Cell PRESS
Open ACCESS

EXTENDED EXPERIMENTAL PROCEDURES

RNA and Protein Analysis

Total RNA was prepared from ESC and EB using Trizol (Invitrogen). RNA was tested by RT-PCR using Superscript III (Invitrogen) according to standard procedures. Quantitative real-time RT-PCR was performed using SYBR green and specific primers (sequences available from the authors on request).

For Western blot analysis of protein expression, ESCs or EB were boiled in 2x concentrated sample buffer containing SDS and β-mercaptoethanol. Protein extracts were cleared by centrifugation, separated by SDS-PAGE, and transferred to nitrocellulose. Blot membranes were blocked in TBST-5% nonfat dry milk and probed with antibodies to Dido3 (generated in our lab), Oct4 (1:400; Santa Cruz) or HA (MAB 1:1000; Covance).

For sections of embryo analysis, uteri were isolated at the indicated days post *coitum*, fixed in 4% paraformaldehyde (4 h), dehydrated, paraffin-embedded and sections were incubated on slides (37°C, overnight), deparaffinized, rehydrated, and used for immunohistochemistry.

For immunofluorescence, ESCs were fixed with 4% formaldehyde in PBS, permeabilized with 0,2% NP-40 and stained with antibodies for H3K4me3, H3T3ph, H3T6ph and Dido.

Dido3 Expression Constructs and Transfection

For reconstitution experiments, we cloned cDNA of the full-length, N-terminal region of Dido3 (DidoNT and DidoPHDm) into the pCAGGS-HA-tagged mammalian expression vector in front of an IRES site and puromycin-resistance cassette. ESCs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol; 24 hr after transfection, puromycin (1 μg/ml) was added to culture medium and cells were selected for puromycin resistance for at least 1 week. Pooled colonies and/or single colonies were picked, expanded and tested for overexpression of the HA-Dido3; HA-DidoNT or HA-DidoPHDm.

Cloning and Protein Purification

Wild-type Dido3 PHD (aa 266-325 and aa 260-325) was cloned from full length human Dido3 cDNA (Open Biosystems) into a pDEST15 vector containing a glutathione S-transferase (GST) tag and a PreScission cleavage site at the N-terminal end. Point mutants were prepared using the Stratagene QuickChange XL Site Directed Mutagenesis kit according to the manufacturer's instructions. Wild-type and point mutant proteins were expressed in Rosetta2 (DE3) pLysS in either Luria Broth or M19 minimal media supplemented with 50 μM $ZnCl_2$ and $^{15}NH_4Cl$ or $^{15}NH_4Cl$ and ^{13}C-glucose. Cells were induced with 1 mM IPTG, grown for 16 hr at 18°C, and harvested by centrifugation at 6k rpm. Cells were lysed by sonication in lysis buffer containing 20 mM Tris pH 7.5 at 4°C, 150 mM NaCl, 3 mM dithiothreitol, 0.05% NP-40, 1 mM PMSF, DNaseI and clarified by centrifugation at 15k rpm for 30 min. Proteins were purified on glutathione agarose beads (Pierce® cat# 16101) and the GST-tag cleaved using PreScission protease for at least overnight at 4°C. Cleaved proteins were concentrated into 20 mM Tris pH 7.5, 150 mM NaCl, and 3 mM dithiothreitol. For crystallization, the protein was further purified on HiPrep 16/60 Sephacryl S-100 column. PHF3 PHD (aa 717-774) was cloned from full length PHF3 cDNA (Open Biosystem) and unlabeled and ^{15}N-labeled proteins were purified using similar protocol.

Glutathione-S-transferase pull-down experiments with Dido3 fusion proteins in the pEBG vector were carried out as described (Prieto et al., 2009). To generate a destabilized PHD domain mutant, zinc-coordinating residues corresponding to C285 and H290 from the murine Dido1 sequence were mutated to alanine by site-directed mutagenesis. As a reciprocal control, C285 and H290 were interchanged, generating a PHD mutant capable of coordinating zinc.

Data Sets for Histone Modifications

Analyzed *Drosophila melanogaster* ChIP-seq data sets for the histone modifications H3K4me1, H3K4me3, H3K9ac, H3K27me3 and H3K9me3 were downloaded from the National Human Genome Research Institute model organism Encyclopedia of DNA Elements website (www.modENCONDE.org). ChIP seq reads were visualized with the integrated genome viewer using the Dm9 genome built. The GEO accession numbers for the data sets are: GSM439448 (H3K27me3), GSM439457 (H3K9me3), GSM401409 (H3K4me1), GSM401407 (H3K27ac), GSM400656 (H3K4me3).

NMR Spectroscopy and Sequence-Specific Resonance Assignments

NMR experiments were recorded at 298K on a Varian INOVA 600 MHz spectrometer equipped with a cryogenic probe. For backbone assignments, uniformly ^{15}N- and ^{13}C-labeled Dido3 PHD (aa 260-325) was used to collect triple-resonance HNCACB (Wittekind and Mueller, 1993), CBCA(CO)NH (Grzesiek and Bax, 1992), C(CO)NH (Grzesiek et al., 1993), and HC(CO)NH (Grzesiek et al., 1993). The NMR data were processed with nmrPipe (Delaglio et al., 1995) and analyzed with nmrDraw. Initial sequence specific assignments were obtained using the PINE program and were verified and completed using CcpNMR Analysis v1.6 (Vranken et al., 2005).

Chemical shift perturbation experiments were carried out using 0.1 mM uniformly ^{15}N-labeled wild-type or mutated Dido3 PHD. The ^{1}H,^{15}N heteronuclear single quantum coherence (HSQC) spectra were recorded in the presence of increasing concentrations

of 12-mer histone tail peptides (synthesized by the University of Colorado Denver Biophysics Core Facility.) K_d values were calculated by a nonlinear least-squares analysis in Kaleidagraph using the following equation:

$$\Delta\delta = \frac{\Delta\delta_{max}\left(([L]+[P]+K_d) - \sqrt{([L]+[P]+K_d)^2 - 4[P][L]}\right)}{2[P]},$$

where [L] is concentration of the peptide, [P] is concentration of the protein, $\Delta\delta$ is the observed chemical shift change, and $\Delta\delta_{max}$ is the normalized chemical shift change at saturation. Normalized (Grzesiek et al., 1996) chemical shift changes were calculated using the equation $\Delta\delta = \sqrt{(\Delta\delta H)^2 + (\Delta\delta N/5)^2}$, where $\Delta\delta$ is the change in chemical shift in parts per million (ppm).

Fluorescence Spectroscopy

Spectra were recorded at 25°C on a Fluoromax-3 spectrofluorometer (HORIBA). The samples containing 0.5 μM Dido3 PHD (aa 260-325) in PBS buffer and progressively increasing concentrations of the histone peptide were excited at 295 nm. Emission spectra were recorded over a range of wavelengths between 315 and 405 nm with a 0.5 nm step size and a 1 s integration time. Three scans were averaged and recorded. K_d values were calculated using a non-linear least-squares analysis, using the following equation:

$$\Delta I = \frac{\Delta I_{max}\left(([L]+[P]+K_d) - \sqrt{([L]+[P]+K_d)^2 - 4[P][L]}\right)}{2[P]},$$

where [L] is the concentration of the histone peptide, [P] is the concentration of Dido3 PHD, ΔI is the observed change of signal intensity, and ΔI_{max} is the difference in signal intensity of the free and bound states of the PHD finger. K_d represents the average of three separate experiments, with error computed as the standard deviation between those values.

Peptide Microarray

Peptide synthesis and validation, microarray fabrication, effector protein hybridization and detection, and data analysis were performed as previously described (Rothbart et al., 2012) with the following modification. Each peptide listed in Table S1 was spotted in triplicate eight times per array. Triplicate spots were averaged and treated as a single value for subsequent statistical analysis.

HADDOCK

Modeling of the Dido3 PHD-H3T3phK4me3 and Dido3 PHD-H3K4me3 complexes was performed using the flexible docking program HADDOCK (www.haddocking.org). The H3T3phK4me3 peptide was generated using Coot. Three intermolecular hydrogen bonds formed with Ala1 of the peptides were used as restrains. The lowest energy structures from the top clusters were analyzed for each run.

Confocal Scanning Fluorescence Microscopy

Antibodies against mouse Dido3 have been described before (Prieto et al., 2009; Trachana et al., 2007), antibodies against human Dido3 (order# AF6047) were from R&D Systems (Minneapolis, MN), the antibody against γ-tubulin (GTU-88) from Sigma, and antibodies against α-tubulin (AB7291), acetylated tubulin (AB24610), H3K4me3 (AB12209), H3K9me3 (AB8898), H3T3ph (AB78351) and H3T6ph (AB14102) from Abcam (Cambridge, UK). To analyze protein localization, MEFs, 3T3 or HT29 cells were seeded on glass coverslips and grown in DMEM containing 10% FCS until 70% confluent. For immunofluorescence, cells were rinsed once in PBS, fixed in PBS containing 4% formaldehyde for 5 min, and permeabilized in PBS with 0.5% Triton X-100 for 5 min. After blocking in PBS containing 1% bovine serum albumin (1 hr, room temperature), cells were incubated with primary antibodies (1 hr, room temperature). Cells were then washed, incubated with secondary antibodies (Jackson Immunoresearch, West Grove, PA), washed again, and mounted in Prolong Gold antifade (Life Technologies, Grand Island, NY). Confocal microscopy was performed using an IX81 microscope (Olympus, Japan) with sequential excitation of fluorophores. Representative images corresponding to single confocal layers are shown in the figures.

SUPPLEMENTAL REFERENCES

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., and Bax, A. (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR *6*, 277–293.

Grzesiek, S., and Bax, A. (1992). Improved 3D triple-resonance NMR techniques applied to a 31-kDa protein. J. Magn. Reson. *96*, 432–440.

Grzesiek, S., Anglister, J., and Bax, A. (1993). Correlation of backbone amide and aliphatic side-chain resonances in C-13/N-15-enriched proteins by isotropic mixing of C-13 magnetization. J. Magn. Reson. *101*, 114–119.

Grzesiek, S., Stahl, S.J., Wingfield, P.T., and Bax, A. (1996). The CD4 determinant for downregulation by HIV-1 Nef directly binds to Nef. Mapping of the Nef binding surface by NMR. Biochemistry *35*, 10256–10261.

Rothbart, S.B., Krajewski, K., Strahl, B.D., and Fuchs, S.M. (2012). Peptide microarrays to interrogate the "histone code". Methods Enzymol. *512*, 107–135.

Vranken, W.F., Boucher, W., Stevens, T.J., Fogh, R.H., Pajon, A., Llinas, M., Ulrich, E.L., Markley, J.L., Ionides, J., and Laue, E.D. (2005). The CCPN data model for NMR spectroscopy: development of a software pipeline. Proteins *59*, 687–696.

Wittekind, M., and Mueller, L. (1993). HNCACB, A high-sensitivity 3D NMR experiment to correlate amide-proton and nitrogen resonances with the alpha-carbon and beta-carbon resonances in proteins. J. Magn. Reson. *101*, 201–205.

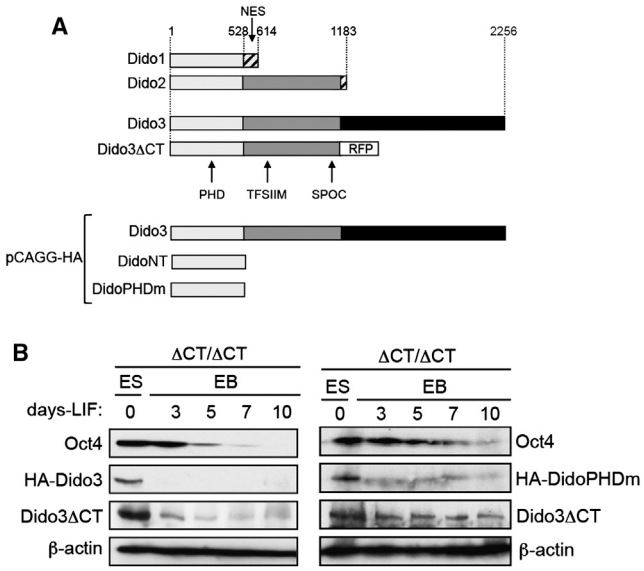

Figure S1. The Dido3 PHD Finger Is Essential in Promoting Differentiation, Related to Figure 1

(A) Schematics of the Dido isoforms and constructs used in this study. NES: nuclear export signal.

(B) ΔCT/ΔCT ESCs expressing HA-tagged full-length Dido3 or HA-DidoNT-PHDm were treated as in Figure 1D and analyzed by Western blot for expression of Oct4, endogenous Dido3ΔCT and overexpressed HA-Dido3 and HA-DidoPHDm; β-actin was used as loading control.

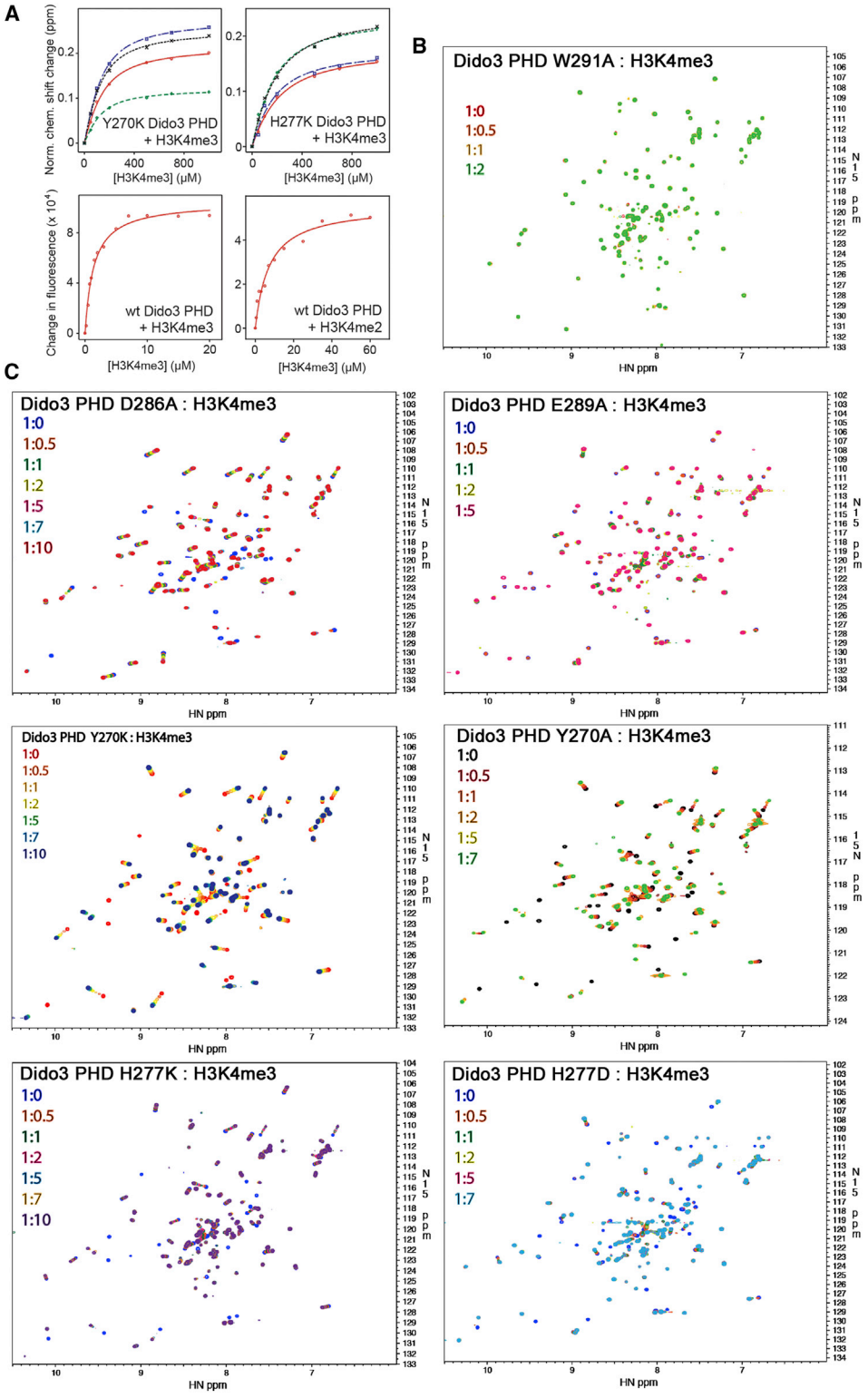

Figure S2. The Dido3 PHD Finger Binds Specifically to H3K4me3, Related to Figure 4

(A) Representative binding curves used to determine the K_d values by tryptophan fluorescence and NMR.

(B and C) Superimposed $^{1}H,^{15}N$ HSQC spectra of the indicated Dido3 PHD mutants recorded as H3K4me3 peptide was titrated in.

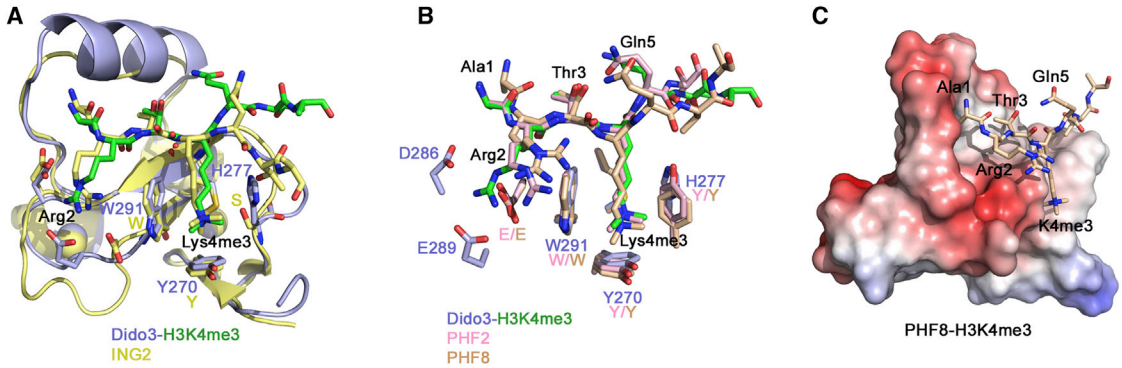

Figure S3. The Molecular Mechanism of H3K4me3 Recognition by Dido3 PHD, Related to Figure 4

(A) Overlays of the structures of the H3K4me3-bound PHD fingers of Dido3 (blue) and ING2 (PDB: 2G6Q). (B) Overlays of the structures of the H3K4me3-bound PHD fingers of Dido, PHF2 (PDB: 3KQI) and PHF8 (PDB: 3KV4). Shown only: the bound H3K4me3 peptide, the aromatic cage and the acidic residues, which are in contact with Arg2. (C) The structure of the PHF8 PHD-H3K4me3 complex (PDB: 3KV4). The protein surface is colored in line with its electrostatic potential.

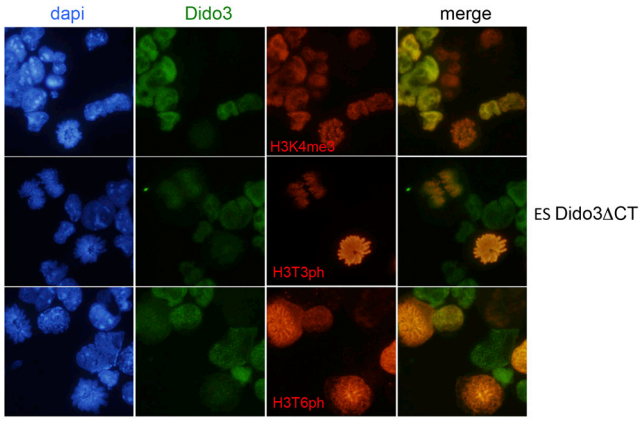

Figure S4. Dido3 Is Excluded from Chromatin in Mitosis in Dido3ΔCT ESCs, Related to Figure 6

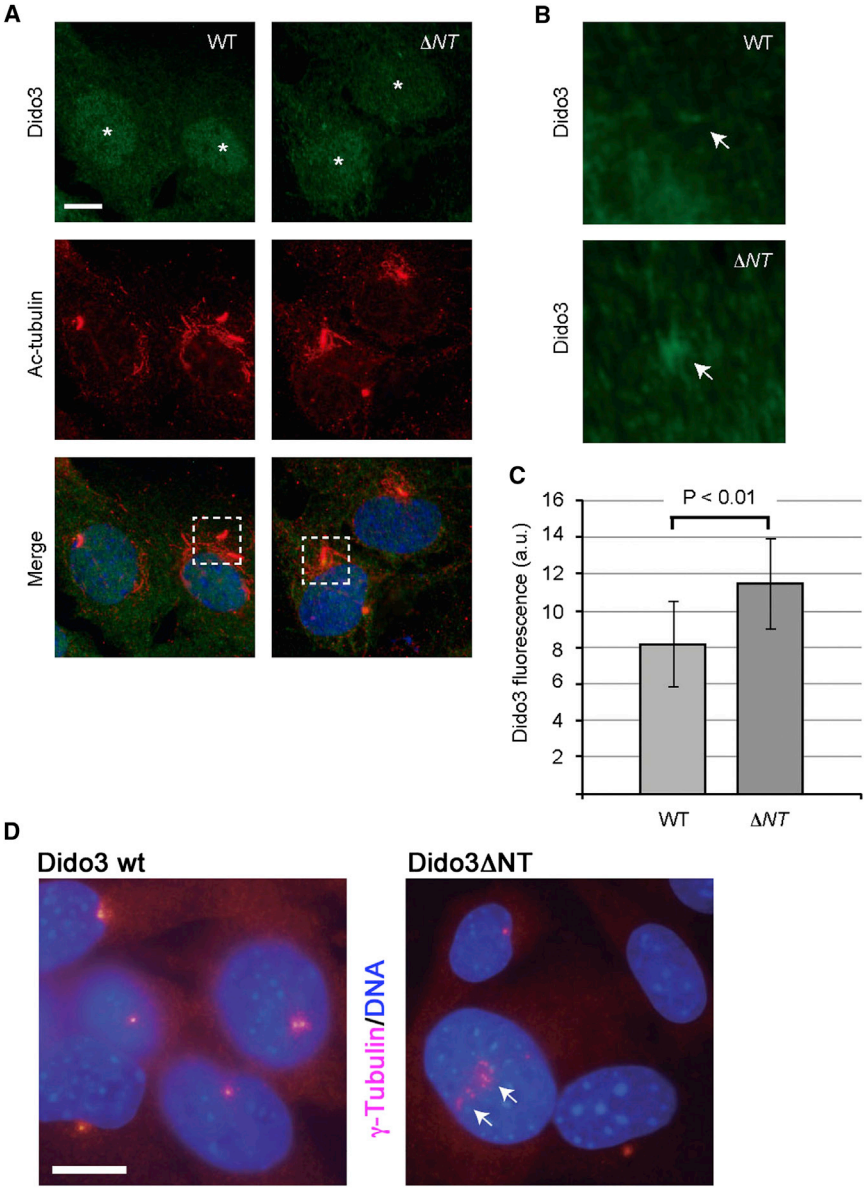

Figure S5. Localization of Dido3 and Dido3ΔNT at the Centrosome, Related to Figure 6

(A) MEFs were seeded on coverslips, labeled with antibodies against Dido3 and acetylated tubulin, and visualized by fluorescence microscopy.

(B) Magnification of the Dido3 signal corresponding to the boxed areas under (A).

(C) Quantitation of centrosomal Dido3 and Dido3ΔNT accumulation. Error bars indicate SD (n = 15).

(D) Overexpression of Dido3 and Dido3ΔNT in MEFs leads to centrosome amplification as evidenced by γ-tubulin labeling (arrows). Scale bars, 10 μm.

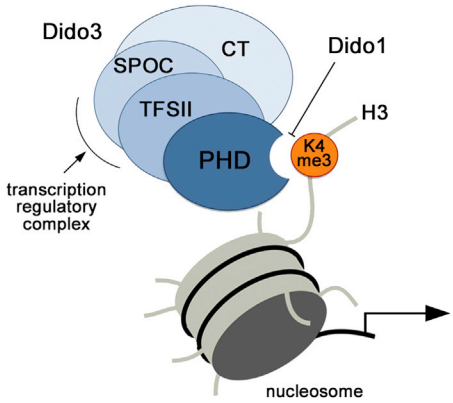

Figure S6. A Model for Dido3 Displacement in ESCs, Related to Figure 6