DIDO3 acts at the interface of RNAPII transcription and chromatin structure regulation

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ABSTRACT

Chromatin structure and organization has a key role in gene expression regulation. Here, we integrated ChIP-seq, RNA-seq, Hi-C, epigenetic, and cancer-related mutations data to get insight into the role of Death Inducer Obliterator gene (*Dido1*) in RNA pol II (RNAPII) transcription and chromatin structure regulation. Analysis of ChIP-seq data of DIDO3, the largest protein isoform of *Dido1*, revealed binding-sites overlap about 70% with RNAPII and H3K36me3 in the mouse genome, but also significant overlap 10-30% with Polycomb, CTCF, H3K4me3, and H3K27ac. Based on this analysis we propose that DIDO3's PHD domain interacts with H3K36me3 posttranslational modification. Integrating multi-omics data we describe how DIDO3 potentially recruit several transcription factors, including RNAPII, and also regulates genes transcribing those same transcription factors. DIDO3 regulation of the genes translated into proteins to which it binds puts DIDO3 in the center of intricate feedback loops. We showed, by using data from a DIDO3 mutant, that DIDO3 C-terminus is responsible for most of these transcriptional regulation, and is also implicated in other very important pathways by regulating genes encoding for Polycomb-accessory proteins, subunits of the SWI/SNF chromatin remodelling, or Set1/COMPASS chromatin modifier complexes. These multi-protein complexes control gene activation or silencing and also play a role in tumour development. DIDO3 C-terminus region and splice-site for alternative DIDO2/DIDO3 protein isoforms tended to accumulate recurrent truncating mutations identified in the TCGA Pan-Cancer dataset. We hypothesize that deregulation of DIDO3, as it happens with large epigenetic complexes and long-range interactions, leads to cell differentiation deficiency and cancer development. Overall, we propose here a molecular mechanism by which DIDO3, favour RNAPII pausing and long-range chromatin interactions.

INTRODUCTION

Death Inducer Obliterator (*Dido1*) is a complex gene expressed in all tissues (1) that encodes the three protein isoforms DIDO1, DIDO2 and DIDO3 (2). Previous bioinformatics analysis and experimental data revealed that these three protein isoforms have a common plant homeodomain (PHD) that binds to histone 3 trimethylated on lysine 4 (H3K4me3) (3,4). DIDO2 and DIDO3 have a transcription elongation factor S II subunit M (TFSII_M) domain, which mediates binding to RNAPII (3,5), and also have a SPOC domain characteristic of the Spen family proteins involved in transcriptional repression (6) (Figure 1). These three domains connect *Dido1* to chromatin-regulation processes; however, their contribution to cell differentiation and cancer remains to be elucidated.

In order to characterize DIDO3 function, and in particular its C-terminal, isoform specific, domain, Fütterer et al. created a DIDO3 mutant with a deletion of its last exon, the sixteenth (DIDO3ΔE16). They show that the mutation was lethal in early embryonic development (7,8), embryos died by gestation day 8.5. The same authors also showed that expressing DIDO3ΔE16 mutant in embryonic stem cells (ESC) impeded cellular differentiation in vitro, but maintained their self-renewal capacity (5,7,8). Both of these phenotypes, early embryonic lethality and in vitro loss of cellular differentiation were also observed altering other proteins linking them to DIDO3 function. *H3f3a* and *H3f3b* is a first example; their deletion causes embryonic lethality, in this case around day 6.5 (9), and defects in differentiation capacity (10–12). These genes encode for the H3.3 variant of the H3 core histone part of the nucleosome. The H3.3 is associated with chromatin dynamics and nucleosome turnover. H3.3 deposition is mediated by the histone regulator A (HIRA), a chaperone complex, that also associates H3.3 with the RNAPII machinery (13), transcription factors (TFs) (14), and replication protein A (15). Another example of phenotype similar to DIDO3ΔE16 has been observed after a homozygous gene deletion for COMPASS core subunits *Ash2l* and *Dpy30*, and PRC2 core subunits *Eed* and *Suz12*, with embryos dying by gestation day 8.5 (16). To be noted that, in this case, those nucleosome posttranslational modifications multiprotein complexes were not linked loss of differentiation in vitro.

As we said before, DIDO3 PHD domain preferentially binds to H3K4me3 (4). However, PHD domain also recognizes the peptide H3₁₋₂₀ with methylation and acetylation patterns at different positions (i.e., K4me3+K9ac, K4me3+K14ac, K4me3+K18ac)(unpublished results communicated by T.G. Kutateladze *et al*). Although H3K4me3, H3K36me3, and H3K27ac marks are associated with genome activation (17), methylation patterns at H3K4 and H3K36 may cross talk with posttranslational modifications at H3K27 in the recruitment of PRC2; thus contributing to gene expression regulation and facultative heterochromatin establishment (18,19). Detailed descriptions of H3K36me3 function in cryptic transcription, alternative pre-mRNA splicing, 3D chromosome organization and DNA damage response can be found elsewhere (17,19). Nevertheless, affinity of binding of DIDO3's PHD domain to the peptide H3₂₁₋₄₄ with simultaneous methylation and acetylation patterns at K27 and K36 has not been tested.

An N-terminal truncation of *Dido1* (*Dido1*ΔNT), results in genomic instability, due to centrosome amplification and centromere localized DNA breaks (20,21), leading to myeloid malignancies (2). In the last years, several independent studies linked genetic alterations in *Dido1* to different cancer types: Melanoma (22), Kaposiform hemangioendothelioma (23), Chronic Myeloid Leukemia (24), Prostate cancer (25), Head and neck cancer (26), Hepatic neuroendocrine tumours (27), Colorectal cancer (28), Bladder cancer (29), Renal cell carcinoma (30), and Esophageal cancer (31,32). Some of these studies suggested an important role of epigenetic modifications and chromatin remodelling processes in cancer development. Interestingly, a cancer-related phenotype caused by centromere and kinetochore genes overexpression (e.g., *Cenpa* and its chaperone *Hjrp*, *Cenpm*) and protein mislocalization into noncentromeric regions, increases genome instability through centromere misregulation, increases levels of DNA damage, and correlates with poor patient prognosis (33,34). However, the molecular details behind these associations and *Dido1* disruption are not fully understood yet.

In the present work, we report that DIDO3 has a pleiotropic role in RNAPII transcription and chromatin structure regulation, presumably, mediated by participation in large protein complexes. There we underpin the role of DIDO3 chromosome instability in ESC self-renewal and differentiation, assembly of H3.3/CENP-A chaperone complexes, and chromatin structure regulation.

MATERIALS AND METHODS

Software

The following software was used in this study: ChIPseeker (35), clusterProfiler (36), and BEDTools (37) to analyse ChIP-seq data and generate binding site annotations; PyRanges (38) for comparison of genomic intervals; Networkx (39) to represent graphs; TADbit (40) to obtain chromatin three-dimensional interaction matrices, topologically associated domains (TADs) and A/B compartments from Hi-C experiments; Metascape (41) to provide a comprehensive gene list annotation, functional enrichment, and interactome analysis over 40 independent knowledge bases; Epicorr v1.20 (42) to find regions of the human genome epigenetically correlated

with the Dido1 locus; and RStudio (www.rstudio.org) to run R/Bioconductor packages (e.g. [org.Mm.eg.db](http://org.mmm.org), [TxDb.Mmusculus.UCSC.mm10.knownGene](http://txdb.mmusculus.org), [ggplot2](http://ggplot2.tidyverse.org), [edgeR](http://edgeR.bioconductor.org)).

Databases

Cancer associated variants were retrieved from Pan-Cancer TCGA dataset using the cBioPortal tool (www.cbioportal.org) (43). We mined publicly available TCGA transcriptome data via the University of Alabama web portal (UALCAN; <http://ualcan.path.uab.edu>) (44). Protein codes and functional annotations were extracted from UniProtKB (www.uniprot.org) (45), Pfam (<http://pfam.xfam.org/>) (46), and PROSITE (<https://prosite.expasy.org>) (47).

Data processing

ChIP-seq data analysis. The genomic coordinates for HA-DIDO3 binding (GSE85029) were converted to the UCSC mouse genome build mm10 using liftOver (<http://genome.ucsc.edu/cgi-bin/hgLiftOver>). BED files were imported to RStudio and annotated using the R/Bioconductor package ChIPseeker (35). The promoter region was set to -1 kb to 200 bp of the TSS. We also used the Bioconductor packages [org.Mm.eg.db](http://org.mmm.org) and [TxDb.Mmusculus.UCSC.mm10.knownGene](http://txdb.mmusculus.org) for peak annotations. Significance of overlap between ChIP-seq data sets was calculated using the `enrichPeakOverlap` function implemented in ChIPseeker, setting the number of random permutations (`nShuffle`) of the genomic locations to 10000. Genomic coordinates for the overlapped (or intersect) sites were calculated with the `intersectBed` function implemented in BEDTools (37). PyRanges (38) was also used for the comparison of genomic intervals.

RNA-seq and differential gene expression analysis. RNA-seq data for DIDO3 and DIDO3ΔE16 in mouse embryonic stem cells (GSE152346) and mouse embryonic fibroblasts (SRP150516 and PRJNA476070) were processed as described in (8). Briefly, Burrows-Wheeler aligner BWA-MEM 0.7.15 (<http://bio-bwa.sourceforge.net>) was used to align paired-end reads to the UCSC mouse genome build mm10 with standard settings. Alignments were converted to BAM format and de-duplicated with Picard tools 2.9.0 (<http://broadinstitute.github.io/picard/>). To quantify relative expression of transcripts, we ran StringTie 1.3.3 (48) and calculated the transcripts per million (TPM) reads. Sample scaling and statistical analyses were performed with the R package edgeR (49). Transcripts with TPM >0 in all samples were kept for downstream analysis. Differentially expressed genes with an absolute value of log2 fold change ≥0.7 and a false-discovery rate (FDR) <0.05 were considered statistically significant.

Protein-protein interaction, gene ontology and pathway analysis. The complete list of genes with HA-DIDO3 binding sites detected in ChIP-seq, and those with gene expression alterations in RNA-seq data were submitted to Metascape (41) using the multi-gene-list and meta-analysis option. Metascape compiles protein interactions from BioGRID (<https://thebiogrid.org>) and provides a protein-protein interaction network along with sub-networks of highly connected proteins (modules) identified by the MCODE clustering algorithm. Gene ontology (50) and pathway enrichment analysis was also done with Metascape. The core set of default ontologies (i.e. GO processes, KEGG pathways, Reactome gene sets, canonical pathways, CORUM complexes) was applied for enrichment analysis. The hypergeometric test and Benjamini-Hochberg p-value correction algorithms were used by Metascape to identify all ontology terms that contain a statistically greater number of genes in common with the input list than expected by chance.

Hi-C data processing. All Hi-C data collected from GEO were first transformed to FASTQ files using parallel-fastq-dump. Raw reads were processed with TADbit (40) for read quality control, read mapping, interaction detection, interaction filtering, and matrix normalization. Valid interactions were then used to generate genome-wide interaction maps at 100 kb, 50 kb, 20 kb, and 5kb to segment the genome into the so-called A/B compartments, TADs, and perform a meta-analysis, respectively. A/B compartments were calculated using normalized and decay corrected matrices by calculating the first component of a principal component analysis of chromosome-wide matrices of the Pearson product-moment internal correlation as described in (51) and implemented in TADbit (40). TADs were next identified at 50 kb resolution with the TAD border detection method implemented in TADbit (40). Chromatin loop were called using the Mustache software (52) run at 10 kb resolution with default parameters but lowering the sparsity threshold to 0.8 and increasing the p-value threshold to 0.5. Aggregate peak analysis (APA) consists of extracting small submatrices (here 21 by 21 cells at 5 kb resolution) from the genomic Hi-C matrix at the intersection of a specific pair of genomic coordinates (53). Once the submatrices corresponding to all input pairs of genomic coordinates are extracted, these submatrices are averaged into a single APA. The interactions in this APA represent the average interactions between two lists of input coordinates. We then transformed the Cartesian coordinates of the APA into polar coordinates summing distance to the center in both X- and Y-axes (Figure 2E for an example between a list of coordinates of DIDO3 ChIP-seq peaks and a list of coordinates of H3K36me3 ChIP-seq peaks). The distance to the center in terms of number of 5 kb bins is then used to separate interaction cells in two categories, close to the center (less or equal to 5 bins), away from the center (the rest). The interactions in cells within these two categories were then compared using a T-test of independence in order to assess how strong is the interaction enrichment in the center. We interpret significant

enrichment in the center of the APA as an indicator of tendency to colocalize in the nucleus, between the proteins or marks in the input lists of coordinates.

Computational analysis of epigenetically correlated regions. In order to look for regions of the human genome epigenetically correlated with *Dido1* locus we used the epigenomic datasets compiled by the Roadmap Epigenomics Consortium (54). We downloaded the 127 “consolidated epigenomes”, for which the chromatin states are given in a 15-state vocabulary for all 200 bp windows (https://egg2.wustl.edu/roadmap/web_portal/chr_state_learning.html). The 127 epigenomes include different tissues, embryonic stem cells, cancer cell lines, different developmental stages, etc. For these epigenomes, detailed information on different proxies of the epigenetic state is provided at a base-pair resolution, such as histone acetylation, DNA methylation, DNase accessibility or RNA-seq expression. For each 200bp genomic region, the ChromHMM method (55) is used to collapse all these experimental epigenetic proxies into a single “epigenetic state” with 15 possible values: Active TSS (TssA), Flanking Active TSS (TssAFlnk), Transcription at gene 5’ and 3’ (TxFlnk), Strong transcription (Tx), Weak transcription (TxWk), Genic enhancers (EnhG), Enhancers (Enh), ZNF genes & repeats (ZNF/Rpts), Heterochromatin (Het), Bivalent/Poised TSS (TssBiv), Flanking Bivalent TSS/Enhancer (BivFlnk), Bivalent Enhancer (EnhBiv), Repressed PolyComb (ReprPC), Weak Repressed PolyComb (ReprPCWk), and Quiescent/Low (Quies). We downloaded from the URL above the BED files with these chromatin states for the 127 epigenomes. To work at lower resolutions (e.g. 1 kb window), the epigenetic state of a given window is taken as that with the highest frequency among the 200bp segments within the window; a minimum frequency of 80% is required otherwise, the *undefined* state is assigned to that window. In this work, the whole human epigenome was scanned with 1 kb non-overlapping windows. The epigenetic profile of a given window represents those epigenetic states across all samples, and can be seen as a vector of length 127 (epigenomic samples) where each component can take 15 possible values (epigenetic states). Finally, the “epigenetic correlation” between two genome windows (G_i and G_j) is quantified from their corresponding vectors as their “mutual information” (MI), an entropy-based parameter, according to the formula:

$$MI(G_i, G_j) = \sum_{k=1}^{15} \sum_{l=1}^{15} P(G_{ik}, G_{jl}) \cdot \log_2 \frac{P(G_{ik}, G_{jl})}{P(G_{ik}) \cdot P(G_{jl})}$$

Where the sums run for the 15 possible epigenetic states. $P(G_{ik})$ and $P(G_{jl})$ are the frequencies of epigenetic states k and l in windows G_i and G_j respectively, and $P(G_{ik}, G_{jl})$ the frequency of state k in window G_i matching state l in window G_j in the same samples. State *undefined* is ignored for this calculation, and the terms of the sums where either $P(G_{ik})$ or $P(G_{jl})$ are 0 are skipped. Consequently, this parameter has a high value for two windows when their epigenetic profiles correlate.

Results

Previous sequence similarity searches of *Dido1* have proposed its role in chromatin stability, transcriptional regulation and cancer (3,6). But additional experimental results accumulated over years, from our laboratory and others, support the proposed roles for *Dido1* as well as raise new hypotheses (4,5,7,8,20,56–58). It is also known that aberrant chromatin architecture causes transcriptional dysregulation, which is largely associated with cancer development and progression. Transcriptional dysregulation also plays a major role in other cancer hallmarks such as reprogramming of cell metabolism (63). In Figure 1 we summarize the DIDO3 annotations in different resources (Pfam domains, PROSITE motifs, and low-complexity regions).

The combined computational analysis of DIDO3 binding sites (ChIP-seq; GSE85029), transcriptome data of DIDO3ΔE16 (RNA-seq; GSE152346), and protein-protein interactions indicated that CT-domain is critical for the transcription of different subunits in large protein complexes. In Table 1 we defined a set of primary targets, with changes in expression upon DIDO3 deletion (DIDO3ΔE16) and DIDO3 binding over the whole gene, and secondary target with only expression changes without nearby DIDO3 ChIP-seq peak. For more details see Supplementary Table S1 and Supplementary Files 1 and 2. Importantly, some of the protein complexes highlighted in Table 1 regulate chromatin structure and function, including: CCCTC-binding factor (CTCF) through *Bhlhe40*, Cohesin, H3.3 chaperones, Polycomb repressor complex 2 (PRC2), SWI/SNF, and Set1/COMPASS. On the other hand, the unique and disordered CT-domain of DIDO3 (Figure 1) modulates RNA metabolism through enhancing its interactions with proteins (8,58). In fact, interactions between DIDO3 and proteins whose encoding genes contain binding-sites in ChIP-seq (Table 2 and Supplementary Table S1) were confirmed by our group, using proximity ligation assay, co-purification, immunoprecipitation experiments, and proteomics analysis (8,58). Such is the case of splicing factor SFPQ (58), helicase DHX9 that unwinds DNA and DNA:RNA hybrids (8), and chromatin decondensation factor NCL (58).

DIDO3 binding sites contribute to CTCF-mediated long-range chromatin interactions

A re-analysis of ChIP-seq experiments in DIDO3 wild-type ESC (GSE85029) confirmed the co-localization of DIDO3 and RNAPII in the mouse genome. 79% of DIDO3 sites (2269 out of 2888) overlap with RNAPII (Figure 2A and Supplementary Table S2). This result was expected to some extent based on previous work (4). But this analysis also revealed an unexpected overlap of DIDO3 binding sites with H3K36me3 (69%, 2002 intersect sites), CTCF (13%, 360 intersect sites), and PRC2 (8%, 242 intersect sites) with significant p-values (Figure 2A and Supplementary Table S2). This result suggests a role of DIDO3 in chromatin organization as both, CTCF and PRC2 contribute to chromatin long-range interactions (60). Also, CTCF can attract other TFs to chromatin, including tissue-specific transcriptional activators, repressors, cohesin and RNAPII (61,62).

We observed that various DIDO3 primary target genes (e.g., *Dido1*, *Hmga1*, *Klf2*, *Smarcc1*, *Smarcd1*, *Pus3*) listed in Table 1 and Supplementary Table S1, have been associated to intra-chromosomal CTCF-CTCF interactions in mESC (63). CTCF-loops can be affected by a downregulation of *Bhlhe40* a DIDO3 secondary target gene detected in the RNA-seq of DIDO3ΔE16 ESC (Table 1). BHLHE40 is a novel CTCF partner identified by co-immunoprecipitation experiments (64). It has been demonstrated that loss of BHLHE40 in HeLa-S3 cells led to reduction of CTCF binding to its recognition sites (i.e. a consensus sequence containing CpG dinucleotides), and to the disruption of CTCF-mediated long-range chromatin interactions (64). Altogether, considering the CTCF-TF cooperation in actively transcribed genes, and the downregulation of *Bhlhe40*, we suggest that DIDO3 may contribute to the regulation of CTCF-loops at specific loci.

Regulation of cohesin complex formation

Other genomic regions involved in higher-order chromatin organization (i.e. “insulator sites”) have been associated to different functions, including: activation, repression, alternative splicing, and protection from DNA methylation. However, the molecular mechanisms behind these pleiotropic functions remain poorly understood (61). In this scenario of “insulator sites”, we also identified a downregulation of *Mau2* in the RNA-seq of DIDO3ΔE16 ESC (Table 1). The chromatid cohesion factor MAU2 is a protein subunit of the cohesin-loading complex. Protein-coding gene *Mau2* has a DIDO3 binding site in wild-type ESC and its downregulation in DIDO3ΔE16 may affect CTCF-cohesin complex formation. We speculate that the downregulation of *Mau2* could favour the CTCF self-association and clustering through internal RNA-binding regions in CTCF's Zinc fingers 1, 10, and 11 (65), thus affecting CTCF insulation function. Remarkably, RAD21 and SMC3 others subunit of the cohesin-complex also had a DIDO3 binding site in wild-type ESC (Table 2). Proteins MAU2, RAD21, and SMC3 interacted through the NIPBL-MAU2 heterodimer and the cohesin-complex composed of SMC1A, SMC3, RAD21 and STAG1. Taken together, these results point to the molecular interplay between DIDO3 and CTCF-cohesin complex.

Insulator sites showed a strong binding of the CTCF-cohesin complex, and are generally, but not always, flanked by distinct compartments on either side (61). Compartments refer to actively transcribed (open or A-compartment) and transcriptionally repressed (closed or B-compartment) regions. A recent study showed that the decrease of insulator-based long-range interactions causes deregulation of heterochromatic regions and formation of H3K27me3 “micro-domains” (2-8 nucleosomes) in distant euchromatin (66). Downregulation of *Bhlhe40* and *Mau2* in DIDO3ΔE16 ESC may contribute to the deregulation of heterochromatin and the spreading of H3K27me3 to distant gene loci (64). This could be an additional mechanism by which DIDO3 regulates gene expression in an epigenetic and structural context (see results below for TADs and compartments).

DIDO3 may specifically bind to H3K36me3 through its PHD domain

Intriguingly, DIDO3 seems to bind preferentially to two distinct sets of regions (Figure 2A and 2B). First to enhancers and promoters, overlapping with H4K27ac, H3K4me3 and CTCF. This result can be put in the context of DIDO3 binding to its own promoter, and regulating *Dido1* transcriptional activation (5). Second DIDO3 binds to gene body and intergenic regions, overlapping with RNAPII and H3K36me3 respectively. In the intergenic regions, H3K36me3 is significantly isolated from any of the other mark we studied. This isolation of H3K36me3 means that it cannot be a secondary target of DIDO3 through any of the other studied mark, and that this interaction has thus a very high specificity. Overall the significant overlap between DIDO3 and H3K36me3 binding sites (69%, 2002 intersect sites), and most importantly its high specificity in intergenic regions, prompted us to study the amino acid sequence of the PHD domain. The human DIDO3 PHD domain binds H3K4me3, and its crystallographic structure in complex with H3K4me3 is available (PDB ID: 4L7X) (4). Here, we compared the PHD amino acid sequences from mouse DIDO3 and yeast negative regulator of transcription elongation (BYE1), histone demethylase (ECM5), modulator of histone acetylation (NT01), and histone ubiquitination (SNT2) proteins with selective binding of H3K4 or H3K36 tri-methylations (67) (Figure 2C). DIDO3 PHD domain showed the highest similarity (30% sequence identity) with ECM5 and shares most of the key residues in the H3K36me3-binding pocket. According to the alignment and localization of residues in the crystallographic structure of DIDO3, we propose that Y267 in the equivalent position to ECM5-Y1240 could contribute to the H3K36me3 specificity. In this regard, the amino acid sequence flanking the K36 in mouse H3.3 is the most similar to yeast H3 (Figure 2C). All

together, this observation puts DIDO3 in an unexpected place, as H3K36me3 in intergenic regions is related to facultative heterochromatin or DNA damage response (68,69). This hypothesis about H3K36me3 binding is consistent with previous results that link DIDO3ΔE16 mutant to splicing defects (58). H3K36me3 is required for efficient pre-mRNA splicing (70).

DIDO3 regulates nucleosome modifying and histone chaperone machinery complexes

DIDO3 is a chromatin reader that recognizes H3K4me3 through its PHD domain (4), and also has a TF role in the *Dido1* locus (5). As we said before for the CTCF-cohesin complex, we found DIDO3 binding sites in genes coding for different components of nucleosome modifying and histone chaperone machinery complexes, with few of them undergoing expression changes in DIDO3ΔE16 ESC (Tables 1 and 2, and Supplementary Table S1).

The chromatin regulator SETD7, a primary target of DIDO3, illustrates the interconnected network of interactions. SETD7 interacts with Polycomb subunits EED and SUZ12 among other proteins. Of these chromatin and histone binding proteins, SETD7, EED, and SUZ12 had DIDO3 binding-sites in wild-type ESC (Tables 1 and 2), while the coding gene *Setd7* was downregulated in DIDO3ΔE16 ESC (Table 1). Importantly, other coding genes for Polycomb accessory proteins (i.e. *Aebp2*, *Epop*) also had DIDO3 binding sites in wild-type ESC, and were transcriptionally upregulated in DIDO3ΔE16 ESC (Table 1).

Another result connecting our hypothesis with chromatin regulators was that SETD5 mediated trimethylation of H3K36 on active gene bodies (71). SETD5 interacts with the PAF1 complex, a general transcription elongation factor of the RNAPII. The ChIP-seq analysis revealed that H3K36me3 may be bound by DIDO3's PHD domain (69% overlap, and seemingly exclusive overlap in intergenic regions, Figure 2A), which supported a functional relationship between SETD5 and DIDO3. Besides, *Setd5* gene-loss in mice lead to abnormal transcription with impaired RNA maturation and detrimental effects on splicing, and *Setd5* haploinsufficiency impaired the proliferation of neural progenitors and result in behavioural deficits in mice (71). RNA-seq of DIDO3ΔE16 ESC identified a downregulation of *Setd5* (Table 1), and coincidentally, DIDO3ΔE16 mutant produced splicing defects (58), and generated brain abnormalities and behavioural alterations in adult mice (57).

Various protein-coding genes linked to histone H3 modifications, chromatin remodelling, and transcriptional activation (e.g. *H3f3a*, *H3f3b*, *Carm1*, *Kdm2a*, *Nsd1*, and *Mapk11*) were identified in the ChIP-seq and transcriptome data of DIDO3. All these genes had DIDO3 binding-sites in wild-type ESC, while *Carm1* and *Mapk11* were also downregulated in DIDO3ΔE16 ESC (Tables 1 and 2). H3F3A and H3F3B represent an epigenetic imprint of transcriptionally active chromatin. CARM1 methylates histone H3 at Arg-17 leading to transcriptional activation through chromatin remodelling. NSD1 and KDM2A respectively write and erase the dimethylation of H3K36 (our proposed DIDO3 binding site). MAPK11 phosphorylates histone H3, and is a component of the MAP kinase (p38 MAPKs) signal transduction pathway. This pathway regulates chromatin modifiers and remodellers essentials for the initiation of chromatin condensation in mitosis and related to the activation of poised genes (72).

The last example comprised the link between DIDO3 and the regulation of heterochromatin. Based on the analysis of Hi-C (GSE99530), ChIP-seq (GSE85029), and RNA-seq (GSE152346) data in mice ESC we revealed that: i) about 12% (336 positions) of DIDO3 binding sites occurred in heterochromatic regions (Figure 2D), ii) the vast majority of DIDO3 binding sites co-localized in the 3D chromatin structure (Figure 2F), iii) 23-26% (11,467 to 12,852 positions) of CTCF binding sites are located in heterochromatic regions (Figure 2D), iv) DIDO3ΔE16 transcriptionally regulated the Polycomb accessory components *Aebp2* and *Epop*, and v) insulator-based long-range interactions causing deregulation of heterochromatic regions could also be affected by DIDO3ΔE16. In addition, the aggregate peak analysis (53) indicated that DIDO3 binding sites are close to RNAPII, H3K36me3, H3K4me3, and H3K27ac in the chromatin 3D structure of mESC (Figure 2F). The 3D spatial interaction of these proteins is more prominent in the DIDO3ΔE16 downregulated than in upregulated genes (Figure 2F, last two rows).

We detected DIDO3 binding sites in *Cenpa*, *Cenph*, *Cenpv* and *Hjurp*, reinforced by an observed upregulation of *Cenpb* and *Cenpm* in DIDO3ΔE16 ESC (Table 1). These protein-coding genes are involved in the centromere-associated protein network and the histone H3 chaperone complex of CENP-A variant. We also observed a DIDO3 binding site in wild-type ESC and a downregulation of *Trim28* in DIDO3ΔE16 ESC (Table 1). TRIM28 is a scaffold protein of the heterochromatin protein 1 (CBX5/HP1A) present in the pericentric heterochromatin in the mouse centromere (73). Interestingly this observation can be linked to previous results based on that centromeric DNA is actively transcribed by RNAPII, and it is the presence of H3K4me1, H3K4me2, H3K36me2 and H3K36me3 that promotes RNAPII activity, and is essential for HJURP targeting and CENP-A assembly (74,75).

DIDO3 CT-domain affects gene expression of Polycomb components and different subunits of chromatin modifiers and remodeller complexes

Polycomb mediates long-range chromatin interactions between distal genes, favouring transcriptional repression (60). Our meta-analysis of gene expression in DIDO3ΔE16 ESC (GSE152346), DIDO3 binding sites (GSE85029), and

protein-protein interaction (PPI) data, revealed that DIDO3 could regulate long-distance chromatin contacts through the Polycomb-mediated interactions (Tables 1 and 2, and Figure 3).

Polycomb repressive complex II (PRC2) regulates pluripotency, differentiation, and tumorigenesis through catalysis of H3K27me3 on chromatin (68). The computational analysis presented here indicated that *Eed*, *Ezh2*, and *Suz12* genes coding for the core subunits of PRC2 have DIDO3 binding sites in ESC, and that their encoded proteins are localized in densely connected regions of the PPI network (Table 2 and Figure 3). Other genes coding for accessory proteins of PRC2 such as *Aebp2*, *Ctbp2*, *Elob* (or *Tceb2*), *Eloc* (or *Tceb1*), *Epop*, *Jarid2*, and *Pcl2* (or *Mtf2*) also had DIDO3 binding sites and some of their encoded proteins were localized as well in densely connected regions of the PPI network (Figure 3). But, more significantly, *Aebp2* and *Epop* were overexpressed in DIDO3ΔE16 ESC, suggesting that DIDO3 acts as a repressor for these genes (Table 1). AEBP2 is a DNA-binding transcriptional repressor specific for RNAPII. EPOP is a scaffold protein between the PRC2/EED-EZH2 complex and the elongin BC (ELOB-ELOC) complex, that plays a key role in genomic regions with both active and repressive chromatin properties — the so-called bivalent regions (76). These results agree with the hypothesis that alterations in the PRC2 recruitment in active chromatin contributes to the high number of overexpressed genes 524 (66%) out of the 785 transcriptionally regulated in DIDO3ΔE16 ESC (Figure 3A). We also speculate that defective gene silencing lead to defective cell differentiation. In concordance with that, DIDO3ΔE16 impairs differentiation capacity in ESC, as has been experimentally determined (5).

Moreover, *Phf1* and *Mtf2* genes encode two H3K36me3 readers that recruits PRC2 to H3K36me3-containing chromatin regions (68). While *Phf1* seems to be indirectly upregulated by DIDO3, as it is downregulated in DIDO3ΔE16 ESC, the gene expression of *Mtf2* was unaltered but presented a DIDO3 mark (Tables 1 and 2).

Other multiprotein complexes that maintain the precise gene expression states and prevent human pathologies are COMPASS and SWI/SNF. A misregulation of COMPASS, SWI/SNF, and PRC2 activities impairs the ratio of activated and repressed genes necessary for the maintenance of cell pluripotency and differentiation. Enhancer regions can be characterized by either high levels of H3K4me1 and H3K27me3 in poised enhancers or by H3K27ac in active enhancers. Indeed, in pluripotent cells it has been suggested that these bivalent regions are enriched at the developmental genes that are kept in a poised transcriptional state, ready to be activated or repressed upon stimulation by pro-differentiation signals. PRC2 mediates interactions between poised enhancers and developmental genes to facilitate their transcriptional induction upon differentiation (76). COMPASS can counteract H3K27me2/3 repressive marks deposited by PRC2, and thus favour H3K27ac. Here, we found that about 25% upregulated and 16% downregulated genes in DIDO3ΔE16 (Figure 3C) were bona fide bivalent genes described in mouse ESC (76). In addition, bivalent genes are significantly upregulated compared to non-bivalent genes (Fisher's test: p=0.005) according to RNA-seq data in DIDO3ΔE16.

The central core of COMPASS and COMPASS-related complexes is called WARD (or WRAD), because it is composed of WDR5, ASH2, RBBP5, and DPY30 proteins. Most of their coding genes *Wdr5*, *Ash2l*, and *Dpy30* had DIDO3 binding sites in wild-type ESC (Table 2). In addition, WDR5 as well as PRC2 core proteins (EED, EZH2, SUZ12) were localized in densely connected regions of the PPI network (Figure 3B). *Setd1b* and *Wdr82* genes that code for proteins of the Set1/COMPASS complex also have DIDO3 binding sites in ESC (Tables 1 and 2). SETD1B is a histone methyltransferase that specifically methylates Lys-4 of histone H3. Its coding gene, *Setd1b*, is overexpressed in DIDO3ΔE16 and thus repressed by DIDO3 in wild-type. It is thus reasonable to assume that this regulation may induce a decrease in H3K4me3 for which DIDO3 is a reader (Table 1). WDR82 is a regulatory component that facilitates histone H3 Lys4 methylation via recruitment of SETD1B to the Ser5 phosphorylated C-terminal domain of RNAPII large subunit. All these data suggested the contribution of DIDO3, and its connection to RNAPII, PRC2, and COMPASS in the regulation of gene expression dynamics during ESC self-renewal and differentiation.

The SWI/SNF (or BAF) complex is an ensemble of chromatin remodelling enzymes of around 15 subunits that hydrolyze ATP to mobilize nucleosomes and free DNA. This complex has a direct role in antagonizing Polycomb-mediated repression. Yet, SWI/SNF appears to also promote repression by Polycomb in stem cells and cancer (77,78). Several genes encoding for SWI/SNF subunits, for example *Arid1a*, *Bcl7b*, *Gltscr1l*, *Phf10*, *Smarca5*, *Smarca41*, *Smarcc1*, and *Smarce1* have DIDO3 binding sites in wild-type ESC. Among them, *Gltscr1l* and *Smarca41* are upregulated, while *Smarcc1* is downregulated in our RNA-seq data from DIDO3ΔE16 ESC (Tables 1 and 2). Specifically, the expression of *Smarcc1* is necessary for heterochromatin formation and chromatin compaction during mouse ESC differentiation (77). Therefore, this finding suggests a deregulation in the mechanism of heterochromatin formation in DIDO3ΔE16 ESC. Downregulation of both *Smarcc1* (SWI/SNF) and *Phf1* (PRC2) exacerbated gene-silencing defects caused by DIDO3ΔE16 in ESC. In the same line of evidences, a recent study reported that rapid SWI/SNF depletion leads to quick redistribution of PRC2 from highly occupied domains to weakly occupied sites genome-wide (78). This resulted in transcriptional derepression, chromatin decompaction, and opposite epigenomic changes to repressed domains (78).

Our PPI-network analysis indicates that SMARCC1 is positioned in the same cluster that core proteins of PRC2 and COMPASS (Figure 3B). As mentioned before, SMARCC1 plays important roles in mouse ESC differentiation by coupling gene repression with global and local changes in chromatin structure (77). As well, protein interactions among SMARCC1 (SWI/SNF), WDR5 (COMPASS), and SUZ12 (PRC2) include the transcription factor POU5F1. *Pou5f1* is a core stemness gene and a key regulator of self-renewal and differentiation in ESC. Previous experimental results from our laboratory demonstrated that gene expression of *Pou5f1* and *Dido1* are coordinated (8). Again, these results together supported the functional relationship we found among genes with DIDO3 binding sites in wild-type ESC, those with expression changes in DIDO3ΔE16 ESC, and regulation of the chromatin structure mediated by PRC2, COMPASS and SWI/SNF complexes.

DIDO3 contributes to chromatin structure regulation

As we said in the introduction, DIDO3ΔE16 ESCs did not differentiate in vitro, but maintained their self-renewal capacity (7,8). Here, we also investigated the relationship between DIDO3 binding around transcription start sites (TSS) of up- and downregulated genes in DIDO3ΔE16 ESC (Figure 3D left pane). The frequency of DIDO3 binding sites around TSS -50kb upstream to +50kb downstream shows a similar distribution for upregulated and downregulated genes. We observe that the binding of DIDO3 in TSS is significantly higher than random in genes up- or down-regulated upon DIDO3 deletion of exon 16. However we observed that this increased specificity is comparable to other actors for which we had ChIP-seq (Figure 3D right panels). According to this, we hypothesize that DIDO3 is not the primary determinant, but it could be an accessory subunit for the RNAPII transcription activation and repression mechanisms.

Based on these results, we studied the relationships between DIDO3 binding sites, chromatin loops (e.g., CTCF and Polycomb), and TAD boundaries. The combined analysis of ChIP-seq (GSE85029, GSM2645517, GSM2645511, GSM2645495, GSM2645513, GSM2645515, GSM2645497, GSE120393, GSM2645509) and Hi-C data in mESC (GSE99530) shows enrichment for DIDO3, RNAPII, H3K36me3, H3K4me3, H3K27ac, and CTCF binding sites inside the chromatin loops (Figure 4A). Interestingly DIDO3 was found to be the mark most enriched in the *inside* of loops, between anchor points (positive coordinates in Figure 4A). In previous observations DIDO3 was overlapping to other marks, but here it can be differentiated from CTCF sites maintaining the loop and even, to a lesser extent, from RNAPII. We also defined six classes of chromatin loops (i.e., all, Polycomb, enhancer-promoter, CTCF, CTCF+enhancer-promoter, and CTCF-only) and analysed the enrichment of DIDO3, RNAPII, H3K36me3, H3K4me3, and H3K27ac within these classes in comparison with a random distribution (Figure 4B). This comparison indicated a significant enrichment for DIDO3 and RNAPII binding sites in the inside of “CTCF only” loops but not in “Polycomb” loops (Figure 4B). And following our observation about the presence of DIDO3 in the inside of chromatin loops, in Figure 4B we can narrow in this pattern to “CTCF only” loops suggesting that it can be a consequence of the physical occupancy of the CTCF and perhaps associated proteins. In the context of TAD boundaries (Figure 4C) we observed a significant enrichment CTCF protein but also of RNAPII and histone marks related to transcriptional activity (H3K4me3, H3K27ac, H3K36me3). Interestingly, among the proteins and histone marks measured, DIDO3 is, in proportion, the most enriched in TAD boundaries.

Finally, we also checked the distribution of DIDO3 marks in chromatin compartments. Overall, and as suggested by previous observations, DIDO3 marks were enriched in active A compartments (Figure 4D). We observed a similar distribution when looking at genomic bins (100 kb resolution) containing genes activated or repressed upon deletion of exon 16 of DIDO3 (DIDO3ΔE16) (Figure 4E). Up- and down-regulated genes seem to be respectively less and more in A compartments, but this small difference is hard to associate to the presence of DIDO3 marks as it may just be a consequence of their definition. For instance: down regulated genes show a tendency to be more A compartment, but probably because to be detected as “down-regulated” they need to show a decrease and thus start with a minimum level of activity.

Similar epigenetic patterns suggest a co-regulatory role of DIDO3 and chromatin-associated complexes.

Previously in this work, we identified some important chromatin regulation mechanisms altered by DIDO3ΔE16 in mouse. Now we investigated these regulation mechanisms in human cells, and searched for correlated chromatin marks over the *Dido1* locus and other regions genome-wide. For this, we applied the Epicorr v1.20 methodology (see Materials and Methods for details).

The *Dido1* locus shows a variable epigenetic pattern across samples in a part of the locus (~61,545 kb-61,555 kb in chromosome 20) (Figure 5 top panel). Scanning all 1 kb windows within this fragment against all 1 kb windows of the 24 human chromosomes we identified 167 pairs of genomic regions with correlation score higher than 0.7 (a complete list of the regions is available at http://csbg.cnb.csic.es/pazos/DIDO1_epigen/). Among these correlated regions, we found enhancers, promoters, and coding regions associated to the human genes *ARID1A*, *BCL11A*, *CENPA*, *CENPE*, *CENPF*, *HJURP*, and *CTBP2* (Figure 5 and Supplementary Table S3). Interestingly, the mouse homologous genes of *ARID1A*, *CENPA*, *HJURP*, and *CTBP2* also had DIDO3 binding sites in wild-type ESC (Table 2).

We know that *Dido1* produces the same three protein isoforms by alternative splicing in mouse and human cells (2). In fact, mouse and human DIDO3 sequences are 75% identical. All together, these results indicate that there is a co-regulatory role of DIDO3, HJURP/CENP-A chaperone complex, SWI/SNF, and PRC2 in both the mouse and human cells.

CENP-A is a centromere marker associated with chaperone HJURP in mammals, which facilitates deposition into chromatin (79,80). Additionally, active RNAPII machinery is physically associated with CENP-A chromatin at early G1, as demonstrated in HeLa cells (81). Since DIDO3 binds to the RNAPII (5) and 78.6% of its binding sites co-localize with the RPB1 subunit of RNAPII (Figure 2A and supplementary table S2), it is reasonable to think that DIDO3 CT-domain and RPB1 C-terminus domain cooperate in the assembly of a large macromolecular complex. DIDO3 CT-domain contains repetitive sequences and low-complexity regions that are commonly found in protein interfaces (Figure 1). Unfortunately, detailed structural data about the DIDO3 CT-domain in large protein complexes is not available yet.

To further analyse these 167 regions with an epigenetic pattern correlated to that in *Dido1* locus, we retrieved the genes matching these regions with bedtools (37). For that we downloaded from the NCBI the genome assembly our epigenomic data is based on (GRCh37.p13). Any gene overlapping, totally or partially, with the genome windows of interest was retrieved and a “functional enrichment analysis” was carried out with the topGO R package (<https://bioconductor.org/packages/topGO/>), using the Gene Ontology (50) annotations for the human genes downloaded from the NCBI. Among the list of significant terms we found two main categories: *i*) cell adhesion and signalling (in orange in supplementary file 3), and *ii*) cell division and chromosome segregation (in green in supplementary file 3). These two categories match the DIDO3 mutants phenotype in centromere localized DNA breaks (21) and in cellular differentiation defects (5,8). These results in human and mouse strengthen the link between DIDO3 and centromere transcription, but also with the CTCF-cohesin complex in sister chromatid cohesion.

Recurrent alterations in different cancer types accumulate in the DIDO3 splice-site and CT-domain

It is known that deregulation of chromatin structure is associated with cancer development (59,60). On the other hand, *Dido1* gene alterations have been recently reported in small cohorts of cancer patients (Table 3). However, *Dido1* is not classified as a potential cancer driver gene. We studied the distributions of *Dido1* alterations, and the mutational pattern of other chromatin regulatory genes in a large cohort of patients. For this, we used the cBioPortal tool and the TCGA PanCancer dataset to study about 10,528 tumour samples with mutation, structural variants, and copy number alterations from 32 different studies. About 10,439 of the samples have mutations, and 9,896 have both mutations and copy number alterations.

The analysis revealed that only 415 (4%) out of 10,439 queried samples have mutations in *Dido1* (Figure 6A left panel). A total of 535 mutations (462 missense, 54 truncating, 16 splice-site, and 3 structural variants) were identified. The number of tumours with copy-number alterations in *Dido1* was even lower: 211 (2%) out of 9,896 queried samples, containing 208 amplifications and 3 deletions (Figure 6A right panel and Supplementary File 4). In addition, we investigated alterations in *Dido1* gene expression in tumour samples. In general, the number of samples with gene expression data was lower in normal samples, and thus presents a higher variability (Supplementary Table S4). This high variability may be the reason why even-though 13 out of the 24 tumour types presented significant expression difference when compared to normal, these differences were over-expressions in tumour in only 9 of them. These result suggest either that *Dido1* has a tumour specific mechanisms of action or, and being more conservative because of the high variability of the data, that *Dido1* presents a similar average expression across the 24 tumour types (Figure 6B).

We also studied the distribution of mutations that were identified in at least two different samples along the DIDO3 amino acid sequence. Out of the 6 truncating mutations in *Dido1*, 4 are positioned in the DIDO3 CT-domain, after the PHD domain, and may thus be comparable to the DIDO3ΔE16 mutant (p.G1745Afs*119, p.P1908Lfs*111, p.P2034Rfs*240, and the splice site p.X1181_splice in Figure 6C).

Other genes coding for different components of the CTCF-cohesin, PRC2, SWI/SNF, Set1/COMPASS, and centromere-associated protein network showed gene expression changes upon DIDO3ΔE16. Each of these macromolecular complexes coordinate their activities to regulate chromatin structure and gene expression dynamics (60,73). Remarkably, according to the TCGA-PanCancer dataset, tumour samples with truncating mutations in the DIDO3 CT-domain (black dots, Figure 6C) also accumulate mutations in genes such as: *BHLHE40* and *MAU2* (CTCF-cohesin); *AEBP2*, *EPOP*, and *PHF1* (PRC2); *BICRAL*, *SMARCA1*, and *SMARCC1* (SWI/SNF); *SETD1B* (Set1/COMPASS); *CENPB* and *CENPM* (centromere protein network), *SETD5*, *SETD7*, and *TRIM28* (chromatin regulators). These genes are the human homologs of DIDO3 primary and secondary target genes in mouse ESC (Table 1). Except for *CENPM*, *EPOP* and *SETD7*, the remaining genes accumulated significantly more mutations in tumour samples with truncating mutations in the DIDO3 CT-domain (Fisher Exact test, $P < 0.001$,

according to cBioPortal)(Figure 6D). Taken together, these observations point again to the molecular interplay between DIDO3 and large macromolecular complexes that regulates chromatin structure and gene expression dynamics in mouse and human species.

DISCUSSION

Chromatin modifiers, TFs, CTCF-cohesin, and RNAPII are key regulators in gene expression mechanisms that ensure cellular differentiation (82–84). In the RNA-seq data, we observed an enrichment of genes coding for multi-protein complexes related to chromatin remodellers, chromatin binding, and histone modifiers among the upregulated and downregulated genes. Many of these multi-protein complexes interact with RNAPII and are at the crossroads of gene regulation and genome 3D architecture.

A possible feedback loop between RNAPII transcription and DIDO3 targets

RNAPII transcription is not only restricted to protein-coding and a large set of non-coding genes, but also widespread in intergenic regions (85). Accurate regulation of gene expression and repression mechanisms is essential for cell differentiation and cancer development processes. DIDO3 binds to the RNAPII (5) and DIDO3ΔE16 mutant shows altered RNA splicing and transcription termination in mESC and MEF (8,58,86). Our analysis of ChIP-seq data indicates a significant overlap between the DIDO3, RNAPII, H3K36me3, H3K27ac, H3K4me3, and CTCF in mESC. Among the strongest colocalization signals is the colocalization between DIDO3 and H3K4me3, which is in agreement with experimental data about the specificity of the PHD domain (4). But we also found a significant overlap between DIDO3 and the repressive complex PRC2. However our analysis was not able to discern which of these interactions were due to direct interactions or to common targets. For instance the overlap between H3K4me3 and PRC2 is completely unexpected (87), and can only be explain because both may target promoter regions. In order to refine this analysis of colocalization, we divided the genome in sub-regions (promoter regions and intergenic regions). By this mean we were able to isolate some interesting signals. In intergenic regions, DIDO3 was no longer colocalizing with any mark or protein related to transcriptional repression suggesting that its colocalization with PRC2 at promoters may be, as it is also probably the case for H3K4me3, circumstantial. This analysis also revealed an interesting correlation: in intergenic regions DIDO3 and H3K36me3 colocalize significantly, while, at the same time, H3K36me3 is significantly separated from any other mark or protein tested. This result put H3K36me3 as a strong candidate to be a target of DIDO3. Following this hypothesis we compared DIDO3's PHD domain to other PHD domains with a H3K36me3 specific pocket and found a high sequence similarity. We believe that both of these observations suggest strongly that DIDO3 selectively recognizes and binds to H3K36me3.

Based on our prediction that DIDO3 recognizes H3K36me3, we can reasonable describe its role in RNA splicing and transcription termination (8,58,86). This claim is not simple to explain assuming that DIDO3's PHD domain binds to H3K4me3 (4). H3K36me3 has been associated with cryptic transcription, alternative pre-mRNA splicing, and 3D chromatin organization (19,70). On the other hand, numerous studies have shown that CTCF mediates RNAPII pausing (62,85,88) and RNA splicing events (89,90), however the determinants of such distinct functions are still unknown. We hypothesize that the DIDO3 CT-domain not only alters interactions with splicing factor SFPQ (58) and helicase DHX9 (8), but also may contribute to the recruitment of other proteins crucial for the RNAPII processivity (e.g. HDAC1, CDK12, PAF1). Interestingly we also observed a possible feedback loop between protein subunits in large RNAPII-associated complexes and their coding genes, with a DIDO3 binding site. Our analysis of ChIP-seq data revealed that several of these actors coding genes were harbouring a DIDO3 binding site (RNAPII largest subunit RPB1 - *Polr2a*, splicing factor *Sfpq*, helicase *Dhx9*, H3K27ac specific histone deacetylase *Hdac1*, cyclin-dependent kinase *Cdk12* which phosphorylates the C-terminal domain of RPB1 and regulates transcription elongation, RNAPII-associated factor *Paf1* also involved in transcription elongation). Even if we were not able to find significant differences in gene expression in DIDO3ΔE16, we believe that they could be involved in this feedback loop. These findings raise new hypotheses that should be tested experimentally.

Different researchers have studied the interplay between RNAPII pausing and chromatin structure regulation (91). Here we postulate that DIDO3 contributes to RNAPII pausing and also to chromatin structure regulation.

Role of DIDO3 in 3D chromatin structure

The analysis of 3D colocalization performed through the aggregate peak analysis (APA) revealed similar patterns to that observed by overlapping ChIP-seq peaks in the linear genomic sequence. DIDO3 colocalizes mostly with indicators of transcriptional activity, as H3K4m3, H3K27ac, H3K36me3, RNAPII, active promoter and enhancers. To a lesser extent, but also significantly we found DIDO3 colocalizing with repressive Polycomb and H3K27me3. Finally, we found less interaction between DIDO3 and CTCF. This last observation contrasts with previous results showing a very clear overlap between CTCF bindings and DIDO3 bindings, suggesting that the interaction between DIDO3 and CTCF could be circumstantial (i.e. DIDO3 binds near promoters that happens to be close from CTCF-CTCF loop anchors). This observation about the CTCF-DIDO3 link can also be related to the overlap of DIDO3 with

CTCF-CTCF anchor points, the presence of CTCF at anchor points is negatively correlated with the presence of DIDO3, which seems to be displaced towards the inside of the loop (25 kb to 100 kb). To be noted that this trend is not specific to DIDO3, we observed the same behaviour for RNAPII and histone marks related to active transcription, however this displacement is the strongest for DIDO3. With respect to DIDO3's location at TAD borders, we observed a clear enrichment, again following CTCF proteins and transcriptional activity. Finally, with respect to the location of genes marked with DIDO3 within A/B compartments, we observed an expected enrichment in A compartments. When dividing genes with DIDO3 marks into up- and down-regulated genes we observed that up-regulated genes in DIDO3ΔE16 were more prone to be in B compartments while down-regulated genes, more in the active compartment type. As up-regulated genes must start at low expression level to detect increases in expression, and down-regulated must start expressed to detect repression, what we observed was surprising in the sense that even up-regulated genes (with currently low expression) were significantly far from B-compartments. This observation that genes potentially directly down regulated by DIDO3 are in A-compartments is in favour of a very dynamic regulation.

DIDO3 function as a transcription factor

Although the exact mechanism underlying DIDO3 and DIDO3ΔE16 mutant remains to be characterised, several possibilities can be proposed based on previous studies and our combined analysis of ChIP-seq, RNA-seq, and Hi-C data in mESC and MEF.

(1) *RNAPII pausing*. The RNAPII elongation rate can be controlled by chromatin 3D organisation and influence alternative splicing decisions. Interestingly, DIDO3 may participate in a transcription-coupled methylation/deacetylation mechanism allowing induced genes to be more rapidly repressed when their expression is no longer required. As we said before, the H3K36me2 specific methyltransferase *Nsd1* and the H3K27ac specific deacetylase *Hdac1* had a DIDO3 binding site, as well as primary or secondary targets histone methyltransferases specific *Setd7* (H3K4me1), *Setd1b* (H3K4me2/3), *Setd5* (H3K36me3). A second possible function of DIDO3 in a transcription-coupled methylation/deacetylation mechanism could be to reassemble a repressive chromatin configuration that switches off cryptic promoter-like sequences within transcribed regions, and repress antisense transcription in coding regions (92). This assumption is based on the predicted capacity of DIDO3 to bind H3K36me3 and its putative interaction with HDAC1. Both, H3K36me3 and histone deacetylase complexes provide a type of negative feedback to attenuate regulation of genes that must be not only activated but also repressed with the proper kinetics (19,93). Once the DIDO3 PHD-domain recognizes H3K36me3, DIDO3 CT-domain (1080a.a. in mouse) in association with the C-terminal domain of RPB1 (377a.a. in mouse Supplementary FigureS1 in (94)) can recruit HDAC1 erasing acetylation on H3K27ac; thus preventing transcription initiation from cryptic promoter-like sequences within the body of genes. In addition, our ChIP-seq analysis indicates that the gene coding for PAF1, a putative DIDO3 interactor that modulates the RNAPII-elongation rates, also had a DIDO3 binding site in wild-type ESC. Other genes coding for proteins potentially involved in DIDO3 interactions, and required for pre-mRNA splicing and related to spliceosome assembly (*Prpf4*, *Prpf6*, *Prpf38b*, *Prpf39*, *Srsf1*, *Srsf10*, *Srsf11*) also had a DIDO3 binding site.

(2) *Regulation of PRC2 guidance and DNA methylation*. PRC2 is a protein complex that silences gene expression via epigenetic modifications of chromatin. But recently, single-molecular force spectroscopy experiments and computational simulations support the formation of a cooperative PRC2-mediated DNA loop (i.e. hairpin-like) and the implication of core subunits EZH2, SUZ12 and accessory AEBP2 (95). This study suggests that accessory proteins may regulate the genomic location of PRC2. Our combined analysis indicates that *Ezh2*, *Suz12* and *Aebp2* had DIDO3 binding sites, and moreover, *Aebp2* is overexpressed in DIDO3ΔE16 mutant cells. Concerning DNA methylation, another transcriptional silencing mechanism, it has been suggested that DNA (cytosine-5)-methyltransferases DNMT3A and DNMT3B bind to H3K36me3 epigenetic mark (96,97). In addition, DNMT3B interacts with the PRC2/EED-EZH2 complex (98,99). In the ChIP-seq data we detected DIDO3 binding sites on *Dnmt3b*, *Kdm2a*, and *Hdac1*. Histone demethylase KDM2A modify H3K36. Therefore, a cross talk between DIDO3 and H3K36me3 seems to potentially have a regulating role on DNA methylation through the complexes HDAC1-DNMT3A-DNMT3B, putting DIDO3 in the centre of the repressive marking of the chromatin.

(3) *Regulation of SWI/SNF chromatin regulation complex*. The ATP-dependent chromatin remodelling complexes (i.e. SWI/SNF) regulate the CTCF-cohesin-mediated chromatin interactions (100). SWI/SNF modulates the accessibility of naked DNA, can slide nucleosomes along DNA, and eject octamer histone core or histone dimers from chromatin. Then SWI/SNF facilitates the generation of nucleosome-free DNA, necessary for initiation of transcription, and in general binding of proteins such as CTCF. In this work we report the DIDO3 binding and transcription regulation by DIDO3ΔE16 of different components of SWI/SNF (*Arid1a*, *Bcl7b*, *Smarcc1*, *Smarcad1*, *Smarca5/Snf2h*, *Smarce1*, *Gltscr11/Bicral*, *Phf10*).

(4) *Misregulation of centromere associated proteins*. In the ChIP-seq and RNA-seq data of mESC, *Cenpa* and *Incenp* had a DIDO3 binding site, while *Birc5* is an overexpressed secondary target gene. Previous experiments with

DIDO3 mutants found double-strand DNA breaks adjacent to centromeres, and related this finding to mitotic spindle defects (21). Other protein-coding genes involved in the centromere-associated protein network (i.e., *Cenpb*, *Cenph*, *Cenpm*, *Cenpv*, *Hjurlp*, *Mis18bp1*, *Dnmt3b*) also had DIDO3 binding sites and/or were up-/down-regulated in DIDO3ΔE16 mutant cells. In addition, our computational analysis in different human cells indicates a correlated pattern of chromatin marks over the *DIDO1* locus and *CENPA*, *CENPE*, *CENPF*, and *HJURP* genomic regions.

(5) *Regulation of Cohesin loading factors*. Cohesin rings are needed to attach the sister chromatids before they are segregated. A misregulation of cohesin-loading or cohesin-release processes may cause mitotic defects due to precocious sister chromatid separation. Our combined analysis of ChIP-seq and RNA-seq data revealed that *Mau2* and *Wapl*, two cohesin-loading factors, had a DIDO3 binding site in wild-type mESC and *Mau2* was downregulated in DIDO3ΔE16 mutant cells.

CONCLUSION

Overall, our findings indicate that DIDO3 is a keystone of RNAPII processivity that operates at the interface of transcription and chromatin structure regulation. We put together a selection of experimental results from our laboratory and others, and propose a new target for DIDO3 (H3K36me3). We also place DIDO3 in the spotlight of the chromatin structure regulation from the level of: *i*) promoter enhancer loops, *ii*) potential indirect interaction with CTCF, *iii*) regulation of cohesin loading complexes, *iv*) regulation of SWI/SNF, *v*) regulation of Polycomb specificity, *vi*) prevention/reparation of double strand breaks, and *vii*) regulation of proteins involved in chromosome segregation. In order to exactly understand the mechanisms allowing DIDO3 to regulate or at least intervene in these many aspects of RNAPII processivity and chromatin structure further experimental studies are needed. We believe that our work highlights the relevance of DIDO3 by proposing and confirming its implication in pathways essential to cellular differentiation, and related to cancer development.

DATA AVAILABILITY

The datasets supporting the conclusions of this article are accessible on the Gene Expression Omnibus (GEO) platform under accession number: GSE85029 (HA-DIDO3), GSM2645517 (Rpb1), GSM2645511 (H3K36me3), GSM2645495 (H3K4me3), GSM2645513 (H3K27ac), GSM2645497 (H3K27me3), GSM2645515 (CTCF), GSE120393 (PRC2), GSM2645509 (Ring1B) for ChIP-seq experiments; GSE152346 (DIDO3 and DIDO3ΔE16 in mouse embryonic stem cells) for RNA-seq experiments; and GSE99530 (mESC), GSE121087 and GSE115983 (MEF) for Hi-C experiments. RNA-seq data for DIDO3 and DIDO3ΔE16 in mouse embryonic fibroblasts is accessible on the NCBI sequence read archive with accession number SRP150516 and PRJNA476070. The complete list of correlated genomic regions identified by Epicorr v1.20 is available at http://csbg.cnb.csic.es/pazos/DIDO_epigen/.

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Table and Figure Legends

Table 1. Primary and secondary target genes with expression changes between DIDO3ΔE16 and WT ESC

^(a) Gene position is represented by chromosome (Chr) number and strand directions in parenthesis (forward (+) and reverse (-)); ^(b) Unique MACS2 identifiers of ChIP-seq peak according to the HA-DIDO3 dataset (GSE85029); ^(c) Gene expression changes, log2 fold change (log₂FC) and adjusted p-values (FDR) between DIDO3ΔE16 and WT ESC, were calculated from RNA-seq data in edgeR. ^(d) UniProtKB annotations (www.uniprot.org). Hyphens (-) indicate that ChIP-seq peaks were not detected.

Table 2. Genes with DIDO3 binding-sites and their proteins would have DIDO3 interactions

^(a) Gene position is represented by chromosome (Chr) number and strand directions in parenthesis (forward (+) and reverse (-)); ^(b) Unique MACS2 identifiers of ChIP-seq peak according to the HA-DIDO3 dataset (GSE85029); ^(c) UniProtKB annotations (www.uniprot.org).

Table 3. Cancer types with *Dido1* genetic alterations reported in the literature

^(a) The two alleles present at each *Dido1* locus contain the genetic variant. Hyphens (-) indicate that heterozygosity is not provided.

Figure 1. Scheme of protein isoforms produced by alternative splicing of the *Dido1* gene

Amino acids start and end positions for the Pfam domains, PROSITE motifs, and low-complexity regions are indicated in parenthesis using the numbering scheme of the mouse sequence (UniProtKB accession number: Q8C9B9). Experimentally determined protein interactions are also shown at bottom. PHD: PHD finger; TFIIS_M: Transcription factor S-II central domain; SPOC: Spen paralogue and orthologue C-terminal domain; NLS_BP: Bipartite nuclear localization signal; ZF_PHD_1 and ZF_PHD_2: Zinc finger PHD-type; TFIIS_CENTRAL: TFIIS central domain; CCT: CCT domain; ARG_RICH: Arginine-rich region; PRO_RICH: Proline-rich region; THR_RICH: Threonine-rich region; GLU_RICH: Glutamic acid-rich region; ASP_RICH: Aspartic acid-rich region. Rojas et al (3) predicted a dPHD domain of approximately 25 amino acids -not shown here- and positioned 67 residues downstream of the PHD domain. The annotations about protein domains, motifs, and low-complexity regions are publicly available at: <http://pfam.xfam.org/> and <https://prosite.expasy.org> (last accessed on May 12, 2021).

Figure 2. DIDO3 binding sites intersection with RNAPII, CTCF, Polycomb, and histone H3 modifications

(A) Distribution of intersects peaks (i.e., DIDO3, RNAPII, CTCF, Polycomb, and H3 modifications) over chromosomes. (B) Percentage of intersects peaks across promoters and intergenic regions. (C) Sequence comparison among PHD domains and histone H3 variants in yeast and mouse. We also show the PHD 3D-structure and the position of key residues in the peptide-binding pocket. (D) Percentage of DIDO3 and CTCF peaks in euchromatin and heterochromatin regions. (E) Example of APA in polar coordinates showing the average interaction enrichment between DIDO3 and H3K36me3 coordinates (F) Colocalization of DIDO3 binding sites with other ChIP-seq marks in the 3D chromatin structure of mouse ESC. First row show DIDO3 marks against other marks with not filters. Second row shows the interaction between marks near any gene. Third row shows the interaction between marks near gene upregulated in DIDO3ΔE16. Fourth row shows the interaction between marks near gene downregulated in DIDO3ΔE16. Proximity between gene and mark requires any overlap inside the same 5 kb bin.

Figure 3. DIDO3 target genes coding for Polycomb subunits and chromatin modifiers are interconnected.

(A) Circlos plot of the genes detected in ChIP-seq and RNA-seq data. (B) Protein-protein interactions network and densely connected sub-networks detected by MCODE. (C) Percentage of bivalent genes up- and down-regulated in DIDO3ΔE16 ESC. (D) Frequency of DIDO3 binding sites around TSS (-50kb upstream to 50kb downstream) of the up- and down-regulated genes compare to a random distribution.

Figure 4. DIDO3 is tightly linked to chromatin structure.

Active promoter category are promoter regions with both H3K27ac and H3K4me3 marks, *enhancers* regions enriched in H3K27ac at least 40 kb away from any gene position of ChIP-seq marks with respect to loop anchor points. Positive coordinates represent the inside of loops (between loop anchor points). (A) Overall position of ChIP-seq marks around loop anchor points. Notches in boxplots represent 95% confidence of the median after 1.000 bootstraps. (B) Enrichment of ChIP-seq marks around loop anchor points with 100 bootstraps error band. In grey are shown the result of 100 randomizations of the ChIP-seq mark coordinates. *all* are any loop detected by the software Mustache. *Enhancer-promoter* loops are loops with anchor points away from PRC2 or Ring1B (> 20 kb) and close to H3K27ac or H3K4me3 (< 40 kb). *CTCF* loops are loops with anchor points away from PRC2 or Ring1B (> 20 kb) and close to CTCF marks (< 20 kb). *CTCF + enh-prom* loops are loops with anchor points away from PRC2 or Ring1B (> 20 kb), close to H3K27ac or H3K4me3 (< 40 kb) and close to CTCF marks (< 20 kb). *CTCF only* loops are loops with anchor points away from PRC2 or Ring1B or H3K27ac or H3K4me3 (> 20 kb), and close to CTCF marks (< 20 kb). (C) Same as (A) with TAD borders detected by TADbit. (D) Distribution of compartment scores in all 100 kb genomic bins (left), bins containing up regulated genes in DIDO3ΔE16, down regulated genes in DIDO3ΔE16, bins with DIDO3 ChIP-seq mark, bins with the mark and upregulated genes, and finally (right) bins with the mark and down regulated genes. (E) Changes in A/B compartment score in mESC and mEF highlighting in black bins with DIDO3 mark in mESC, in red bins with the mark and genes with significant expression increase in mESC, and, in blue bins with the mark and genes with significant expression decrease in mESC.

Figure 5. Genomic regions with informative epigenetic patterns

The epigenetic state of each 200 bp portion (small boxes) is indicated with colors. For details, see legend at the top left panel. The two discussed *Dido1* regions (1000 bp windows) are highlighted in large boxes, surrounded by their epigenomic neighborhood. The names of the epigenetic sample groups are on the top right corner, colored using the tissue-based color schema of the Roadmap Epigenomics Consortium.

Figure 6. ***Dido1* genetic alterations in the TCGA-PanCancer dataset.** (A) Bar plot of the *Dido1* missense mutations, copy-number alterations, and predictions of gene fusions. (B) Box plot of *Dido1* mRNA expression in normal and tumour samples according to the UALCAN web-portal (<http://ualcan.path.uab.edu>). Significantly different TPM values (Student's t-test, $P < 0.001$) in normal-vs-tumour samples were indicated with an asterisk (*). (C) Lollipop plots showing the distribution of coding variants (i.e., missense, nonsense and frameshift variants) across DIDO3 functional domains. (D) Mutation frequency of DIDO3 primary and secondary targets in tumor samples with "truncating mutations" in the DIDO3 CT-domain. (*) Indicates statistical significance (Fisher Exact test, $P < 0.001$).

Table 1

<i>Gene</i>	<i>Chr</i> ^(a)	<i>ChIP-seq</i> ^(b)	<i>RNA-seq</i> ^(c)	<i>Description</i> ^(d)
Primary target gene				
<i>Setd7</i>	Chr3 (-)	#1718	log ₂ FC = -0.86 FDR = 1.16e-02	Histone methyltransferase (H3K4me1)
<i>Carm1</i>	Chr9 (+)	#2735	log ₂ FC = -0.81 FDR = 1.73e-03	Histone methyltransferase (H3R17me2)
<i>Dido1</i>	Chr2 (-)	#1664 to #1671	log ₂ FC = -4.81 FDR = 1.21e-11	H3K4me3 reader, RNA pol II associated
<i>Hmga1</i>	Chr17 (+)	#1174, #1175	log ₂ FC = -1.56 FDR = 2.77e-10	Nucleosome phasing
<i>Mau2</i>	Chr8 (-)	#2626, #2627	log ₂ FC = -3.03 FDR = 3.24e-16	Cohesin-loading complex (chromatid cohesion factor)
<i>Trim28</i>	Chr7 (+)	#2336	log ₂ FC = -0.81 FDR = 1.73e-03	Scaffold protein for the heterochromatin protein 1 (CBX5/HP1A)
<i>Aebp2</i>	Chr6 (+)	#2299, #2300	log ₂ FC = 2.12 FDR = 8.72e-08	Polycomb accessory protein (PRC2)
<i>Epop</i>	Chr11 (-)	#509, #508	log ₂ FC = 1.00 FDR = 1.44e-08	Polycomb accessory protein (PRC2)
<i>Set1b/Setd1b</i>	Chr5 (+)	#2148	log ₂ FC = 1.01 FDR = 2.7e-03	Set1/COMPASS complex protein, methyltransferase activity (H3K4me2/3)
<i>Smarcc1</i>	Chr9 (+)	#2838	log ₂ FC = -1.25 FDR = 1.51e-04	SWI/SNF complex, chromatin remodeling
<i>Smarcad1</i>	Chr6 (+)	#2225	log ₂ FC = 0.92 FDR = 2.02e-02	SWI/SNF complex, chromatin remodeling
<i>Gltscr1l/Bicral</i>	Chr17 (-)	#1242	log ₂ FC = 0.88 FDR = 3.35e-04	SWI/SNF complex, chromatin remodeling
Secondary target gene (downstream)				
<i>Bhlhe40</i>	Chr6 (+)	-	log ₂ FC = -1.44 FDR = 3.46e-07	Novel CTCF binding partner
<i>Birc5/Survivin</i>	Chr11 (+)	-	log ₂ FC = 7.76 FDR = 3.35e-03	Recruitment of chromosome passage protein complex (CPC) to centromeres
<i>Cenpb</i>	Chr2 (-)	-	log ₂ FC = 0.81 FDR = 2.19e-03	Centromere-associated protein
<i>Cenpm</i>	Chr15 (-)	-	log ₂ FC = 0.73 FDR = 1.21e-02	Centromere-associated protein
<i>Mapk11</i>	Chr15 (-)	-	log ₂ FC = -0.95 FDR = 1.82e-02	Phosphorylate histone H3 and the Nucleosomal protein HMGN1
<i>Pcl1/Phf1</i>	Chr17 (+)	-	log ₂ FC = -1.71 FDR = 4.2e-02	Polycomb accessory protein (PRC2), H3K36me3 reader
<i>Setd5</i>	Chr6 (+)	-	log ₂ FC = -4.17 FDR = 3.28e-26	Histone methyltransferase (H3K36me3)

Table 2

<i>Gene</i>	<i>Chr</i> ^(a)	<i>ChIP-seq</i> ^(b)	<i>Description</i> ^(c)
<i>Set</i>	Chr2 (+)	#1490, #1491	Nucleosome assembly and histone chaperone
<i>Ep300</i>	Chr15 (+)	#969	Histone acetyltransferase (H3K122ac, H3K27ac)
<i>Hdac1</i>	Chr4 (-)	#1915	Histone deacetylase (H3K27ac)
<i>Hsp90aa1</i>	Chr12 (-)	#678, #679, #680	Modulate histone deacetylases and participate in the eviction of histones from the promoter region
<i>H3f3a</i>	Chr1 (-)	#143	Histone H3.3
<i>H3f3b</i>	Chr11 (-)	#556, #557, #558	Histone H3.3
<i>Hjurp</i>	Chr1 (-)	#82	H3 chaperone complex of CENP-A variant
<i>Cenpa</i>	Chr5 (+)	#2062	Centromere-associated protein, CENP-A variant
<i>Cenph</i>	Chr13 (-)	#808, #809, #810	Centromere-associated protein
<i>Cenpv</i>	Chr11 (-)	#403	Centromere-associated protein
<i>Mis18bp1</i>	Chr12 (-)	#635	Required for recruitment of CENP-A to centromeres
<i>Dnmt3b</i>	Chr2 (+)	#1592	DNA methyltransferase 3B (CpG in pericentromeric). Interacts with centromere-associated protein CENP-C
<i>Cdca8</i>	Chr4 (-)	#1900	Chromosomal passenger complex (CPC) protein
<i>Incenp</i>	Chr19 (-)	#1402, #1403	Chromosomal passenger complex (CPC) protein
<i>Kdm2a</i>	Chr19 (-)	#1362, #1363, #1364	Histone demethylase (H3K36me2)
<i>Nsd1</i>	Chr13 (+)	#747, #748	Histone methyltransferase (H3K36me2)
<i>Morf4l2/Mrgx</i>	ChrX (-)	#2885	Binds H3K36me2/3, yeast Eaf3 homolog
<i>Psip1</i>	Chr4 (-)	#1858, #1859, #1860	Binds H3K36me3, recruits splicing factors
<i>Ncl</i>	Chr1 (-)	#76, #77, #78, #79	Induces chromatin decondensation by binding to histone H1
<i>Snw1</i>	Chr12 (-)	#670	Spliceosome component, yeast Prp45 homolog
<i>Prpf4</i>	Chr4 (+)	#1856	Pre-mRNA splicing, U4/U6-U5 tri-snRNP complex
<i>Prpf6</i>	Chr2 (+)	#1682	Pre-mRNA splicing, U4/U6-U5 tri-snRNP complex
<i>Prpf38b</i>	Chr3 (-)	#1782	Pre-mRNA splicing
<i>Prpf39</i>	Chr12 (+)	#631, #632, #633	Pre-mRNA splicing
<i>Rad21</i>	Chr15 (-)	#935	Cohesin complex
<i>Smc3</i>	Chr19 (+)	#1441	Cohesin complex
<i>Wapl</i>	Chr14 (+)	#836	Regulates cohesin loading onto chromatin
<i>Eed</i>	Chr7 (-)	#2469, #2470	Polycomb core protein (PRC2)
<i>Ezh2</i>	Chr6 (-)	#2215	Polycomb core protein (PRC2)
<i>Suz12</i>	Chr11 (+)	#464	Polycomb core protein (PRC2)
<i>Ctbp2</i>	Chr7 (-)	#2559	Polycomb accessory protein (PRC2)
<i>Elob/Tceb2</i>	Chr17 (-)	#1150	Polycomb accessory protein (PRC2)
<i>Eloc/Tceb1</i>	Chr1 (-)	#22	Polycomb accessory protein (PRC2)
<i>Jarid2</i>	Chr13 (+)	#736, #737	Polycomb accessory protein (PRC2)
<i>Pcl2/Mtf2</i>	Chr5 (+)	#2115 to #2118	Polycomb accessory protein (PRC2)
<i>Wdr5</i>	Chr2 (+)	#1476, #1477	COMPASS complex, core protein (WARD)
<i>Ash2l</i>	Chr8 (-)	#2603, #2604	COMPASS complex, core protein (WARD)
<i>Dpy30</i>	Chr17 (-)	#1272, #1273	COMPASS complex, core protein (WARD)
<i>Wdr82</i>	Chr9 (+)	#2827, #2828, #2829	Set1/COMPASS complex protein
<i>Arid1a</i>	Chr4 (-)	#1933, #1934	SWI/SNF complex, chromatin remodeling
<i>Bcl7b</i>	Chr5 (+)	#2169	SWI/SNF complex, chromatin remodeling
<i>Smarca5/Snf2h</i>	Chr4 (+)	#1962, #1963	SWI/SNF complex, chromatin remodeling
<i>Smarca1</i>	Chr11 (-)	#525	SWI/SNF complex, chromatin remodeling
<i>Phf10</i>	Chr17 (-)	#1117	SWI/SNF complex, chromatin remodeling

Table 3

Cancer type	Cohort size	Genetic alteration	Heterozygosity ^(a)	Reference
Melanoma	n = 8	<i>Dido1</i> expression	-	25
Kaposiform Hemangioendothelioma	n = 1	p.E49*	Homo	26
Chronic Myeloid Leukemia	n = 60	<i>Dido1</i> expression	-	27
Prostate	n = 281	<i>Dido1</i> expression	-	28
Head and neck	n=279	loss-of-function mutations, CNVs	-	29
Hepatic neuroendocrine	n=22	p.D850H, p.I749M	-	30
Colorectal	n=51	p.R1145H	-	31
Bladder	n=20	<i>Dido1</i> expression	-	32
Kidney clear cell renal cell	n=34	<i>Dido1</i> expression	-	33
Esophageal squamous cell	n=50	<i>Dido1</i> expression	-	34
	n=50	<i>Dido1</i> expression	-	35

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Supplementary Table and Files

Supplementary Table S1. Genes with expression changes between DIDO3ΔE16 and WT ESC, and putative DIDO3 interactions in large protein complexes

^(a) Gene position is represented by chromosome (Chr) number and strand directions in parenthesis (forward (+) and reverse (-)); ^(b) Unique MACS2 identifiers of ChIP-seq peak according to the HA-DIDO3 dataset (GSE85029); ^(c) Gene expression changes, log2 fold change (log₂FC) and adjusted p-values (FDR) between DIDO3ΔE16 and WT ESC, were calculated from RNA-seq data in edgeR. ^(d) UniProtKB annotations (www.uniprot.org). Hyphens (-) indicate that ChIP-seq peaks or gene expression changes were not detected.

Supplementary Table S2. DIDO3 binding sites intersection with RNAPII, CTCF, Polycomb, and H3 modifications

^(a) Query ChIP-seq sample, ^(b) Target ChIP-seq sample, ^(c) Number of query peaks, ^(d) Number of target peaks, ^(e) Number of overlapped peaks between query and target, ^(f) Percentage of overlapped peaks, ^(g) the genomic region analysed, ^(h) calculated p-value by ChIPseeker, ⁽ⁱ⁾ p-value correction (FDR) according to the Benjamini and Hochberg method. The values were obtained using the ChIPseeker command `enrichPeakOverlap(queryPeak=file1, targetPeak=file-list, TxDb=TxDb.Mmusculus.UCSC.mm10.knownGene, pAdjustMethod="BH", nShuffle=10000, chainFile=NULL, verbose=FALSE)` and a number of randomly permutations in the genomic locations of 10000. +++ Overlap more than expected (Boferroni corrected p-value < 0.001). ++ Overlap more than expected (Boferroni corrected p-value < 0.01). + Overlap more than expected (Boferroni corrected p-value < 0.05). --- Overlap less than expected (Boferroni corrected p-value < 0.001). -- Overlap less than expected (Boferroni corrected p-value < 0.01). - Overlap less than expected (Boferroni corrected p-value < 0.05). NS non-significant overlap.

Supplementary Table S3. Correlated genomic regions identified by Epicorr v1.20

^(a) Genomic regions are represented by chromosome number and start-end positions; ^(b) Epicorr v1.20 correlation score above 0.7; ^(c) UniProtKB annotations (www.uniprot.org). ^(d) Chromatin states that overlap in the two correlated regions. Acronyms: enhancers (Enh); genic enhancers (EnhG); strong transcription (Tx); weak transcription (TxWk).

Supplementary Table S4. List of cancer types, number of tumor and normal samples, and statistical significance in the gene expression

The statistical significance (P-values) of normal-vs-tumour differences in gene expression levels was estimated by Student's t-test according to the UALCAN web-portal (<http://ualcan.path.uab.edu>). Significantly different TPM values (P < 0.001) were highlighted in bold.

Supplementary File 1. Summary of the RNA-seq analysis

Supplementary File 2. ChIPseeker annotations for the DIDO3 ChIP-seq dataset

Supplementary File 3. Gene Ontology enrichment analysis for the genes within the correlated regions identify by Epicorr v1.20

Supplementary File 4. *Dido1* genomic alterations annotated in TCGA-PanCancer Atlas studies

Supplementary Table S1

<i>Gene</i>	<i>Chr</i> ^(a)	<i>ChIP-seq</i> ^(b)	<i>RNA-seq</i> ^(c)	<i>Description</i> ^(d)
Primary target gene				
<i>Jade1</i>	Chr3 (+)	#1708 to #1713	log ₂ FC = -5.91 FDR = 2.74e-04	Histone acetylation (H4K5ac, H4K8ac, H4K12ac), HBO1 complex
<i>Wdr33</i>	Chr18 (+)	#1300	log ₂ FC = 0.96 FDR = 6.47e-04	RNA pol II associated (mRNA 3'-end processing)
<i>Dhx16</i>	Chr17 (+)	#1220, #2021	log ₂ FC = -1.07 FDR = 6.49e-04	mRNA splicing
<i>Mbd3</i>	Chr10 (-)	#226, #227, #228	log ₂ FC = 0.81 FDR = 1.02e-03	Methyl-CpG-binding domain protein
<i>Pus3</i>	Chr9 (+)	#2744, #2745, #2746	log ₂ FC = 3.94 FDR = 2.3e-09	tRNA pseudouridine synthase
<i>Klf2</i>	Chr8 (+)	#2656, #2657	log ₂ FC = 0.7 FDR = 2.95e-03	RNA pol II associated (transcription factor)
<i>Sall1</i>	Chr8 (-)	#2682	log ₂ FC = -1.13 FDR = 6.27e-04	Transcription repressor (pluripotency and differentiation)
Secondary target gene (downstream)				
<i>Suv420h1/Kmt5b</i>	Chr19 (+)	-	log ₂ FC = -2.2 FDR = 9.52e-06	Histone methyltransferase (H4K20me2/3)
<i>Foxd3</i>	Chr4 (+)	-	log ₂ FC = 1.5 FDR = 1.85e-05	Maintenance of pluripotent cells
<i>Otx2</i>	Chr14 (-)	-	log ₂ FC = -2.16 FDR = 2.76e-03	Differentiation associated gene
<i>Sugp1</i>	Chr8 (+)	-	log ₂ FC = -1.07 FDR = 2.46e-02	mRNA splicing
<i>Foxj2</i>	Chr6 (+)	-	log ₂ FC = -0.99 FDR = 4.85e-02	Transcription coactivator
<i>Zfp462</i>	Chr4 (+)	-	log ₂ FC = -0.82 FDR = 1.94e-02	(pluripotency and differentiation)
Putative DIDO3 interactions in large protein complexes				
<i>Nanog</i>	Chr6 (+)	#2269	-	Transcription factor (pluripotency)
<i>Rpa2</i>	Chr4 (+)	#1925	-	Replication protein A complex
<i>Suv39h1</i>	ChrX (-)	#2850	-	Histone methyltransferase (H3K9me2/3), constitutive heterochromatin at pericentromeric
<i>Trrap</i>	Chr5 (+)	#2186	-	NuA4 histone acetyltransferase complex (H2A, H4). SWR1-like complex (remove H2A.Z/H2AZ1)
<i>Ep400</i>	Chr5 (-)	#2124	-	NuA4 histone acetyltransferase complex (H2A, H4). SWR1-like complex (remove H2A.Z/H2AZ1)
<i>Tet1</i>	Chr10 (-)	#194, #195, #196	-	Promote histone H2B GlcNAcylation
<i>Npm1</i>	Chr11 (-)	#355, #356, #357	-	Involved in histone assembly and centrosome duplication
<i>Cdk12</i>	Chr11 (+)	#521, #522, #523	-	Phosphorylates RNAPII CTD, transcription elongation
<i>Dhx9</i>	Chr1 (-)	#122, #123	-	Unwinds R-loops; Binds dsDNA, ssDNA, dsRNA, ssRNA and poly(A)-containing RNA
<i>Dhx30</i>	Chr9 (-)	#2837	-	RNA-dependent helicase
<i>Ddx39b</i>	Chr17 (+)	#1214	-	Cotranscriptional and nonscheduled R-loop removal
<i>Setx</i>	Chr2 (+)	#1482	-	Modulate RNAPII binding to chromatin. R-loop resolution at G-rich pause sites
<i>Sirt3</i>	Chr7 (-)	#2566, #2567	-	NAD ⁺ -dependent deacetylase. Involved in stabilizing heterochromatin and senescence
<i>Paf1</i>	Chr7 (+)	#2381	-	PAF1 complex. Modulate RNAPII elongation rates and ESC pluripotency
<i>Cpsf4</i>	Chr5 (+)	#2187, #2188	-	CPSF complex (pre-mRNA 3'-end formation)
<i>Cpsf6</i>	Chr10 (-)	#282, #283, #284, #285	-	CFIm complex (pre-mRNA 3'-end cleavage and polyadenylation)
<i>Pabpc1</i>	Chr15 (-)	#924	-	Binds the poly(A) tail of mRNA (pre-mRNA splicing and mRNA stability)
<i>Pabpn1</i>	Chr14 (+)	#854, #855	-	Involved in the 3'-end formation of mRNA precursors. Stimulates poly(A) polymerase
<i>Pnrc2</i>	Chr4 (-)	#1945, #1946, #1947	-	Involved in nonsense-mediated mRNA decay
<i>Sfpq</i>	Chr4 (+)	#1903, #1904, #1905	-	mRNA splicing
<i>Smg5</i>	Chr3 (+)	#1744	-	Involved in nonsense-mediated mRNA decay
<i>Upf1</i>	Chr8 (-)	#2631	-	RNA-dependent helicase (nonsense-mediated decay of mRNAs containing premature stop codons)
<i>Zc3h12</i>	Chr4 (-)	#1901	-	Endoribonuclease (mRNA decay). Cleaves mRNA harboring a stem-loop located in their 3'-UTRs.

Supplementary Table S2

PyRanges							
qSample ^(a)	tSample ^(b)	qLen ^(c)	tLen ^(d)	Overlap (%) ^{(e)(f)}	All ^(g)	Promoters ^(g)	Intergenic ^(g)
DIDO3	RNAPII	2 888	29 041	2 269 (78.6%)	+++	+++	+++
DIDO3	H3K36me3	2 888	33 970	2 002 (69.3%)	+++	+++	+++
DIDO3	H3K27ac	2 888	29 284	1 036 (35.9%)	+++	+++	+++
DIDO3	H3K4me3	2 888	43 051	811 (28.1%)	+++	+++	+++
DIDO3	CTCF	2 888	49 203	360 (12.5%)	+++	+++	+++
DIDO3	PRC2	2 888	23 646	242 (8.4%)	+++	+++	NS
DIDO3	RING1B	2 888	10 116	41 (1.4%)	NS	+++	---
DIDO3	H3K27me3	2 888	19 812	22 (0.8%)	---	NS	---
RNAPII	H3K36me3	29 041	33 970	16 326 (56.2%)	+++	+++	---
RNAPII	H3K27ac	29 041	29 284	14 402 (49.6%)	+++	+++	+++
RNAPII	H3K4me3	29 041	43 051	16 511 (56.9%)	+++	+++	+++
RNAPII	CTCF	29 041	49 203	7 174 (24.7%)	+++	+++	---
RNAPII	PRC2	29 041	23 646	3 385 (11.7%)	+++	+++	---
RNAPII	RING1B	29 041	10 116	3 871 (13.3%)	+++	+++	---
RNAPII	H3K27me3	29 041	19 812	2 310 (8.0%)	+++	+++	---
H3K36me3	H3K27ac	33 970	29 284	8 067 (23.7%)	+++	+++	---
H3K36me3	H3K4me3	33 970	43 051	9 286 (27.3%)	+++	+++	---
H3K36me3	CTCF	33 970	49 203	6 792 (20.0%)	+++	+++	---
H3K36me3	PRC2	33 970	23 646	1 557 (4.6%)	---	+++	---
H3K36me3	RING1B	33 970	10 116	611 (1.8%)	---	NS	---
H3K36me3	H3K27me3	33 970	19 812	449 (1.3%)	---	---	---
H3K4me3	H3K27ac	43 051	29 284	18 893 (43.9%)	+++	+++	+++
H3K4me3	CTCF	43 051	49 203	11 714 (27.2%)	+++	+++	+++
H3K4me3	PRC2	43 051	23 646	10 043 (23.3%)	+++	+++	+++
H3K4me3	RING1B	43 051	10 116	10 236 (23.8%)	+++	+++	+++
H3K4me3	H3K27me3	43 051	19 812	9 754 (22.7%)	+++	+++	+++
H3K27ac	CTCF	29 284	49 203	6 848 (23.4%)	+++	+++	+++
H3K27ac	PRC2	29 284	23 646	2 368 (8.1%)	+++	+++	---
H3K27ac	RING1B	29 284	10 116	2 636 (9.0%)	+++	+++	NS
H3K27ac	H3K27me3	29 284	19 812	1 121 (3.8%)	NS	+++	---
CTCF	PRC2	49 203	23 646	3 671 (7.5%)	+++	+++	+++
CTCF	RING1B	49 203	10 116	3 574 (7.3%)	+++	+++	+++
CTCF	H3K27me3	49 203	19 812	4 279 (8.7%)	+++	+++	+++
PRC2	RING1B	23 646	10 116	7 638 (32.3%)	+++	+++	+++
PRC2	H3K27me3	23 646	19 812	8 009 (33.9%)	+++	+++	+++
RING1B	H3K27me3	10 116	19 812	7 142 (70.6%)	+++	+++	+++
ChIPseeker							
qSample ^(a)	tSample ^(b)	qLen ^(c)	tLen ^(d)	N_OL ^(e)	N_OL (%qLen) ^(f)	p-value ^(h)	p-adjust ⁽ⁱ⁾
HA-DIDO3	RNAPII	2 888	29 041	2 290	79.3%	9.9e-05	1.6e-04
HA-DIDO3	H3K36me3	2 888	33 970	2 019	69.9%	9.9e-05	1.6e-04
HA-DIDO3	H3K27ac	2 888	29 284	1 050	36.4%	9.9e-05	1.6e-04
HA-DIDO3	H3K4me3	2 888	43 051	829	28.7%	9.9e-05	1.6e-04
HA-DIDO3	CTCF	2 888	49 203	368	12.7%	4.6e-03	6.9e-03
HA-DIDO3	PRC2	2 888	23 646	287	9.9%	9.9e-05	1.6e-04
HA-DIDO3	RING1B	2 888	10 116	41	1.4%	0.2	0.2
HA-DIDO3	H3K27me3	2 888	19 812	22	0.8%	0.9	0.9

Supplementary Table S3

<i>Region1</i> ^(a)	<i>Gene</i>	<i>Region2 (Dido1)</i> ^(a)	<i>Score</i> ^(b)	<i>Description</i> ^(c)	<i>Chromatin states</i> ^(d)
1:27,038,001-27,039,000	<i>ARID1A</i>	20:61,545,001-61,546,000	0.782	SWI/SNF complex, chromatin remodeling	Enh, EnhG, Tx, TxWk
2:60,772,001-60,773,000	<i>BCL11A</i>	20:61,545,001-61,546,000	0.8	TF associated with the SWI/SNF complex	Tx, TxWk
2:27,015,001-27,016,000	<i>CENPA</i>	20:61,545,001-61,546,000	0.704	Centromere-associated protein network	Tx, TxWk
4:104,089,001-104,090,000	<i>CENPE</i>	20:61,545,001-61,546,000	0.726	Centromere-associated protein network	Tx, TxWk
1:214,811,001-214,812,000	<i>CENPF</i>	20:61,545,001-61,546,000	0.809	Centromere-associated protein network	Enh, Tx, TxWk
1:214,814,001-214,815,000	<i>CENPF</i>	20:61,545,001-61,546,000	0.778	Centromere-associated protein network	Tx, TxWk
1:214,812,001-214,813,000	<i>CENPF</i>	20:61,545,001-61,546,000	0.742	Centromere-associated protein network	Enh, Tx, TxWk
1:214,805,001-214,806,000	<i>CENPF</i>	20:61,545,001-61,546,000	0.734	Centromere-associated protein network	Enh, Tx, TxWk
1:214,819,001-214,820,000	<i>CENPF</i>	20:61,545,001-61,546,000	0.729	Centromere-associated protein network	Tx, TxWk
2:234,759,001-234,760,000	<i>HJURP</i>	20:61,545,001-61,546,000	0.708	H3 chaperone complex of CENP-A variant	Tx, TxWk
2:234,747,001-234,748,000	<i>HJURP</i>	20:61,545,001-61,546,000	0.704	H3 chaperone complex of CENP-A variant	EnhG, Tx, TxWk
10:126,832,001-126,833,000	<i>CTBP2</i>	20:61,550,001-61,551,000	0.857	Polycomb accessory protein (PRC2)	Enh, TxWk
10:126,820,001-126,821,000	<i>CTBP2</i>	20:61,550,001-61,551,000	0.743	Polycomb accessory protein (PRC2)	Enh, TxWk

Supplementary Table S4

Abbreviation	Cancer type	Tumor	Normal	Statistical significance (gene expression)
ACC	Adrenocortical carcinoma	79	0	n.a.
AML	Acute myeloid leukemia	173	0	n.a.
BLCA	Bladder urotelial carcinoma	408	19	2.5×10^{-01}
BRCA	Breast invasive carcinoma	1097	114	2×10^{-02}
CESC	Cervical squamous cell carcinoma	305	3	2.9×10^{-01}
CHOL	Cholangiocarcinoma	36	9	3.6×10^{-11}
COAD	Colorectal adenocarcinoma	286	41	9.9×10^{-13}
DLBC	Lymphoid Neoplasm Diffuse Large B-cell Lymphoma	48	0	n.a.
ESCA	Esophageal carcinoma	184	11	6.8×10^{-03}
GBM	Glioblastoma multiforme	156	5	4.2×10^{-01}
HNSC	Head and neck squamous cell carcinoma	520	44	1.9×10^{-04}
KICH	Kidney Chromophobe carcinoma	67	25	9.6×10^{-05}
KIRC	Kidney renal clear cell carcinoma	533	72	2.6×10^{-01}
KIRP	Kidney renal papillary cell carcinoma	290	32	9.3×10^{-01}
LGG	Brain Lower Grade Glioma	513	0	n.a.
LIHC	Liver hepatocellular carcinoma	371	50	1.6×10^{-12}
LUAD	Lung adenocarcinoma	515	59	1.2×10^{-04}
LUSC	Lung squamous cell carcinoma	503	52	3.9×10^{-01}
MESO	Mesothelioma	87	0	n.a.
OV	Ovarian serous cystadenocarcinoma	305	0	n.a.
PAAD	Pancreas adenocarcinoma	178	4	3.9×10^{-01}
PCPG	Pheochromocytoma and paraganglioma	179	3	1.9×10^{-01}
PRAD	Prostate adenocarcinoma	497	52	5.6×10^{-03}
READ	Rectal adenocarcinoma	166	10	1.7×10^{-03}
SARC	Sarcoma	260	2	$< 1 \times 10^{-12}$
SKCM	Skin cutaneous melanoma	472	1	n.a.
STAD	Stomach adenocarcinoma	415	34	2.1×10^{-07}
TGCT	Testicular Germ Cell Tumors	150	0	n.a.
THCA	Thyroid carcinoma	505	59	1.9×10^{-12}
THYM	Thymoma	120	2	1.6×10^{-02}
UCEC	Uterine Corpus Endometrial Carcinoma	546	35	1.6×10^{-12}
UCS	Uterine Carcinosarcoma	57	0	n.a.
UVM	Uveal Melanoma	80	0	n.a.