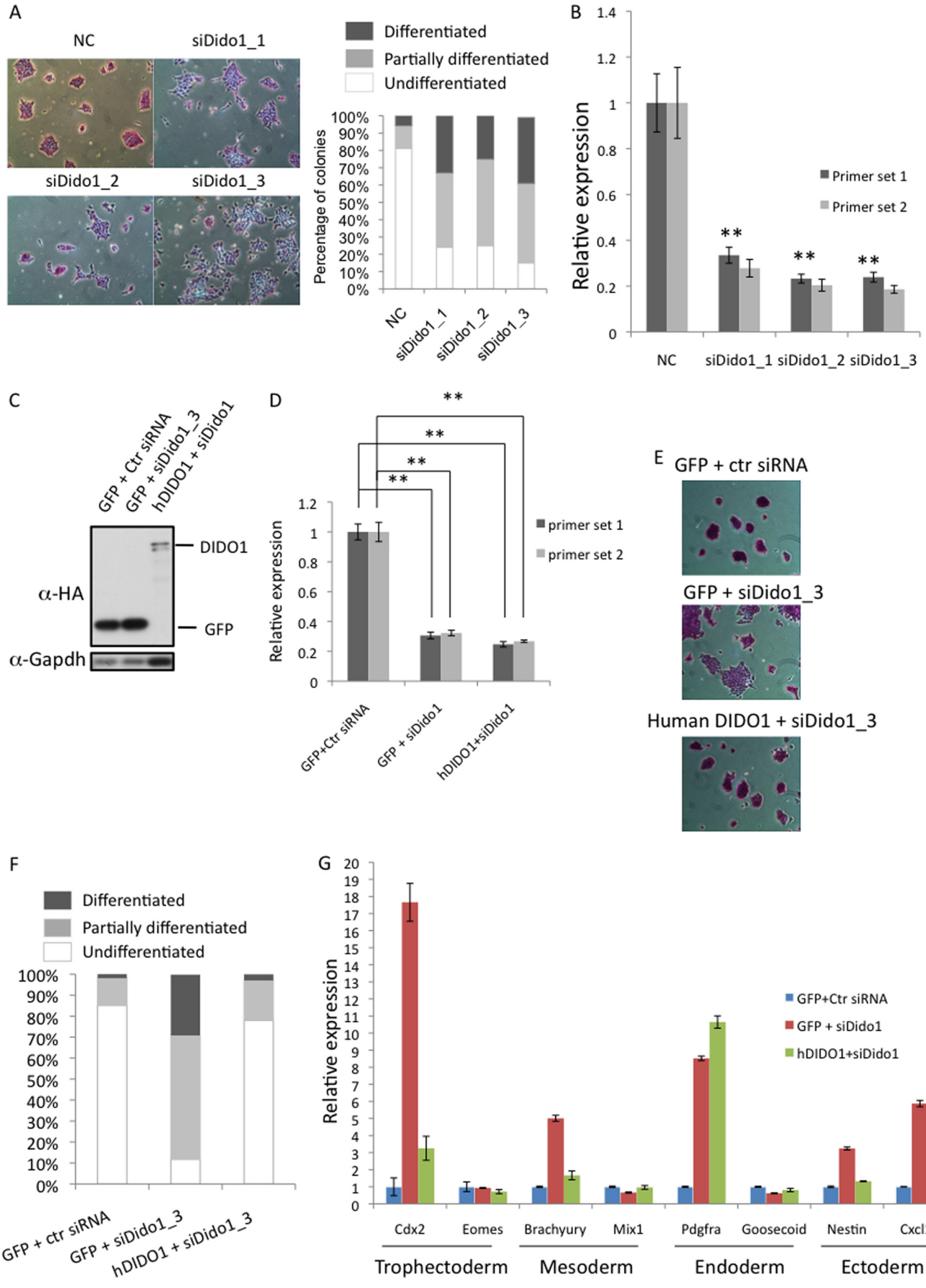


FIGURE 2. **Nanog and Oct4 positively regulate *Dido1* gene expression.** *A*, mouse ES cells stably overexpressing GFP or Nanog were examine by RT-qPCR for *Dido1* mRNA expression. *Error bars* indicate S.D. ($n = 3$). *B*, ChIP experiments were performed using antibodies against endogenous Nanog and Oct4 and primers targeting the promoter region of *Dido1* locus. Positions for the primers are indicated. Rabbit IgG was used as a negative control. *Error bars* indicate S.D. ($n = 3$). *C*, mouse ES cells were transiently transfected with siRNA oligonucleotides against *Oct4* for 2 days before being used for RT-qPCR analysis with primers for *Oct4* and *Dido1*. A scramble siRNA oligonucleotide (*NC*) served as a negative control. *Error bars* indicate S.D. ($n = 3$). *D*, RT-qPCR analysis was similarly carried out for mouse ES cells transiently transfected with siRNA oligonucleotides for *Nanog*. A scramble siRNA oligonucleotide (*NC*) served as the negative control. *Error bars* indicate S.D. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ 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


A
NC
siDido1_1
siDido1_2
siDido1_3
Differentiated
Partially differentiated
Undifferentiated
Percentage of colonies
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
NC
siDido1_1
siDido1_2
siDido1_3
B
Relative expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Primer set 1
Primer set 2
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NC
siDido1_1
siDido1_2
siDido1_3
C
GFP + Ctr siRNA
GFP + siDido1_3
hDIDO1 + siDido1
DIDO1
α-HA
GFP
α-Gapdh
D
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Relative expression
1.2
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0.8
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primer set 1
primer set 2
GFP+Ctr siRNA
GFP + siDido1
hDIDO1+siDido1
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GFP + siDido1_3
Human DIDO1 + siDido1_3
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Differentiated
Partially differentiated
Undifferentiated
100%
90%
80%
70%
60%
50%
40%
30%
20%
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GFP + ctr siRNA
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1
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GFP+Ctr siRNA
GFP + siDido1
hDIDO1+siDido1
Cdx2
Eomes
Brachyury
Mix1
Pdgfra
Goosecoid
Nestin
Cxcl12
Trophectoderm
Mesoderm
Endoderm
Ectoderm

similar to GFP-expressing cells with control knockdown (Fig. 3*E*). 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. 3*F*). In DIDO1 rescue cells, trophectoderm, 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. 4*A*). 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. 4*B*). 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. 4*C*). 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. 4*D*).

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. 4*E*, 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. 4*F*). 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 apoptosis 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 assemble 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,

FIGURE 3. **Depletion of Dido1 in ES cells induces differentiation.** (A) Mouse ES cells were transiently transfected with 3 siRNA oligonucleotides against *Dido1* and examined by alkaline phosphatase staining. A scramble siRNA oligonucleotide (*NC*) served as a negative control. Quantification is shown on the *right*. *B*, knockdown efficiency of the siRNA oligonucleotides from *A* was determined by RT-qPCR using two sets of primers for Dido1. *C*, mouse ES cells stably expressing HA-tagged GFP and DIDO1 were transiently transfected with an siRNA oligonucleotide against *Dido1* from *A* (siDido1_3) and examined by Western blotting with the indicated antibodies after two rounds of siRNA transfection. GAPDH served as a loading control. A scramble siRNA oligonucleotide (*Ctr*) served as a negative control for RNAi. *D–G*, these cells were examined by RT-qPCR (*D*) using two primer sets specific for Dido1, for alkaline phosphatase staining (*E* and *F*), and by RT-PCT for the expression of differentiation markers (*G*). *Error bars* indicate S.D. ($n = 3$). **, $p < 0.01$.

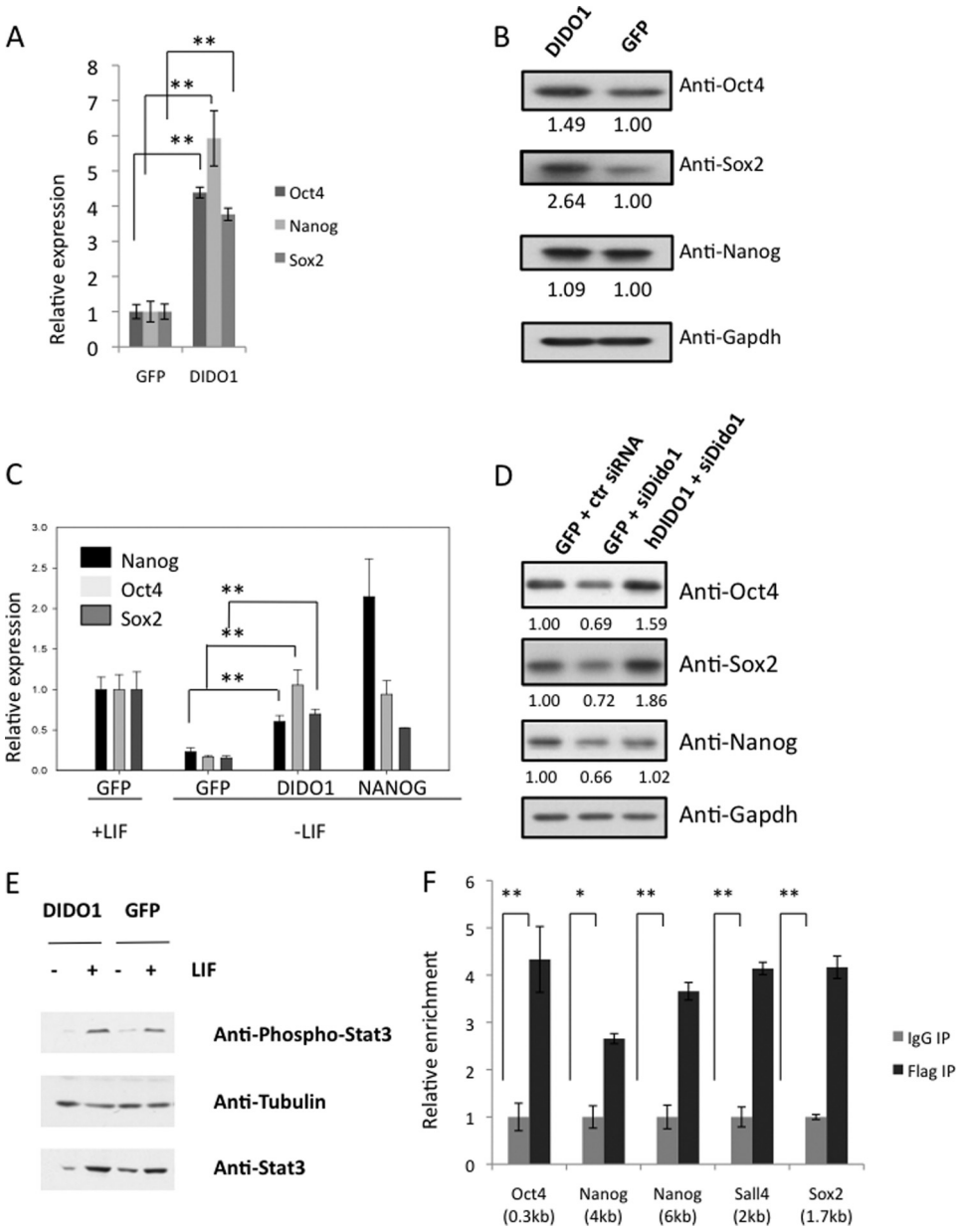

FIGURE 4. **Dido1 feedback regulates the expression of pluripotency factors.** *A* and *B*, mouse ES cells stably expressing DIDO1 were cultured in the presence of LIF and examined for the expression of *Oct4*, *Sox2*, and *Nanog* by RT-qPCR (*A*) and Western blotting (*B*). Cells overexpressing GFP were used as controls. *Error bars* indicate S.D. ($n = 3$). GAPDH was used as a loading control. *C*, mouse ES cells stably expressing GFP (negative control), Nanog (positive control), and DIDO1 were cultured in the absence of LIF. mRNA expression of *Oct4*, *Nanog*, and *Sox2* was analyzed by RT-qPCR. *Error bars* indicate S.D. ($n = 3$). *D*, control, *Dido1* knockdown, and *Dido1* knockdown cells rescued with human DIDO1 expression were cultured in the presence of LIF and examined by Western blotting with the indicated antibodies. GAPDH was used as a loading control. *E*, cells from *A* were cultured in the presence or absence of LIF and examined by Western blotting with the indicated antibodies. Anti-tubulin antibodies were used as loading controls. *F*, mouse ES cells expressing FLAG-tagged DIDO1 were analyzed by ChIP using anti-FLAG antibodies. Rabbit IgG was used as a control. Primers specific for the indicated promoter regions of *Oct4*, *Nanog*, *Sall4*, and *Sox2* were used for qPCR. *Error bars* indicate S.D. ($n = 3$). **, $p < 0.01$. *IP*, immunoprecipitation.

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.

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