ZFP281 ORCHESTRATES INTERCONVERSION OF PLURIPOTENT STATES BY ENGAGING EHMT1 AND ZIC2

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ABSTRACT

Developmental cell fate specification is a unidirectional process that can be reverted in response to injury or experimental reprogramming. Whether differentiation and de-differentiation trajectories intersect mechanistically is unclear. Here, we performed comparative screening in lineage-related mouse naïve embryonic stem cells (ESCs) and primed epiblast stem cells (EpiSCs), and identified the constitutively expressed zinc finger transcription factor (TF) Zfp281 as a bi-directional regulator of cell state interconversion. We showed that subtle chromatin binding changes in differentiated cells translate into activation of the histone H3 lysine 9 (H3K9) methyltransferase Ehmt1 and stabilization of the zinc finger TF Zic2 at enhancers and promoters. Genetic gain- and loss-of-function experiments confirmed a critical role of Ehmt1 and Zic2 downstream of Zfp281 both in driving exit from the ESC state, and in restricting reprogramming of EpiSCs. Our study reveals that cell type-invariant chromatin association of Zfp281 provides an interaction platform for remodeling the cis-regulatory network underlying cellular plasticity.

INTRODUCTION

Mammalian development is a hierarchical process that coordinates organismal growth with increasing cellular differentiation. The lineage progression of the few pluripotent cells in the blastocyst towards the many specialized cell types in the mature embryo is by and large unidirectional. However, fully differentiated cells can be de-differentiated into induced pluripotent stem cells (iPSCs) by ectopic expression of the transcription factors (TFs) Oct4, Sox2, Klf4 and c-Myc (Takahashi & Yamanaka, 2006). Reprogramming of somatic cells into iPSCs requires erasure of the entire developmental history of a somatic cell, but whether this depends on the reversal of developmental hierarchies is unclear (Ladewig *et al*, 2013; Takahashi & Yamanaka, 2015).

Transcriptional and epigenomic profiling of the reprogramming process has revealed an ordered series of events that include the transient and sequential activation of late and early developmental genes (Cacchiarelli *et al*, 2015; Amlani *et al*, 2018; Takahashi *et al*, 2014). Although the specific trajectory is dictated by the identity of the starting somatic cell type (Nefzger *et al*, 2017; Jackson *et al*, 2016) and the experimental regime (Chantzoura *et al*, 2015; Stuart *et al*, 2019), iPSC formation may involve the reversion of natural developmental mechanisms (Takahashi & Yamanaka, 2015). Consistent with this possibility, a mesenchymal-to-epithelial transition is necessary for iPSC formation (Samavarchi-Tehrani *et al*, 2010; Li *et al*, 2010), while the converse epithelial-to-mesenchymal transition is crucial for embryogenesis, e.g. during gastrulation and neural crest formation (Acloque *et al*, 2009). Although it is debated if these observations reflect a shared developmental intermediate (Raab *et al*, 2017), they suggest that de-differentiation and differentiation employ common mechanisms in opposite directions. Here, we systematically and functionally examine this concept using naïve pluripotent embryonic stem cells (ESCs) and primed pluripotent epiblast stem cells (EpiSCs) (Smith, 2017).

ESCs and EpiSCs are developmentally related derivatives of mouse embryonic day (E) 3.75-4.5 blastocysts (Boroviak *et al*, 2014) and E5.5-8.0 embryos (Tesar *et al*, 2007; Brons *et al*, 2007; Osorno

et al, 2012), respectively. ESCs cultured in the presence of two inhibitors (2i) resemble naïve pluripotent cells of the pre-implantation epiblast (Boroviak *et al*, 2014), while primed pluripotent EpiSCs cultured in the presence of FGF2 and Activin A (FA) resemble cells of the late gastrula (Osorno *et al*, 2012; Tsakiridis *et al*, 2014; Kojima *et al*, 2014). Upon *in vitro* differentiation, ESCs progress through a transient post-implantation epiblast-like (EpiLC) cell state that is amenable to EpiSC derivation (Zhang *et al*, 2010; Hayashi *et al*, 2011). Conversely, activation of just one TF, such as Stat3, Klf4, or Esrrb, is sufficient to reprogram EpiSCs into naïve pluripotent EpiSC-derived iPSCs (Epi-iPSCs) in the presence of 2i (Yang *et al*, 2010; Guo *et al*, 2009; Festuccia *et al*, 2012). The interconvertibility of ESCs and EpiSCs thus provides an experimental system to explore if de-differentiation includes the reversion of differentiation mechanisms.

Using a large-scale loss-of-function reprogramming screen in sensitized EpiSCs we identify the zinc finger TF Zfp281 as a prominent bidirectional ESC-EpiSC transition regulator. We show that Zfp281 exhibits stable chromatin association and drives ESC progression through differentiation-specific interaction with Ehmt1 and Zic2. Genomic analysis revealed activation of Ehmt1 and enrichment of Zic2 at Zfp281-bound cis-regulatory elements (CREs) that are associated with developmental transcription in EpiLCs and EpiSCs. Zfp281, therefore, establishes and stabilizes cell fate commitment to safeguard the unidirectionality of pluripotent state transitions.

RESULTS

Zfp281 is a bidirectional ESC-EpiSC transition regulator

We hypothesized that mechanisms common to differentiation and de-differentiation may be encoded in genes that both promote exit from the naïve ESC state and impair reprogramming of EpiSCs. ESC differentiation drivers have been determined in several genetic loss of function screens (Betschinger *et al*, 2013; Li *et al*, 2018; Leeb *et al*, 2014; Guo *et al*, 2011; Westerman *et al*, 2011), but it is unknown if those also inhibit reprogramming of EpiSCs into naïve pluripotency. We therefore set out to systematically identify reprogramming roadblocks using a large-scale endoribonuclease-prepared small interfering RNA (esiRNA) loss-of-function screen (Ding *et al*, 2009). We made use of O4GIP$_{GY118F}$ EpiSCs expressing green fluorescent protein (GFP) and Puromycin N-acetyl-transferase under the regulatory sequences of the *Oct4* gene (Guo *et al*, 2009), and a Stat3 activating receptor (GY118F) responsive to granulocyte colony stimulating factor (Gcsf) driven by a constitutive promoter (Yang *et al*, 2010). Upon exposure to Gcsf and 2i for 4 days (d), O4GIP$_{GY118F}$ EpiSCs gave rise to self-renewing Epi-iPSCs at an efficiency of roughly 0.1% (Figure **EV1A**), thus providing a sensitized setup to identify reprogramming inhibitors. O4GIP$_{GY118F}$ EpiSCs were transfected with esiRNAs targeting 9540 transcripts and control esiRNAs targeting Luciferase (Luc) and the GY118F downstream effector Stat3 in 384 well plates (Figure **1A**). The next day, reprogramming was induced by changing to 2i and Gcsf. After 4d, we selected Epi-iPSCs in the presence of Puromycin, and quantified viability with a fluorescent assay after 3-4d. The screen was performed in duplicate and Z scores were calculated per plate (Table **EV1**). Positive (Stat3 esiRNA), but not negative (non-targeting Luc esiRNA and no esiRNA) controls induced negative Z scores (Figure **EV1B**). Screen hits with average Z scores < -2 included ribosome

and proteasome subunits, Stat3 and Oct4 (Figure **1B**), and were strongly enriched for functions associated with RNA maturation and translation using gene ontology (GO) analysis (Figure **EV1C**). These therefore contain genes required for reprogramming and/or cell survival. Screen hits with positive Z scores, conversely, are expected to inhibit reprogramming and/or proliferation. Among the 146 hits with an average Z score > 2, the zinc finger TF *Zfp281* and the E3 ubiquitin ligase *Fbxw7* scored highest. Zfp281 and Fbxw7 have previously been shown to restrict iPSC generation from somatic cells (Fidalgo *et al*, 2012; Buckley *et al*, 2012; Okita *et al*, 2012; Fidalgo *et al*, 2016), thus suggesting successful identification of reprogramming roadblocks.

To determine if any of the 146 genes also drive ESC differentiation, we compared our screen hits with those from two previous large-scale ESC differentiation loss-of-function studies (Betschinger *et al*, 2013; Li *et al*, 2018) (Figure **1C**). Zfp281 and the cytochrome c oxidase subunits Cox5a and Cox6c scored strongest in all screens. For validation, we depleted each of them by siRNA transfection in independent GY118F-expressing Oct4 reporter 796.4 EpiSCs (Yang *et al*, 2010) and also included siRNAs targeting Fbxw7 and Tcf7l1 as controls (Figure **1B**, **C**). Knockdown of Fbxw7 and Zfp281, but not of Cox5a, Cox6c or Tcf7l1 increased reprogramming (Figure **1D**, **EV1D**). Therefore, Cox5a and Cox6c are false-positive or cell line-dependent screen hits, and we focused our further efforts on Zfp281. Consistent with previous findings (Fidalgo *et al*, 2016), Epi-iPSCs derived by Zfp281 depletion expressed the naïve TFs Esrrb, Klf4, Nr0b1 and Tbx3, and reduced levels of the primed markers Oct6, Fgf5, Sox3 and Dnmt3b (Figure **EV1E**), suggesting successful reversion to the pluripotent ground state.

To quantify the dynamics of this process, we used self-renewal in 2i as a proxy for acquisition of Epi-iPSC identity. Compared to controls, Zfp281 depletion dramatically increased the colony forming capacity of single cells after 2d and 4d of Gcsf addition (Figure **1E**), indicating accelerated and more efficient reprogramming of EpiSCs. Gcsf supplementation was essential and co-depletion of Stat3 abolished Epi-iPSC formation from O4GIP$_{GY118F}$ EpiSCs in the presence of Gcsf (Figure **EV1F**). Similarly, Leukemia inhibitory factor (Lif), which activates Stat3 in EpiSCs (Yang *et al*, 2010), was required for reprogramming of Zfp281-depleted OEC2 EpiSCs (Figure **1F**). To test if Zfp281 acts only in the context of active Stat3, we used conditional expression of Esrrb or Klf4 in O4GIP EpiSCs through addition of Doxycycline (Dox) to induce reprogramming. In the absence of extrinsic Lif we observed an increase in Dox-induced Epi-iPSC colonies upon knockdown of Zfp281 (Figure **1G**), suggesting that Zfp281 functions independent of the specific reprogramming regime. Taken together these findings demonstrate that the vast majority of cell state transition regulators act unidirectionally. Zfp281, in contrast, acts bidirectionally as it drives ESC differentiation and inhibits reprogramming of EpiSCs. Notably, this is inverse to the activity of reprogramming TFs, e.g. Klf4 and Esrrb, that induce and consolidate the naïve ESC state (Yamane *et al*, 2018; Festuccia *et al*, 2012; Guo *et al*, 2009; Martello *et al*, 2012; Niwa *et al*, 2009).

Zfp281 promotes exit from naïve pluripotency independent of Tet1 and Tet2

To characterize the function of Zfp281 in ESC differentiation, we inactivated the gene in naïve RGd2 ESCs that contain a destabilized GFP protein downstream of the *Rex1* (*Zfp42*) promoter (Figure **EV2A**, **B**) which allows near real-time tracking of cell state transition (Kalkan *et al*, 2017): GFP is

homogeneously expressed in 2i and up to 16 hours (h) after 2i withdrawal (GFP_{high}) (Kalkan *et al*, 2017) before becoming progressively downregulated (GFP_{low}) as ESCs exit from self-renewal (Figure **EV2C**). In 2i, reporter expression in two independent *Zfp281* knockout (KO) clones was similar to the parental wildtype cell line (*WT*) and an untargeted wildtype sibling clone (*Zfp281* WT) (Figure **EV2D**). In contrast, 32h and 72h after 2i withdrawal, 30% and less than 1% of *WT* cells were GFP_{high}, while 75% and 10% of *Zfp281* KO cells maintained high GFP expression, respectively. Consistent with impaired exit from the ESC state, 10% of *Zfp281* KO cells formed colonies in 2i after 72h of differentiation (Figure **2A**). This phenotype was reverted by transgenic Zfp281 expression (Figure **2B**). Resistance to exit self-renewal was also observed in KO cells generated in a different ESC line (Figure **EV2A, B, E**), and in EpiLC (Hayashi *et al*, 2011) and embryoid body (EB) differentiation regimes (Figure **2A**, **EV2D**). *Zfp281* mutant cells maintained *Rex1* reporter expression and self-renewal even after lengthy periods in the absence of 2i (Figure **2A**, **EV2D**), demonstrating that differentiation resistance is persistent.

Differentiating *Zfp281* KO cells expressed varied levels of the *Rex1* reporter (Figure **EV2D**) and formed colonies in 2i less efficiently than naïve pluripotent ESCs. E.g. 32h after 2i withdrawal, mutant cells displayed only 40% of the self-renewal capacity of ESCs (Figure **EV1F**). To test if this reduction is linked to population heterogeneity, we purified GFP_{high} and GFP_{low} cells at 32h using fluorescence-activated cell sorting. As expected (Kalkan *et al*, 2017), sorted *WT* GFP_{low} cells were largely committed to differentiation and unable to generate clones in 2i (Figure **2C**). In contrast, *Zfp281* KO GFP_{low} cells formed colonies almost as efficiently as GFP_{high} cells. Rex1 downregulation and exit from the ESC state is, thus, disconnected in *Zfp281* mutants. However, the efficiency with which GFP_{high} cells formed colonies after 32h of 2i withdrawal was lower than of GFP_{high} cells after 24h (Kalkan *et al*, 2017) and of ESCs (Figure **2C**). This was irrespective of genotype, suggesting a gradual decline of self-renewal during differentiation both in *Zfp281* mutant and *WT* GFP_{high} cells. The reduced clonogenicity of *Zfp281* KO populations compared to ESCs may therefore be consequential to impaired progression of an advanced cell state with limited self-renewal capacity and independent of population heterogeneity. In fact, GFP_{low} cells in long-term differentiated *Zfp281* mutants re-established GFP_{high} expression within a few days (Figure **EV2G**), revealing reversibility of the GFP_{low} state in the absence of *Zfp281*. To test sufficiency, we generated naïve RGd2 cells conditionally overexpressing Zfp281 under Dox regulation (Figure **EV2H**). Dox-treatment in the presence of 2i induced silencing of the *Rex1* reporter and loss of self-renewal in a subset of cells (Figure **2D**, **EV2I**). Zfp281 is therefore required and sufficient for exit from naïve pluripotency.

A previous study showed that differentiation of Serum/Lif-cultured ESCs is accompanied by up-regulation of Zfp281, which in turn destabilizes metastable pluripotency by binding to the methylcytosine hydroxylase Tet1 and transcriptionally suppressing Tet2 (Fidalgo *et al*, 2016). If the same mechanisms were to regulate exit from naïve pluripotency downstream of Zfp281, loss of *Tet1* should induce the same phenotype as loss of *Zfp281*, and loss of *Tet2* the opposite. We therefore generated *Tet1*, *Tet2* and *Zfp281*/*Tet2* KO alleles in naïve RGd2 ESCs (Figure **EV2J, K**). Strikingly, the extinction of the *Rex1* reporter and self-renewal was similar in differentiating *WT* cells and *Tet1* and *Tet2* mutants, while absence of *Tet2* in *Zfp281*/*Tet2* KO cells did not revert resistance to differentiation caused by absence of *Zfp281* alone (Figure **2E**, **EV2L**). We furthermore noted only modest changes in Zfp281 mRNA or

protein during ESC differentiation, and across existing RNA sequencing (RNA-seq) datasets of EpiLC and EpiSC differentiation (Buecker *et al*, 2014; Factor *et al*, 2014; Bao *et al*, 2018) and epiblast development (Boroviak *et al*, 2015) (Figure **2F**, **EV2M**). Zfp281 has also been reported to repress *Nanog* transcription through interacting with the NuRD complex in Serum/Lif-cultured ESCs (Fidalgo *et al*, 2012). However, Nanog mRNA was unchanged in naïve *Zfp281* KO ESCs or EpiSCs depleted of Zfp281 by siRNA transfection (Figure **EV2N**, **O**). Furthermore, knockdown of Nanog did not restore differentiation in ESCs depleted of Zfp281 while it partially did so, as expected (Pereira *et al*, 2006), in ESCs depleted of Tcf7l1 (Figure **EV2P**). Taken together these results suggest that Zfp281 is expressed fairly constantly during exit from naïve pluripotency and drives differentiation independent of Tet1, Tet2 and Nanog.

Zfp281 acts independent of cell state-exclusive chromatin association

To identify the transcriptional defects causing differentiation resistance, we performed RNA-seq of *WT* and *Zfp281* KO cells in 2i, and 16h and 32h after 2i withdrawal ($WT_{2i,16h,32h}$ and $Zfp281_{2i,16h,32h}$) (Table **EV2**). The expression of several naïve and primed pluripotency markers was perturbed in $Zfp281_{16h}$ and $Zfp281_{32h}$ cells (Figure **EV3A**), confirming impaired silencing of naïve identity in *Zfp281* mutants. k-means clustering of mRNAs that significantly changed during *WT* differentiation or in *Zfp281* KO cells (2495 genes) identified six gene clusters (Figure **3A**, **B**): Clusters 1-4 (1898 genes) contain the majority of genes that were differentially transcribed in WT_{32h} cells and of which a subset was already regulated in WT_{16h} cells. Comparison with external EpiLC (Buecker *et al*, 2014) and EpiSC (Factor *et al*, 2014; Bao *et al*, 2018) expression datasets, which were not employed in the clustering analysis, revealed persistence of the bulk transcriptional changes established in WT_{32h} cells, suggesting that clusters 1-4 contribute to pluripotent cell state progression. Clusters 5 and 6 (597 genes), in contrast, contain genes that were mostly unchanged in WT_{32h} cells, but transiently regulated in WT_{16h} cells and differentially expressed in EpiSCs. Clusters 5 and 6 may therefore act in gastrulation stage epiblast development and/or EpiSCs.

Clusters 1 and 2 were largely unaffected in differentiating *Zfp281* KO cells, whereas the repression and induction, respectively, of cluster 3 and 4 genes was blunted in both $Zfp281_{16h}$ and $Zfp281_{32h}$ cells (Figure **3A**, **B**). Cell state-specific comparison revealed that this was predominantly due to deregulation during differentiation (Figure **3C**). Although we can't exclude that the transcriptional defects in $Zfp281_{32h}$ cells were influenced by cell state heterogeneity (Figure **EV2D**), perturbed expression of cluster 3 and 4 genes in $Zfp281_{16h}$ cells, a timepoint at which downregulation of Rex1 reporter expression (Kalkan *et al*, 2017) and exit from self-renewal (Figure **EV2C**, **F**) has not yet commenced, suggests a direct role of Zfp281 in regulating these genes. Zfp281 may therefore drive exit from naïve pluripotency through controlling gene clusters 3 and 4, which contain the naïve pluripotency TFs *Klf4*, *Klf5* and *Nr0b1*, and the primed markers *Sox3* and *Dnmt3b*, respectively (Table **EV2**), and are enriched for generic developmental terms using GO analysis (Figure **EV3B**). Conversely, cluster 5 and 6 genes were similarly mis-expressed in $Zfp281_{2i}$, $Zfp281_{16h}$ and $Zfp281_{32h}$ cells, notably with directionalities that are inverse to the changes observed in EpiSCs. GO analysis revealed significant enrichment of regulators of cell adhesion, which is critical for cell polarization (Ebnet *et al*, 2018) that initiates lumenogenesis

after exit from naïve pluripotency (Shahbazi *et al*, 2017). To test if Zfp281 controls polarization, we generated spheroids in Matrigel as described before (Shahbazi *et al*, 2017). *WT* ESCs formed polarized spheroids with expanded lumens that were encircled by apical F-actin, while *Zfp281* KO cells grew as unpolarized and disorganized cellular aggregates that were morphologically similar to ESCs (Figure **3D**). Although we cannot exclude that this is consequential to impaired exit from self-renewal, regulation of cluster 5 and 6 genes by Zfp281 may therefore contribute to cell polarization and cavity formation during ESC differentiation.

Oct4, similar to Zfp281, is expressed at equal levels in ESCs and EpiLCs, but occupies distinct CREs in the two cell states (Buecker *et al*, 2014). To determine if Zfp281 acts through cell state-specific chromatin association, we profiled its genome localization in WT_{2i} and WT_{32h} cells using chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq) (Table **EV2**). *De novo* motif finding identified the consensus CCCCTCCCCC motif in 82.4% of 23756 peaks (Figure **EV3C**), which is similar to results obtained in Serum/Lif ESCs (Fidalgo *et al*, 2016). Surprisingly, Zfp281 occupancy in WT_{2i} and WT_{32h} cells was as highly correlated (Pearson's correlation coefficient R = 0.81) as between replicates (R= 0.84 and R=0.85, respectively), with only few peaks exclusively detected in any of the two cell states (Figure **3E**, **EV3D**). A lower correlation was observed between WT_{32h} cells and published data for EpiSCs (Huang *et al*, 2017) (R=0.69) and trophoblast stem cells (TSCs) (Ishiuchi *et al*, 2019) (R=0.55), but binding at peaks associated to cluster 1-6 genes was largely unchanged (Figure **EV3E**, **F**), suggesting stable chromatin association also during later pluripotency progression and in lineage-unrelated TSCs. To determine if Zfp281 binds to CREs, we profiled histone H3 lysine 27 acetylation (H3K27ac), a chromatin mark associated with active promoters and enhancers. Comparison of our Zfp281 and H3K27ac with published histone mark ChIP-seq data (Kurimoto *et al*, 2015; Buecker *et al*, 2014) (Figure **EV3G**) identified 7697 Zfp281 peaks proximal to transcriptional start sites (TSSs), of which 54% were at active promoters (co-localization with H3K27ac and H3K4 tri-methylation), and 16059 distal Zfp281 peaks of which 62% were at putative enhancers (co-localization with H3K4 mono-methylation). 38% of the latter were also enriched for H3K27ac, qualifying them as active enhancers. Notably, peaks with slightly increased Zfp281 binding in WT_{32h} cells gained H3K27ac and expression of associated genes during differentiation, while decreased binding was associated with reduced H3K27ac and transcription (Figure **3F**). Despite stable occupancy of target sites, quantitative binding changes of Zfp281 at these sites therefore parallel differences in CRE activity. However, this was similar at peaks linked to Zfp281-insensitive cluster 1/2 and -sensitive cluster 3/4 genes (Figure **3G**), showing that differential binding strength at CREs correlates with differentiation-specific gene expression, but only partially with transcriptionally regulated targets.

Zfp281 interacts with Ehmt1 and Zic2 during ESC differentiation

Since chromatin occupancy was largely unchanged, we reasoned that Zfp281 may control transcription through cell state-specific protein interaction partners. To test this, we performed Zfp281 immunoprecipitations (IPs) coupled to semi-quantitative mass spectrometry (MS) in nuclear extracts of WT_{2i} and WT_{40h} cells, including *Zfp281* KO lysates to control for antibody-specificity (Table **EV3**).

Stringent selection criteria identified the previously reported interactor Nanog (Fidalgo *et al*, 2012) in WT_{2i} cells and several proteins specifically enriched in WT_{40h} cells (Figure **4A**). Strikingly, the latter were transcriptionally induced and the former repressed during differentiation (Figure **4B**), suggesting that differential binding to Zfp281 may, at least in part, be driven by protein abundance. To determine functional downstream effectors, we decided to probe genetic interaction of differentiation-specific interactors with Zfp281 in our conditionally overexpressing ESCs. To this end we depleted selected binding partners using siRNA transfection, induced Zfp281 by Dox treatment and quantified *Rex1* reporter distribution after 32h in 2i (Figure **4C**). As controls, we included siRNAs targeting Zfp281 itself and Tcf7l1. Transfection of Zfp281 but not Tcf7l1 siRNAs blocked emergence of GFP_{low} cells (76% reduction) (Figure **4C**, **EV4A**), thus confirming suitability for identifying genetic Zfp281 interactors. Of all candidates tested individually, only knockdown of Ehmt1 and Zic2 reduced the fraction of GFP_{low} cells (34% and 32%, respectvely), an effect enhanced by simultaneous depletion of both (63%). Conversely, conditional overexpression of Ehmt1 and Zic2 in RGd2 ESCs (Figure **EV4B**, **C**), similar to Zfp281, induced downregulation of the *Rex1* reporter in a subset of cells (Figure **EV4D**). The *de novo* DNA methyltransferases Dnmt3a and Dnmt3b have overlapping functions during embryogenesis (Okano *et al*, 1999) and, hence, may act redundantly. However, simultaneous depletion of Dnmt3a and Dnmt3b by siRNAs or in compound *Dnmt3a*/*3b* KO cells (Figure **EV4E**, **F**) did not impair Zfp281-induced reporter repression (Figure **4C**, **EV4G**), demonstrating that Zfp281 drives differentiation independent of Dnmt3a and Dnmt3b.

Zic2 is a zinc finger TF that represses poised developmental enhancers in Serum/Lif ESCs (Luo *et al*, 2015). Ehmt1 (GLP) is a methyltransferase that can be found in a complex with Ehmt2 (G9a), which both mediate mono- and di-methylation of histone H3 lysine 9 (H3K9me1 and H3K9me2) (Tachibana *et al*, 2005). The genetic interactions in naïve ESCs (Figure **4C**) together with the preferential binding during differentiation observed in both nuclear extracts using IP-MS (Figure **4A**) and whole cell lysates using IP-western blot (Figure **EV4H**), suggests that Ehmt1 and Zic2 are functional downstream effectors of Zfp281.

Ehmt1 and Zic2 regulate ESC differentiation and reprogramming of EpiSCs

We therefore generated individual and compound *Ehmt1* and *Zic2* KO RGd2 ESCs (Figure **EV5A**, **B**). In contrast to wildtype or *Zic2* KO cells, *Ehmt1* and *Ehmt1*/*Zic2* KO cells were spindle-shaped (Figure **EV5C**) and proliferated slowly (Figure **EV5D**). They were not arrested at a specific cell cycle stage (Figure **EV5E**) and did not exhibit downregulation of the *Rex1* reporter in 2i (Figure **EV5F**). After 2i withdrawal for 32h (or 72h), 75% (9%) of *Ehmt1* and 55% (7%) of *Zic2* KO cells maintained GFP expression, increasing to 90% (35%) in *Ehmt1*/*Zic2* compound KO cells (Figure **EV5F**), while 30% (1%) of cells from untargeted sibling clones (*Zic2* WT and *Ehmt1* WT) were GFP_{high}. Correspondingly, 5%, 4% and 12% of *Zic2*, *Ehmt1* and *Ehmt1*/*Zic2* KO cells retained self-renewal after 72h of differentiation (Figure **5A**). *Ehmt1* and *Ehmt1*/*Zic2*, but not *Zic2* KO ESCs, were unable to form polarized spheroids in Matrigel (Figure **5B**). Quantification of this effect was similar to *Zfp281* mutants (Figure **EV5G**). We therefore conclude that *Ehmt1* is required for polarization and that *Ehmt1* and *Zic2* promote exit from self-renewal independently of each other.

In the absence of *Ehmt1*, H3K9me2 was limited to DAPI-rich speckles (Figure **EV5H**), which is reminiscent of the depletion of euchromatic H3K9me2 and its enrichment at pericentric heterochromatin in *Ehmt2* mutants (Tachibana *et al*, 2002). Since exposure to the Ehmt inhibitors A-366 and UNC0642 induced dose-dependent cell lethality (data not shown), we decided to test Ehmt1's enzymatic activity by expressing specific loss of function alleles in *Ehmt1* KO cells: An Ehmt1 protein with mutations in the ankyrin domain ($Ehmt1_{ank}$), responsible for binding to methylated H3K9 *in vitro* (Collins *et al*, 2008), reverted nuclear H3K9me2 distribution (Figure **EV5H**) and resistance to *Rex1* downregulation (Figure **5C**, **EV5I**) to a similar extent as the wildtype protein did. In contrast, substitutions in the SET domain ($Ehmt1_{NH\text{-}LE}$) that perturb Ehmt1 methyltransferase *in vitro* (Tachibana *et al*, 2008) rescued only partially and a small deletion in the SET domain ($Ehmt1_{\Delta NHHC}$) that additionally ablates binding to Ehmt2 completely abolished rescue. Therefore, both catalytic activity of Ehmt1 and formation of a larger methyltransferase protein complex are implicated in ESC transition.

Chemical inhibition and knockdown of Ehmt enzymes in somatic cells enhances reprogramming (Sridharan *et al*, 2013; Rodriguez-Madoz *et al*, 2017; Shi *et al*, 2008). Consistently, depletion of Ehmt1 but also of Zic2 in 796.4 and $O4GIP_{GY118F}$ EpiSCs increased Epi-iPSC formation in the presence of Gcsf (Figure **5D, EV5J**). The effect was modest, but enhanced by the combined knockdown of both. Taken together these results suggest that Ehmt1 and Zic2, similar to Zfp281, drive exit from naïve pluripotency and restrain reprogramming of EpiSCs. Notably, phenotypes induced by co-depletion of Zic2 and Ehmt1 were weaker than elimination of Zfp281, suggesting existence of additional Zfp281 effectors that may include other histone modifying complexes (Huang *et al*, 2017; Zhou *et al*, 2017; Ishiuchi *et al*, 2019).

Overlapping transcriptional functions of Zfp281 and Ehmt1/Zic2

To test if the biochemical and functional interaction with Zfp281 is reflected in similar transcriptional outputs, we profiled mRNA expression in *Ehmt1*, *Zic2* and *Ehmt1*/*Zic2* KO cells in 2i and after 32h of differentiation (Table **EV2**). Principle component (PC) analysis, including *Zfp281* KO, EpiLC (Buecker *et al*, 2014) and EpiSC (Bao *et al*, 2018) datasets, of changes relative to *WT* ESCs identified PC1 to discriminate developmental timing and to separate differentiated cells from ESCs (Figure **6A**). Mutant and wildtype ESCs projected similarly onto PC1 and expressed pluripotency marker genes at similar levels (Figure **EV6A**), confirming their naïve identity. PC2, in contrast, segregated *WT* from *Zfp281* and, in particular, *Ehmt1* KO genotypes. We, indeed, observed 1274 deregulated genes in $Ehmt1_{2i}$ cells that were unchanged in $Zfp281_{2i}$ cells (Figure **EV6B**). These were enriched for homeostatic and cell adhesion GO terms (Figure **EV6B**) and likely contribute to the cellular and polarization phenotypes in *Ehmt1* KO cells.

Progression of $Zfp281_{16h/32h}$, $Ehmt1_{32h}$ *and* $Ehmt1/Zic2_{32h}$ cells along PC1 was impaired when compared to matching *WT* controls (Figure **6A**), which we also observed in PC analysis using blastocyst development datasets (Boroviak *et al*, 2015) (Figure **6B**). In fact, alterations in *Ehmt1* and *Zfp281* KO cells correlated during differentiation (Figure **EV6C**), suggesting similar defects in developmental transcription. This correlation was not strong (R = 0.44), but increased (R=0.57) when only considering cluster 1-6 gene expression (Figure **EV6D**). Although we also noted slight deregulation of clusters 1 and 2, transcriptional defects in clusters 3-6 were similar in $Ehmt1_{32h}$ and $Zfp281_{32h}$ cells (Figure **6C**,

D). Ehmt2-dependent H3K9 di-methylation is associated with gene silencing (Zylicz *et al*, 2015), consistent with the majority of genes showing increased RNA levels in *Ehmt1*$_{2i}$ cells (Figure **EV6B**). Nevertheless, 38% of target genes were downregulated. Changes in the absence of *Ehmt1* are therefore likely consequential to both direct and indirect effects and may also include the contribution of non-histone Ehmt1 substrates (Sim *et al*, 2017) to transcription.

Based on mRNA levels, *Zic2*$_{32h}$ cells were not separated from matching control cells (Figure **6A-D**). This was surprising, since *Zic2* KO cells appeared similarly impaired in exiting self-renewal as *Ehmt1* KO cells (Figure **5A**). However, loss of *Zic2* in *Ehmt1*$_{32h}$ cells enhanced the deregulation of clusters 1-4 during differentiation (Figure **6D**) and induced a shift along PC1 (Figure **6A**, **B**). Linear regression revealed that perturbations in *Ehmt1/Zic2* KO cells were predominantly the sum of alterations in single mutants rather than synergistic (Figure **EV6E**), implying subtle, but functionally relevant changes in *Zic2* KO cells. Ehmt1 and Zic2 therefore regulate transcription independently of each other, aligning with their additive loss-of-function phenotypes in differentiation and reprogramming (Figure **5A**, **D**). Taken together, this analysis demonstrates connected functions of Zfp281 and Ehmt1/Zic2 in gene expression during cell state transition.

Ehmt1 and Zic2 act downstream of Zfp281 on chromatin

To identify direct targets and to explore how those relate to the physical interaction with Zfp281 in differentiated cells, we performed Zic2 and Ehmt1 ChIP-seq, and profiled H3K9me2 as a proxy for Ehmt1 activity. Due to absence of ChIP-seq compatible Ehmt1 antibodies, we inserted an N-terminal Flag-Avi tag at both *Ehmt1* alleles in ESCs expressing the BirA biotin ligase (Figure **EV7A**). This did not perturb exit from self-renewal (Figure **EV7B**), indicating expression of a functional Ehmt1 fusion protein (Bio-Ehmt1). ChIP-seq using Streptavidin beads identified broad Ehmt1-occupied chromatin domains that, consistent with an enzyme-substrate-relationship, scaled with H3K9me2 genome-wide (Figure **EV7C**). To determine overlap with Zfp281, we quantified Ehmt1 and H3K9me2 enrichment at Zfp281-bound and matching unbound control windows (see methods for details). Ehmt1 localization at both sets of regions was unchanged during differentiation or in *Zfp281* KO cells (Figure **EV7D**, **E**), indicating that Zfp281 is not required for Ehmt1 localization on chromatin. In contrast to Ehmt1, H3K9me2 increased in *WT*$_{32h}$ cells and EpiLCs (Kurimoto *et al*, 2015), with a more pronounced increase at Zfp281-occupied loci than control windows (Figure **7A**). To test if these dynamics require Zfp281, we performed immunofluorescence staining which revealed that mutant cells failed to gain H3K9me2 by 32h after 2i withdrawal (Figure **EV7F**). H3K9me2 ChIP-seq in *Zfp281* mutants confirmed that H3K9me2 levels were unaffected in *Zfp281*$_{2i}$ cells but did not increase in *Zfp281*$_{32h}$ cells (Figure **7A**, **EV7E**). Impaired gain of H3K9me2 was observed at both Zfp281-bound and -unbound sites, suggesting that Zfp281 is a differentiation-specific pervasive activator of Ehmt1 during ESC transition. Zic2 ChIP-seq identified 28495 peaks, of which approximately 30% overlapped with Zfp281 (Figure **7B**). These were closer to promoters and enriched for H3K27ac compared to Zic2-only and Zfp281-only peaks (Figure **EV7G, H**), suggesting co-localization of Zfp281 and Zic2 at CREs. In *WT*$_{32h}$ cells, Zic2 increased predominantly at co-bound peaks (Figure **7B**). Although we noted a general reduction of Zic2 on chromatin in *Zfp281* mutants, Zic2 localization was particularly perturbed at co-bound sites in *Zfp281*$_{32h}$

cells (Figure **7B**, **EV7I**). Our findings therefore suggest that Zfp281 engages Ehmt1 and Zic2 during ESC differentiation through chromatin co-occupancy-dependent and -independent mechanisms.

At Zfp281-bound peaks, the gain of H3K9me2 and Zic2 occurred mostly at mutually exclusive sets of genomic loci with reduced and increased Zfp281 binding during differentiation, respectively (Figure **7C**, **D**), indicating that these are sites of direct physical interaction that control transcription of nearby genes. We therefore stratified H3K9me2 and Zic2 binding dynamics at Zfp281 peaks by gene cluster association. H3K9me2 increased predominantly at peaks linked to repressed clusters 1 and 3, and Zic2 at peaks belonging to induced clusters 2 and 4 (Figure **7E**). Surprisingly, the gain in WT_{32h} cells and reduction in $Zfp281_{32h}$ cells was indistinguishable between clusters 1 and 3 (for H3K9me2) and clusters 2 and 4 (for Zic2). Hence, Zfp281 catalyzes H3K9me2 and Zic2 deposition at transition-associated CREs genome-wide and without any qualitative or quantitative specificity for its transcriptional targets in clusters 3 and 4. Why cluster 1 and 2 gene expression is insensitive to perturbation of H3K9me2 and Zic2 dynamics in *Zfp281* KO cells (Figure **3C**, **7E**) remains to be determined. Additional chromatin regulators may be involved, since transcription of clusters 1 and 2 is also less sensitive to *Ehmt1* depletion than of clusters 3 and 4 (Figure **6D**). We therefore propose that Zfp281 drives and stabilizes transition-specific transcription, at least in part, through activation of Ehmt1 at cluster 3 CREs and recruitment or stabilization of Zic2 at cluster 4 CREs.

DISCUSSION

Cellular plasticity in response to injury *in vivo* or TF overexpression *in vitro* is frequently accompanied by the reversal of cellular specialization (Merrell & Stanger, 2016). Although single cell profiling has shown that this process is not a strict inversion of natural development (Gerber *et al*, 2018; Treutlein *et al*, 2016), differentiation and de-differentiation trajectories may mechanistically intersect. We aimed to uncover such plasticity regulators in pluripotent cells and performed loss-of-function screening for genes that both drive exit from ESC self-renewal, and shield EpiSCs from reprogramming into the pluripotent ground state. Within the experimental limitations of this approach we identified only one gene, the TF *Zfp281*. Such exclusivity suggests a prominent role in establishing and maintaining the unidirectionality of pluripotent cell state progression *in vitro*. The former is consistent with perturbed epiblast maturation in *Zfp281* mutant embryos (Huang *et al*, 2017), but if Zfp281 protects cellular identities against de-differentiation *in vivo* remains to be determined. We, however, note that Zfp281 restrains iPSC formation from fibroblasts at a late pre-iPSC stage (Fidalgo *et al*, 2012), supporting the notion that resetting of EpiSCs into naïve pluripotency recapitulates a late phase of somatic cell reprogramming (Dunn *et al*, 2019). Other factors that, similar to Zfp281, drive differentiation and inhibit de-differentiation of cell states not represented in our ESC-EpiSC conversion system are likely to exist.

We showed that Zfp281 is important for robust ESC differentiation. This is reminiscent of lineage-specifying TFs, that are specifically expressed in the lineages they instruct (Graf & Enver, 2009). In adult mice, Zfp281 is indeed transcribed strongest in heart tissue and its overexpression in fibroblasts enhances cardiac reprogramming (Zhou *et al*, 2017). During ESC differentiation, however, Zfp281 neither changes expression nor occupies distinct genomic sites, indicating a facilitating, rather than

specifying, function. Using biochemical, genetic and genomic experiments we provide evidence that Zfp281 directs sequential gene expression through permissive and instructive mechanisms involving physical interaction with Ehmt1 and Zic2. Cluster 5 and 6 genes are differentially expressed in EpiSCs, but transcriptionally primed by Zfp281 throughout differentiation, including in the naïve pluripotent starting cell state. Cluster 5 contains modulators of cell adhesion that may contribute to polarization of post-implantation epiblast cells. However, although *Zfp281* null embryos degenerate during gastrulation, they do form an epithelial egg cylinder (Huang *et al*, 2017), suggesting operation of compensatory mechanisms *in vivo*. Cluster 3 and 4 genes are, in contrast, regulated by Zfp281 predominantly during transition, enriched for developmental functions, and, therefore, likely responsible for extinguishing ESC identity. Strikingly, Zfp281 quantitatively decreases at CREs associated with repressed cluster 3 genes and, *vice versa*, increases at CREs associated with induced cluster 4 genes. Concomitant gain of H3K9me2 and Zic2 at cluster 3 and 4 CREs, respectively, suggests that subtle Zfp281 chromatin binding differences are converted into CRE activity changes by reciprocal activation of Ehmt1 and Zic2. Consistently, embryonic arrest in *Ehmt1* and *Ehmt2* mutant mouse embryos (Tachibana *et al*, 2002; 2005) has been attributed to reduced H3K9me2 deposition at and impaired silencing of developmental enhancers (Zylicz *et al*, 2015), while Zic2 triggers neural plate gene expression in EpiSCs through enhancer activation (Iwafuchi-Doi *et al*, 2012). The molecular mechanisms inducing differential binding of Zfp281 to Ehmt1 and Zic2 remain to be elucidated. Because of similar overexpression phenotypes in ESCs we surmise that protein ratios are rate-determining. Zic2 protein levels, despite unchanged mRNA (Figure **4B**), indeed increase during differentiation, while Ehmt1 persists (Figure **EV4H**). However, Ehmt1 and Ehmt2 interact with additional zinc finger TFs (Shinkai & Tachibana, 2011) that may compete with Zfp281 for binding to Ehmt1 specifically in ground state ESCs. Taken together, we suggest that Zfp281 promotes stable cell state transition by permissively marking cluster 5 and 6 genes for forthcoming developmental regulation, and instructing cluster 3 and 4 gene expression for elimination of naïve pluripotency, the latter through differential engagement with Ehmt1 or Zic2 at CREs.

Zfp281 drives differentiation to and inhibits reprogramming of EpiSCs, and therefore qualifies as a bidirectional cell state regulator with antipodal functions during developmental progression and reversion. If this is because Zfp281-Ehmt1/Zic2 control the same cell state transition and act through the same target genes in mutual directions remains to be determined. We showed that *Zfp281* KO cells exposed to differentiation-promoting conditions are less clonogenic than ESCs and give rise to an equilibrium of $Rex1^{high}$ and $Rex1^{low}$ cell states that features defective coupling of Rex1 downregulation with exit from self-renewal. It is therefore conceivable that Zfp281 acts after an initial commitment step to induce and stabilize irreversible silencing of naïve identity. During reprogramming, Zfp281 may inversely antagonize induction of naïve pluripotency prior to formation of a $Rex1^{high}$ state, which has recently been shown to mark transition intermediates with full ESC self-renewal activity and destined to give rise to Epi-iPSCs (Stuart *et al*, 2019). Acting at a late stage of reprogramming also reconciles our finding of Zfp281 impairing Epi-iPSC formation by STAT3 activation and Esrrb overexpression (Figure **1G**, **EV1F**) with the notion that both regimes proceed along distinct transcriptional trajectories before converging on a $Rex1^{high}$ cell state (Stuart *et al*, 2019). However, Zfp281 depletion in EpiSCs also

causes cell cycle arrest and apoptosis (Fidalgo *et al*, 2016), but not upregulation of naïve pluripotent TFs such as Nanog (Figure **EV2O**), and is not alone sufficient for reprogramming to Epi-iPSCs in the presence of 2i (Figure **1F**). Zfp281 activates transcription of Nodal signaling components, such as the cluster 5 genes *Lefty1* and *Lefty2*, in ESCs, EpiSCs and the post-implantation epiblast (Huang *et al*, 2017). Nodal signaling is required for EpiSC maintenance (Vallier *et al*, 2009), but dispensable for exit from ESC self-renewal (Mulas *et al*, 2017) and somatic cell reprogramming (Ruetz *et al*, 2017). We therefore speculate that Zfp281 controls the EpiSC state and reprogramming through different effector genes. The former through regulation of cluster 5/6 genes, e.g. *Lefty1*, *Lefty2* or cell polarity regulators, and the latter through stabilization of cell state transition-specific cluster 3/4 genes.

In metastable Serum/Lif ESCs, Zfp281 is reported to promote DNA methylation and differentiation by recruiting Tet1 and silencing Tet2 (Fidalgo *et al*, 2016). Upon exit from the naïve ESC state, Zfp281 binds to Dnmt3a, Dnmt3b and Dnmt3l (Figure **4A**), suggesting that Zfp281 controls 5-methylcytosine turnover through regulating DNA methylating and demethylating enzymes. Furthermore, Dnmt1, Dnmt3a and Dnmt3b interact with the Ehmt1-Ehmt2 heterodimer (Epsztejn-Litman *et al*, 2008; Estève *et al*, 2006), H3K9me2 and 5-methylcytosine marks overlap genome-wide (Meyenn *et al*, 2016) and DNA is hypomethylated upon depletion or catalytic inhibition of Ehmt2 in ESCs (Zhang *et al*, 2016b). We, however, showed that Zfp281 drives exit from naïve pluripotency independent of Dnmt3a, Dnmt3b, Tet1 and Tet2. Although the specific contributions of 5-methylcytosine, H3K9me2, and other Ehmt substrates (Sim *et al*, 2017) to pluripotent cell plasticity remain to be determined, our findings suggest that resolution of naïve pluripotency *in vitro* is masked or mechanistically distinct in heterogeneous Serum/Lif ESC cultures. Similarly, Zic2 has previously been reported to act as a repressor in metastable Serum/Lif ESCs (Luo *et al*, 2015), but we detect only minor transcriptional defects in naïve *Zic2* KO ESCs.

Using the paradigm of pluripotent cell state conversion *in vitro*, we here identify, in Zfp281, a regulator of cellular plasticity that modulates CRE activity and transcription without reliance on cell type-restricted expression or chromatin occupancy. We propose that the persistence of Zfp281 at developmental CREs throughout cell state transition provides a molecular platform for ordered remodeling of the cis-regulatory architecture and further consolidation by lineage-specifying TFs. These findings demonstrate that differential gene expression is not a necessary criterion of cellular plasticity regulators and we suggest that this feature may not be limited to pluripotent cells.

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AUTHOR CONTRIBUTIONS

D.M., M.R. and J.B. performed experiments. D.M. and M.B.S. performed bioinformatical analysis, D.H. mass spectrometry, and I.L. spheroid image analysis. M.W., A.S. and F.B. assisted the esiRNA screen. D.M. and J.B. wrote the paper.

CONFLICT OF INTERESTS

The authors declare no conflict of interests

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MAIN FIGURES

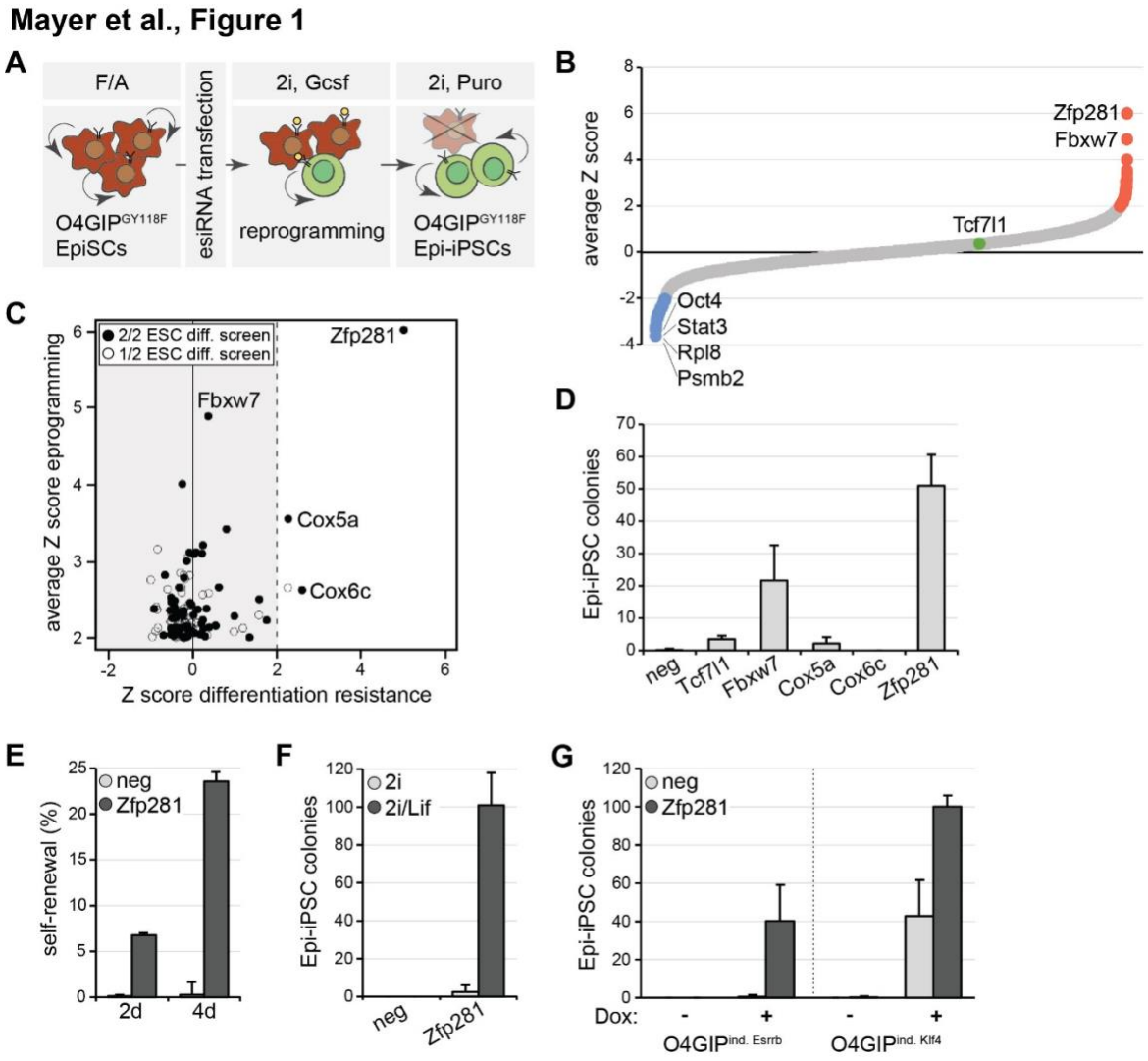

Figure 1: Zfp281 inhibits reprogramming of EpiSCs.

(**A**) Schematic outline of the reprogramming screen. Red indicates O4GiP$_{GY118F}$ EpiSCs and green O4GIP$_{GY118F}$ Epi-iPSCs.

(**B**) Average Z scores of the two screen replicates. Note that esiRNAs targeting Mll1 (Zhang *et al*, 2016a) and Mbd3 (Rais *et al*, 2013) were not included in our library and that Otx2 (Acampora *et al*, 2013) scored below the significance threshold. Screen hits with negative (blue) and positive (red) Z scores (red), and *Tcf7l1* (green) are highlighted.

(**C**) Comparison of reprogramming screen hits with two ESC differentiation screens (Betschinger *et al*, 2013; Li *et al*, 2018)**.** Empty and full circles indicate genes recovered in one and both ESC differentiation screens, respectively.

(**D**) Number of Epi-iPSC colonies derived from 796.4 EpiSCs transfected with indicated siRNAs, stimulated with Gcsf and 2i for 4d, and selected with Puromycin. Average and standard deviation (SD) of 3 experiments performed in duplicates. Negative siRNA (neg).

(**E**) Self-renewal of O4GIP$_{GY118F}$ reprogramming intermediates after 2d or 4d of stimulation with Gcsf and 2i following transfection with indicated siRNAs. Average and SD of 2 experiments performed in duplicates.

(**F**) Number of Epi-iPSC colonies derived from OEC2 EpiSCs transfected with indicated siRNAs, treated for 4d in 2i or 2i/Lif medium, and selected with Puromycin. Average and SD of 2 experiments performed in duplicates.

(**G**) Number of Epi-iPSC colonies derived from O4GIP EpiSCs carrying Dox-inducible Esrrb or Klf4 transgenes after transfection with indicated siRNAs, stimulation with or without Dox for 2d, and selection with Puromycin. Average and SD of 2 experiments performed in duplicates.

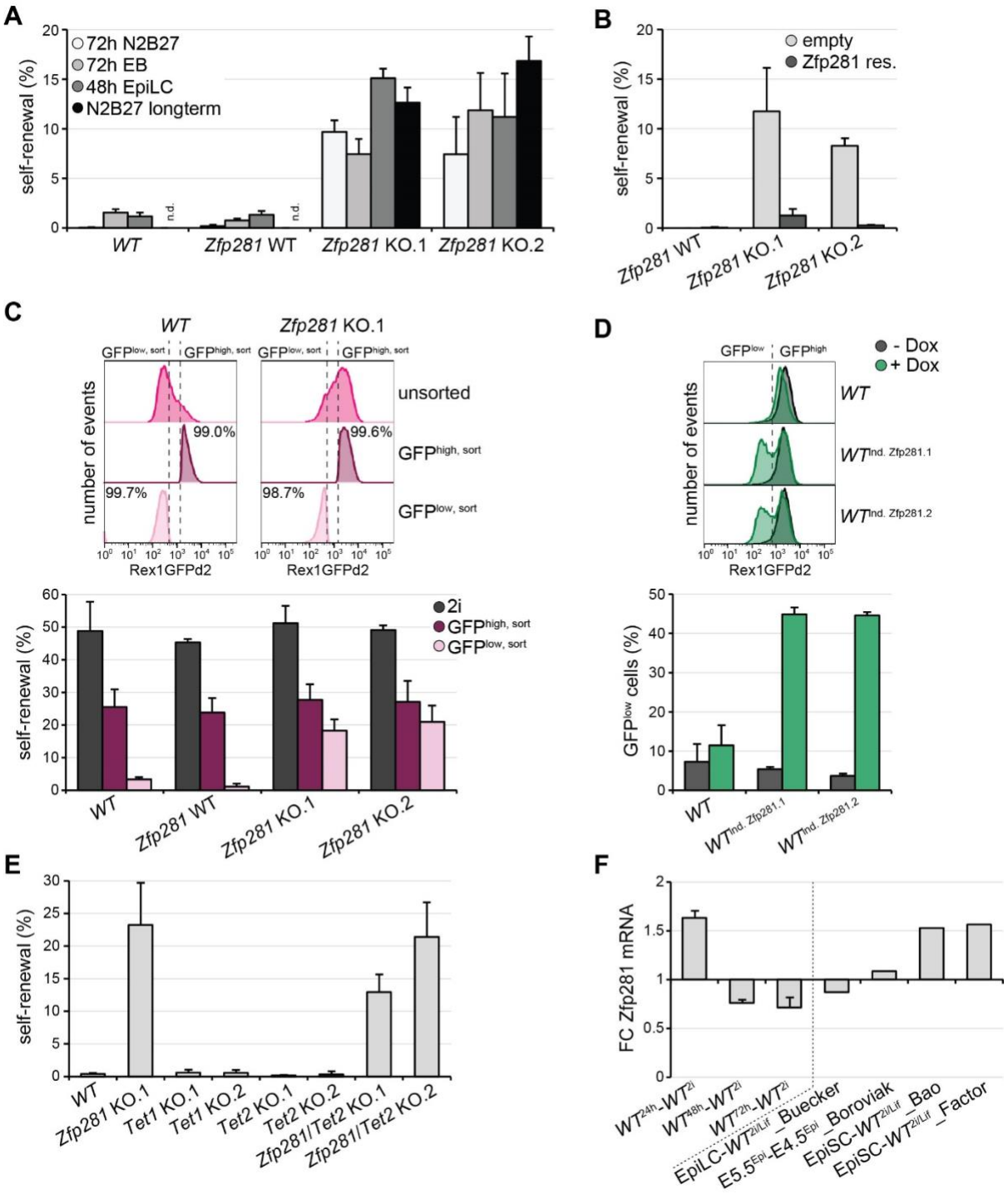

Figure 2: Zfp281 drives exit from naïve pluripotency independent of Tet enzymes.

(**A**, **B**, **E**) Self-renewal in RGd2 ESCs of specified genotypes expressing indicated transgenes (**B**) after differentiation in indicated conditions (**A**) or 72h in N2B27 (**B**, **E**). Average and SD of 2 experiments performed in duplicates. Note that control cells were lost during continuous passaging in N2B27 (**A**). Not determined (n.d.).

(**C**) Representative flow cytometry profiles of *WT* and *Zfp281* KO.1 cells after 32h of 2i withdrawal before (unsorted) and after purification of cells with indicated GFP expression (top panel). Self-renewal

of undifferentiated (2i) and sorted $GFP_{high,sort}$ and $GFP_{low,sort}$ cells of indicated genotypes (bottom panel). Average and SD of 3 experiments performed in duplicates.

(**D**) Representative flow cytometry profiles of control and Zfp281-inducible ESCs (top panel) and quantification of GFP_{low} cells (bottom panel) after 2d in 2i and in the presence (green) or absence (black) of Dox. Average and SD of 2 experiments.

(**F**) Zfp281 mRNA changes during ESC differentiation detected by quantitative PCR (left) and extracted from published RNA-seq datasets (Buecker *et al*, 2014; Factor *et al*, 2014; Boroviak *et al*, 2015; Bao *et al*, 2018) (right). Average and SD of 2 technical replicates (left).

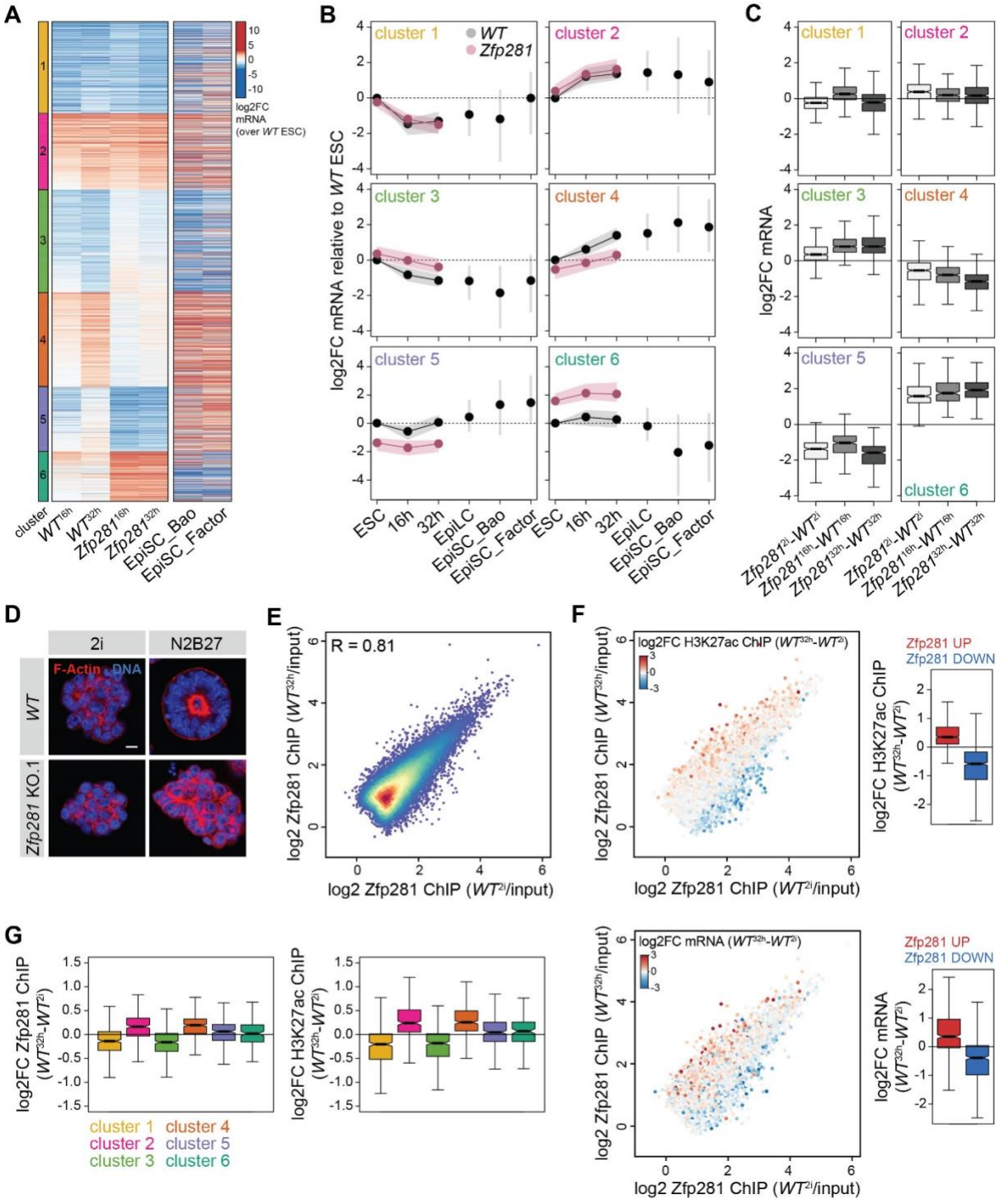

Figure 3: Zfp281 directs sequential gene expression despite stable occupancy of target sites.

(**A**) mRNA log2 fold changes (log2FC) in *WT*16h, *WT*32h, *Zfp281*16h and *Zfp281*32h samples relative to *WT*2i cells, and in EpiSCs relative to *WT*2i/Lif cells (Factor *et al*, 2014; Bao *et al*, 2018). *Zfp281*2i, *Zfp281*16h**,** *Zfp281*32h and *WT*16h and *WT*32h samples were used for k-means clustering.

(**B**, **C**) Quantification of (**A**) including mRNA log2FC in EpiLCs relative to *WT*2i/Lif (Buecker *et al*, 2014) and as indicated (**C**).

(**D**) Representative immunofluorescence staining of spheroids in Matrigel derived from *WT* or *Zfp281* KO.1 ESCs grown in 2i or N2B27 for 3d. Blue: DNA. Red: F-actin. Scale bar is 10µm.

(**E**) Scatter plot comparing Zfp281 log2 ChIP enrichment relative to matched inputs in WT_{2i} and WT_{32h} cells.

(**F**) Same as in (**E**) with dots colored according to H3K27ac ChIP log2FC at the same peaks (top left), and to gene expression log2FC associated with peaks by nearest distance to TSS (bottom left) in WT_{32h} relative to WT_{2i} cells. Quantification of H3K27ac ChIP (top right) and mRNA (bottom right) log2FC at top 1000 Zfp281 peaks with increased (red) or decreased (blue) Zfp281 binding during ESC differentiation.

(**G**) Quantification of Zfp281 (left) and H3K27ac (right) ChIP log2FC in WT_{32h} compared to WT_{2i} cells at Zfp281 peaks assigned to gene clusters 1-6.

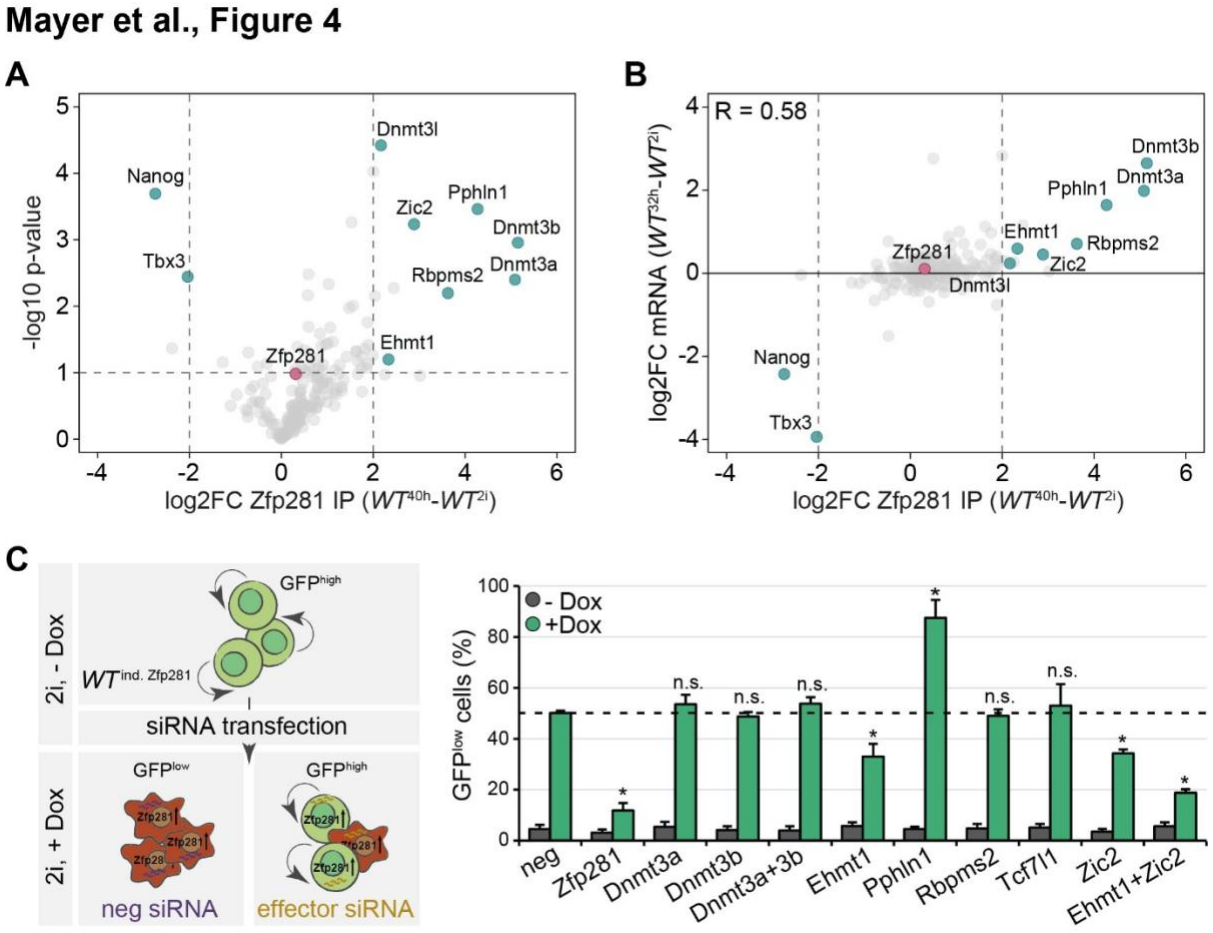

Figure 4: Zfp281 acts by associating with Ehmt1 and Zic2.

(**A**) Cell state-specific Zfp281 interactors in *WT*$_{2i}$ and *WT*$_{40h}$ cells. Pink and cyan mark Zfp281 and selected binding partners, respectively. Quantification is based on 3 biological replicates.

(**B**) Same as (**A**) with mRNA log2FC of differential binding partners during ESC differentiation instead of p-values.

(**C**) Procedure to identify Zfp281 effectors in naïve Zfp281-inducible RGd2 cells (left). Quantification of GFP$_{low}$ *WT*$_{ind. Zfp281}$ cells transfected with indicated siRNAs and incubated for 32h in 2i in the presence (green) or absence (black) of Dox (right). Dashed line marks fraction of GFP$_{low}$ cells in control cells exposed to Dox. Significance was determined using a Wilcoxon Mann-Whitney rank sum test compared to neg control sample. (*) <0.05; not significant (n.s.). Average and SD of 4 experiments.

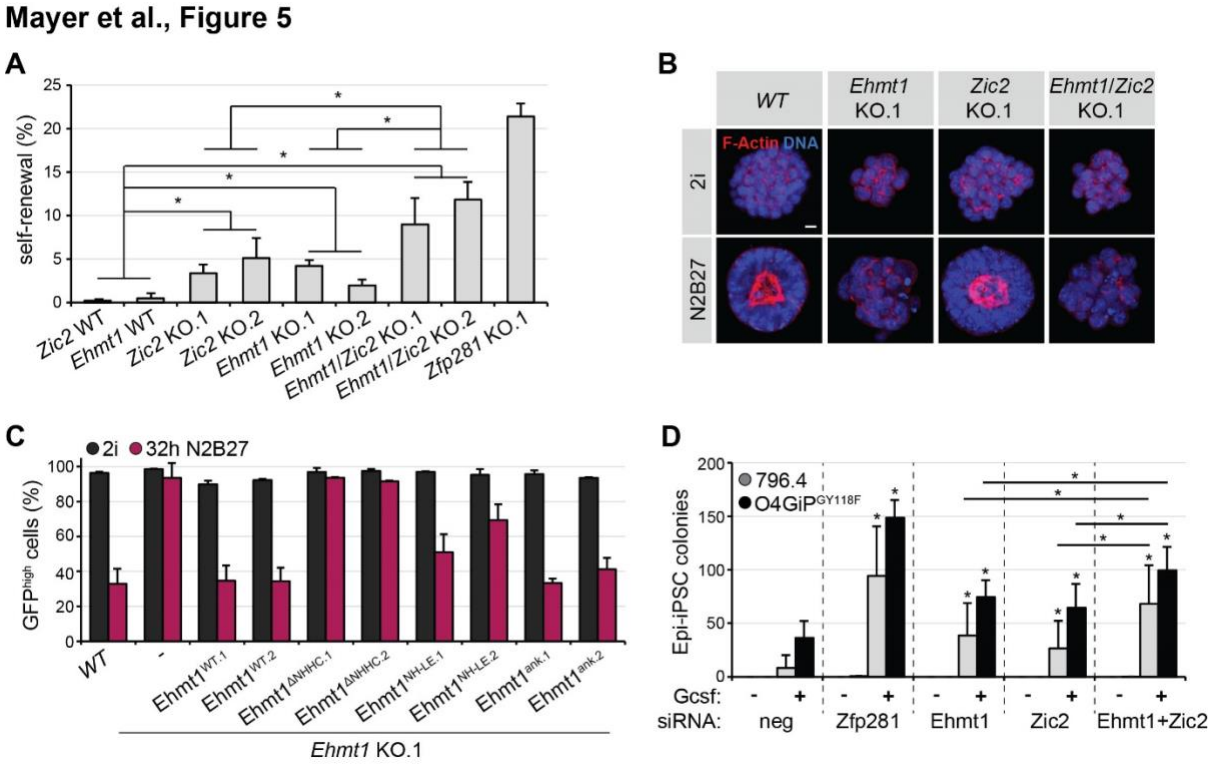

Figure 5: Ehmt1 and Zic2 drive exit from the ESC state and restrict reprogramming of EpiSCs.

(**A**) Self-renewal in cells with indicated genotypes 3d after 2i withdrawal. Significance was determined using a Wilcoxon Mann-Whitney rank sum test comparing the specified genotype groups. (*) <0.05. Average and SD of 2 experiments performed in duplicates.

(**B**) Representative immunofluorescence staining of spheroids in Matrigel derived from indicated genotypes in 2i or N2B27 for 4d. Blue: DNA. Red: F-actin. Scale bar is 10µm.

(**C**) Quantification of GFP_{high} cells in *WT* cells or *Ehmt1* KO clones expressing indicated transgenes in 2i (black) or 32h after 2i withdrawal (pink). Average and SD of 2 experiments.

(**D**) Number of Epi-iPSC colonies derived from 796.4 (grey) and $O4GIP_{GY118F}$ (black) EpiSCs transfected with indicated siRNAs, stimulated with Gcsf and 2i for 4d, and selected with Puromycin. Significance was determined using a Wilcoxon Mann-Whitney rank sum test compared to neg control sample of the respective cell line, or comparing Zic2 and Ehmt1 to Ehmt1/Zic2 depletion. (*) <0.05. Average and SD of 5 experiments performed in duplicates.

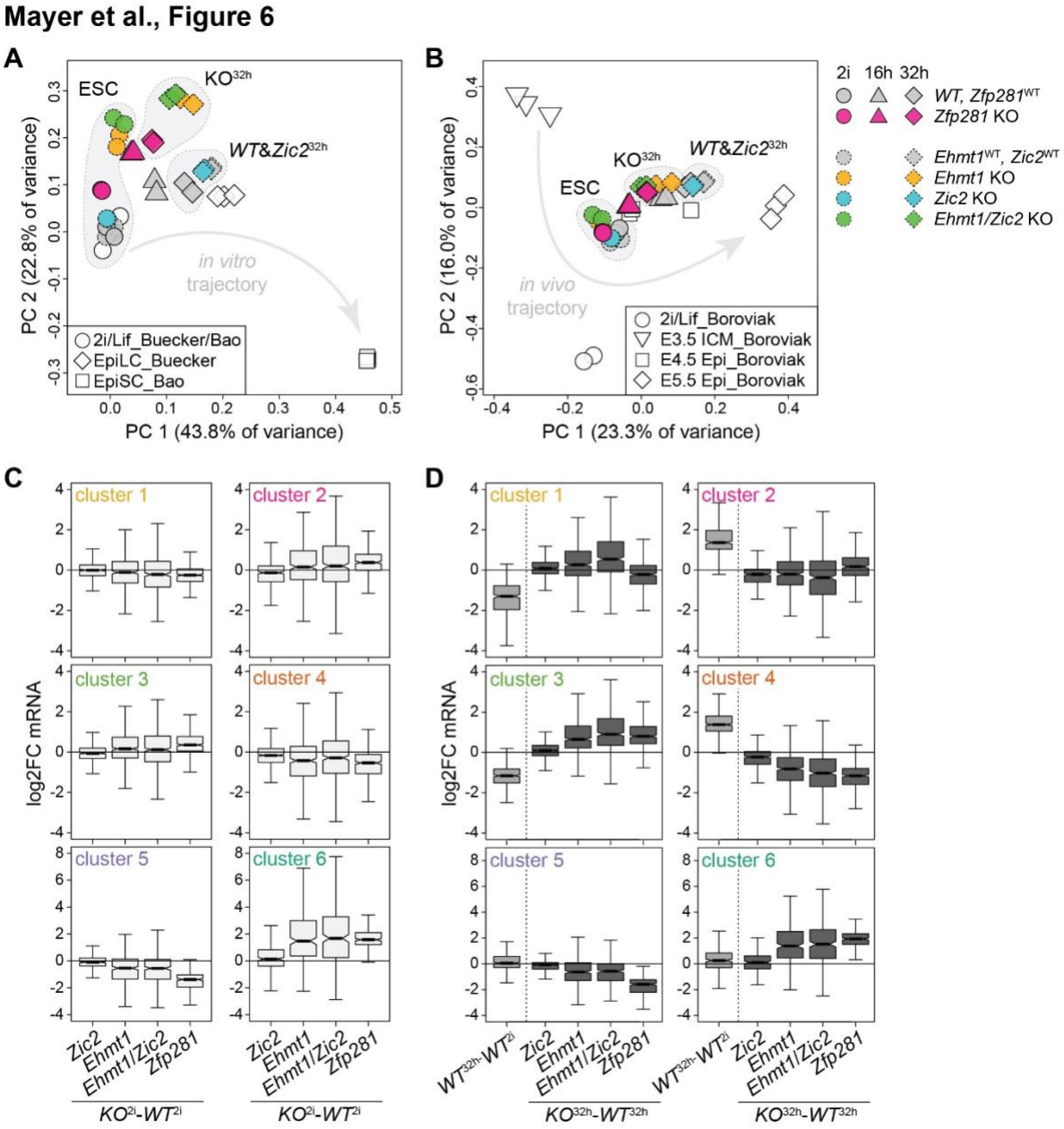

Figure 6: Related transcriptional defects in *Ehmt1*/*Zic2* and *Zfp281* KO cells.

(**A**, **B**) PC analysis of indicated samples normalized to *WT* ESCs (**A**) and all samples (**B**) within each dataset. Arrows indicate developmental trajectories. Full and dashed outlines indicate independent RNA-seq experiments.

(**C**, **D**) Quantification of cell state-specific mRNA log2FC of gene clusters 1-6 between indicated genotypes in 2i (**C**) and 32h after 2i withdrawal (**D**).

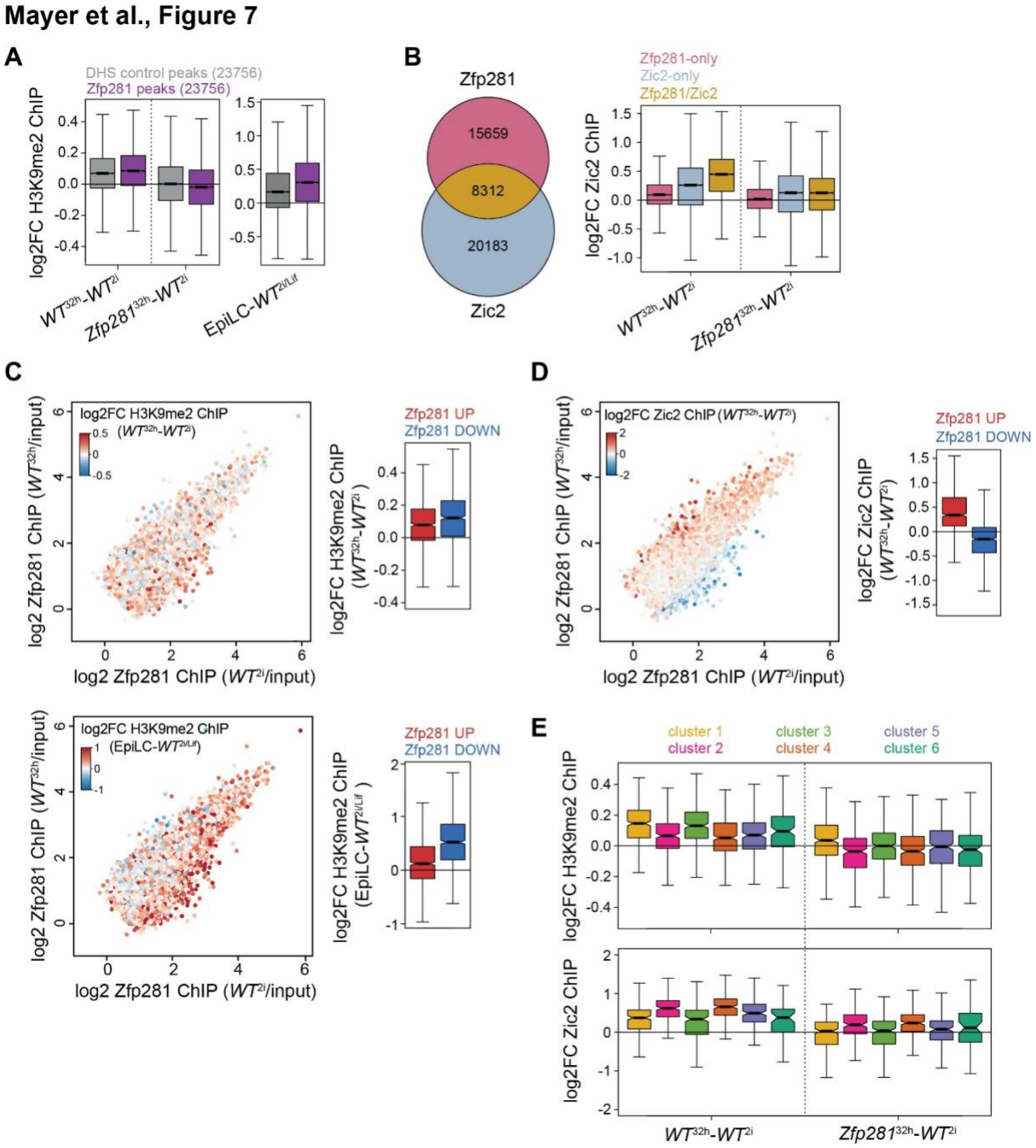

Figure 7: Zfp281 engages with Ehmt1 and Zic2 at developmental CREs.

(**A**) H3K9me2 ChIP log2FC between indicated cell states and genotypes at 10kb windows surrounding Zfp281-bound (purple) or matching DNAse-hypersensitive site (DHS) control peaks (grey).

(**B**) Overlap of Zfp281 and Zic2 ChIP peaks (left) and Zic2 ChIP log2FC between specified cell states and genotypes at indicated peak subsets (right).

(**C**, **D**) Same as in Figure **3F**. Coloring is according to H3K9me2 ChIP log2FC between WT_{32h} and WT_{2i} cells (**C**, top left) and between EpiLCs and $WT_{2i/Lif}$ cells (**C**, bottom left) at Zfp281 peaks extended to 10kb windows, and according to Zic2 ChIP log2FC between WT_{32h} and WT_{2i} cells (**D**, left).

Quantification of corresponding ChIP changes at top 1000 Zfp281 peaks with increased (red) or decreased (blue) Zfp281 binding during ESC differentiation (right).

(**E**) H3K9me2 (top) and Zic2 (bottom) ChIP log2FC between indicated cell states and genotypes at all Zfp281 peaks extended to 10kb windows (top) or Zfp281/Zic2 co-bound peaks (bottom) associated with nearest TSSs of cluster 1-6 genes.

MATERIALS AND METHODS

Cell Culture

ESCs (male and female RGd2 cells containing a Rex1:GFPd2-IRES-Blasticidin (Wray *et al*, 2011), male O4GIP ESCs contain a GFP-IRES-Puromycin transgene under control of an Oct4 regulatory element (Betschinger *et al*, 2013) and male E14 cells) were cultured on plastic coated with gelatin or laminin (Sigma) in N2B27 medium (DMEM/F12 (Life Technologies), Neurobasal (Gibco) supplemented with N2 (homemade) and B-27 Serum-Free Supplement (Gibco), 2mM L-glutamine (Gibco), and 0.1mM 2-mercaptoethanol (Sigma)) with 2i (3µM CHIR99021 and 1µM PD0325901 (Steward lab, Dresden)), and, where indicated, with 1µg/ml Doxycycline (Sigma). EpiSCs (O4GIP and OEC2 (Guo *et al*, 2009) and 796.4 (Yang *et al*, 2010)) were cultured on plastic coated with fibronectin (Millipore) in N2B27 with bFGF (12ng/ml) and ActivinA (20ng/ml) (FA) (Smith lab, Cambridge).

For monolayer differentiation, ESCs were seeded on gelatin-coated plates at 1.5 x 10^4 cells/cm^2 in 2i and the following day 2i was withdrawn. Cells were incubated in N2B27 for 32 hours (h) or 72h, or continuously passaged on laminin-coated plates, as indicated. For EpiLC differentiation for 48h (Hayashi *et al*, 2011), medium was adjusted to FA and 1% knockout serum replacement (Thermo Fisher). For embryoid body (EB) differentiation, ESCs were seeded at 2.5 x 10^4 cells/cm^2 on ultra-low attachment plates (Corning) in Serum media (GMEM (Sigma) supplemented with 10% fetal bovine serum (Sigma), 1mM sodium pyruvate (Gibco), 2mM L-glutamine (Gibco), 0.1mM non-essential amino acids (Gibco), and 0.1mM 2-mercaptoethanol (Sigma)) for 72h. Exit from pluripotency was quantified by measuring GFP fluorescence in RGd2 cells on a LSRII SORP Analyzer (Becton Dickinson) and analyzed using FlowJo (FlowJo, LLC), and by performing self-renewal and commitment assays as described before (Betschinger et al., 2013). Briefly, differentiated RGd2 and E14 cells at indicated time points were plated at clonal density on laminin-coated plates in 2i medium, resulting colonies stained for alkaline phosphatase activity (Sigma) according to the manufacturer's instruction, and counted. RGd2 cells were additionally selected with 10µg/ml Blasticidin (Thermo Fisher). O4GIP cells were differentiated for 72h, treated with 2i medium containing 1µg/ml Puromycin (Gibco) and uncommitted cells quantified after 3 days by adding 1:10 diluted Alamar Blue (Invitrogen) in 2i medium, following by read out on a SpectraMax Gemini EM (Molecular Devices) microplate reader. For cell cycle analysis, cells were fixed in cold 70% ethanol for 30 minutes (min) at 4°C, washed twice with PBS and 0.1% BSA (Sigma), treated with 5µg RNaseA (Thermo Fisher) for 15min at room temperature (RT) and stained with 10µg propidium iodide (Sigma). Cells were analyzed on a LSRII SORP Analyzer and cell cycle distributions determined using FlowJo.

siRNA transfections were performed as described (Betschinger *et al*, 2013) using 16.7nM siRNA (detailed in Table **EV4**) and transfection mixes in OptiMEM (Invitrogen) containing Lipofectamine 2000 or RNAiMAX (Thermo Fisher) for ESCs or EpiSCs, respectively.

For EpiSC reprogramming, cells were plated at 1.5 x 10^4 cells/cm^2 on fibronectin-coated plates in N2B27 with FA. The next day, medium was change to 2i and, as indicated, supplemented with 30ng/ml granulocyte colony stimulating factor (Gcsf) (Peprotech), 10ng/ml Lif (Smith lab, Cambridge) or 1µg/ml Doxycycline. After 4 days (d), medium was changed to 2i with 1µg/ml Puromycin, Epi-iPSC colonies

were stained for alkaline phosphatase activity, and counted. For experiments shown in Figure **1E** and **EV1A**, cells were subjected to self-renewal assays in 2i after 2 and 4d of Gcsf supplementation. For experiments shown in Figure **EV1E,** individual Epi-iPSC colonies were picked and expanded in 2i with Puromycin for further experiments.

Spheroid formation of ESCs was performed as described before (Shahbazi *et al*, 2017). Briefly, ESCs grown in 2i medium were washed in N2B27, 7.5 x 10_3 cells were resuspended in 25µl ice-cold growth factor reduced Matrigel (Corning, 356231), plated dropwise on uncoated 96 well glass plates (Greiner Bio-One), and aggregated in N2B27 with or without 2i for 3 or 4d.

EpiSC screen

O4GIP$_{GY118F}$ EpiSCs were reverse-transfected in fibronectin-coated 384 well plates using mixtures of 50ng esiRNA and 0.075µl Lipofectamine 2000 in 10µl OptiMEM medium. EpiSCs were plated at a density of 5000 cells/well in 80µl N2B27 with FA. Each plate included three negative (Luciferase esiRNA) and two positive (Stat3 esiRNA) control wells. The next day, medium was changed to 2i containing 30ng/ml Gcsf and 4d later to 2i containing 1µg/ml Puromycin. After 3-4d, medium was changed to 2i containing 1/10 vol Alamar Blue (Invitrogen) and cell survival quantified on a SpectraMax M2 (Molecular Devices).

Genome editing

CRISPR/Cas9 genome editing was performed by transient co-transfection of hCas9 and U6-gRNA plasmids (Mali *et al*, 2013) (Addgene plasmids 41815 and 41824) and a dsRed expression plasmid into E14 or female RGd2 ESCs. 2d later, single dsRed positive cells were sorted into gelatin-coated 96 well plates containing Serum media supplemented with 10ng/ml Lif and 2i. Clones were genotyped by sequencing amplified target loci, and by confirming protein absence in Western blots. For generation of knockout (KO) cell lines, two independent KO clones (specified in Figure **EV2A** and **J**, **EV4E** and **EV5A**) and, in the case of *Zfp281*, *Ehmt1* and *Zic2* targeting, one untargeted wildtype sibling clone were kept for further analysis.

N-terminal Flag-Avi tagging of Ehmt1 was performed in *WT* or *Zfp281* KO ESCs constitutively expressing the BirA biotin ligase (see below). The recombination template was generated by cloning homology arms (548bp up- and 618bp downstream of the Ehmt1 transcription start site) into pDONR221 using Gateway technology (Thermo Fisher) and inserting the Flag-Avi sequence by Seamless Cloning (Thermo Fisher). Targeting was performed as above with hCas9, U6-gRNA and dsRed expression plasmids, but included co-transfection of the recombination template, and genotyping the presence of biotinylated Ehmt1 using Western blots.

gRNA sequences, genotyping primers and the Ehmt1 recombination template are specified in Table **EV4**. gRNA sequences targeting *Tet1* and *Tet2* (Wang *et al*, 2013) and *Dnmt3a* and *Dnmt3b* (Domcke *et al*, 2015) have been described.

Immunostaining

Cells seeded on laminin-coated 96 well glass plates were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 10min and spheroids grown in Matrigel for 20min at RT. Samples were blocked for 1h in blocking solution (PBS, 0.1% TritonX (Sigma), 3% donkey serum (Sigma), and 1% BSA) and incubated overnight with primary antibodies (Ehmt1 (Abcam, ab41969, 1:300) and H3K9me2 (Abcam, ab1220, 1:300)) at 4°C. After three washes in washing solution (PBS, 0.1% TritonX), secondary antibodies were added, DNA stained with Hoechst33342 (Life Technologies) and, where indicated, incubated with Alexa Fluor 488 Phalloidin (Life Technologies, A12379, 1:40) for 20min at RT. Images were acquired using a LSM 710 scanning head confocal microscope (Zeiss) at 20x magnification and handled using Fiji and Adobe Photoshop (Adobe). Imaging of spheroids used for quantification in Figure **EV5G** was performed with a Yokogawa CV7000s high throughput confocal microscope at 20x magnification. Images were acquired in confocal mode as z-stack multiplane images over z distance of 50 µm with a 5 µm step size and maximum intensity projections were stored, representatives of which are shown in Figure **5B**.

Molecular biology

Coding sequences for Ehmt1, Esrrb, Klf4 and Zfp281 were amplified from ESC complementary DNA (cDNA) and for BirA biotin ligase from a plasmid (gift of Matyas Flemr, Friedrich Miescher Institute, Basel). For Zic2, the coding sequence was synthesized as a double-stranded gBlock (IDT). Polynucleotides were recombined into pDONR221 using Gateway technology. Ehmt1 point mutations (Ehmt1$_{\Delta NHHC}$: NHHC1198-1201del (Tachibana *et al*, 2008), Ehmt1$_{NH\text{-}LE}$: NH1198-1199LE (Tachibana *et al*, 2008), and Ehmt1$_{ank}$: W872A, W877A, E880A (Collins *et al*, 2008)) were introduced by polymerase chain reaction (PCR). Expression destination vectors were pPB-CAG-DEST-pgk-hph (Betschinger *et al*, 2013) and pPB-TRE-DEST-rTA-pgk-hph (Villegas *et al*, 2019), and GY118F expression vector as described (Yang *et al*, 2010). Stable integration into ESCs or EpiSCs after co-transfection with pBASE (Betschinger *et al*, 2013) was selected in the presence of 150µg/ml HygromycinB (Thermo Fisher).

For relative mRNA quantification, total RNA was isolated from indicated samples using RNeasy Mini Kit (Qiagen) and cDNA prepared using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was performed using TaqMan Fast Universal PCR master mix (Applied Biosystems) with gene specific primers, either using the universal probe library (UPL, Roche) or Taqman system (Applied Biosystems), and a GAPDH probe (Applied Biosystems) for normalization. Oligonucleotide sequences and probes are listed in Table **EV4**.

Protein methods

Cell lysates for Western blotting were generated in RIPA buffer (50mM Tris, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Tx-100, and 0.1% SDS). Primary antibodies were anti-GAPDH (Sigma, G8795, 1:5000), anti-Ehmt1 (Abcam, ab41969, 1:500), anti-Tet1 (Millipore, 09-872, 1:1000), anti-Tet2 (Abcam, ab124297, 1:300), anti-Zic2 (Abcam, ab150404, 1:500), anti-Zfp281 (Bethyl Laboratories, A303-118A, 1:500), and anti-Streptavidin coupled to HRP (Sigma, EV2438, 1:1000).

Nuclear immunoprecipitations (IPs) for Zfp281 were performed in three biological replicates using *WT*$_{2i}$, *Zfp281*$_{2i}$, *WT*$_{40h}$ and *Zfp281*$_{40h}$ cells. Cells were washed with cold PBS, resuspended in 5 packed cell

volume (pcv) of buffer A (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, and 10mM KCl), incubated for 10min on ice, and broke open using a Dounce homogenizer. Nuclei were pelleted at 3300g for 15min at 4°C, resuspended in 3 pcv of buffer B (20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 0.2mM EDTA, and 20% glycerol) supplemented with 420mM NaCl, Complete Mini protease, PhosSTOP phosphatase inhibitors (Roche) and 250U/ml Benzonase (Sigma), and incubated for 30min at 4°C on a rotating wheel. Insoluble material was pelleted at 25000g for 30min at 4°C, and the supernatant diluted with buffer B to a final concentration of 150mM NaCl and including 0.02% NP40. 1% of the supernatant was kept as input sample and the remainder incubated with 10µl Dynabeads ProteinG (Invitrogen) and 1µg Zfp281 antibody (Bethyl Laboratories, A303-118A) for 1h at 4°C on a rotating wheel. Beads were collected on a magnetic rack for 2–3min to remove the supernatant, and washed 4 times in 1ml buffer B containing 150mM NaCl and 0.02% NP40 for 10min each at 4 °C on a rotating wheel. For mass spectrometry, proteins were digested on the beads as described before (Villegas *et al*, 2019).

Whole cell lysate IPs using 1µg Zfp281 antibody (Bethyl Laboratories, A303-118A) were performed as described before (Villegas *et al*, 2019) using Dynabeads.

Chromatin immunoprecipitation (ChIP)

For ChIP of endogenous proteins or histone modifications, 8 x 10_6 cells per IP were fixed for 10min with 1.1% formaldehyde in fixing solution (0.1M NaCl, 1mM EDTA, 0.5mM EGTA, and 50mM HEPES pH 7.5) at RT on a rotating wheel, and neutralized with glycine to a final concentration of 0.125M for 5min at RT. Cells were washed three times with ice cold PBS by spinning at 1600g for 5min at 4°C, incubated for 10min at 4°C on a rotating wheel with 1ml lysis buffer 1 (50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, and 0.25% TritonX), pelleted, and incubated for a further 10min at 4°C in 1ml lysis buffer 2 (10mM Tris pH 8.0, 200mM NaCl, 1mM EDTA, and 0.5mM EGTA). Nuclei were pelleted, resuspended in 140µl shearing buffer (50mM Tris pH 8.0, 10mM EDTA, and 1% SDS), and sonicated in Diagenode 15ml Falcon tubes for 25 cycles (30 seconds ON, 30 seconds OFF) in ice-cold water using a Bioruptor Plus (Diagenode). 10% of sonicated DNA was kept as input sample. Lysates were further pelleted at 14000g for 10min at 4°C and the supernatant diluted 1:10 with ChIP dilution buffer (50mM Tris pH 8.0, 167mM NaCl, 1.1% Tx-100, and 0.11% Na-Deoxycholate). Lysates were precleared over 10µl Dynabeads for 2h and incubated overnight at 4°C on a rotating wheel with the following antibodies: 2µg H3K27ac (Active Motif, 39135), 2µg H3K9me2 (Abcam, ab1220), 2µg Zic2 (Abcam, ab150404), or 2µg Zfp281 (Bethyl Laboratories, A303-118A). The next day, 10µl Dynabeads were added and incubated with lysates for 1h at 4°C on a rotating wheel. Beads were washed with 1ml of the following buffers for 5min each at 4°C: twice with wash buffer 1 (50mM Tris pH 8.0, 0.1% SDS, 0.1% Na-Deoxycholate, 1% TritonX, 150mM NaCl, 1mM EDTA, and 0.5mM EGTA), once with wash buffer 2 (50mM Tris pH 8.0, 0.1% SDS, 0.1% Na-Deoxycholate, 1% TritonX, 500mM NaCl, 1mM EDTA, and 0.5mM EGTA), once with wash buffer 3 (50mM Tris pH 8.0, 250mM LiCl, 0.5% Na-Deoxycholate, 0.5% NP40, 1mM EDTA, and 0.5mM EGTA), and twice with wash buffer 4 (50mM Tris pH 8.0, 10mM EDTA, and 5mM EGTA). Finally, beads were eluted twice with 100µl elution buffer (0.1M $NaHCO_3$, and 1% SDS) for 15min at RT in a shaker at maximum speed, and combined supernatants de-crosslinked overnight by supplementation to 200mM NaCl and continuous shaking at maximum speed at 65°C. The

same procedure was followed for input samples by adjusting the total volume of elution buffer to 200µl and 200mM NaCl. The next day, DNA was purified using MinElute PCR Purification Kit (QIAGEN).

Bio-ChIP for Flag-Avi tagged Ehmt1 was performed as described before (Ostapcuk *et al*, 2018) with minor modifications. Briefly, 8 x 10_6 cells per IP were fixed for 8min with 1% formaldehyde in PBS at RT on a rotating wheel, and neutralized with adjusting glycine to a final concentration of 0.125M and incubation for 1min at RT and for 5min on ice. Cells were washed three times with ice cold PBS and pelleted at 1000g for 5min at 4°C. Cells were lysed in lysis buffers 1 and 2 as described above. Nuclei were washed once in 5ml NUC buffer (15mM HEPES pH 7.5, 60mM KCl, 15mM NaCl, and 0.32mM sucrose) and resuspended in 1ml NUC Buffer supplemented with Complete Mini protease inhibitors, 3.3µl 1M CaCl2, and 2-3µl Micrococcal Nuclease (Cell Signaling, 10011S). Enzymatic activity was induced for 15min at 37°C and shaking at 1000rpm, and stopped by addition of 50µl of STOP solution (250mM EDTA, and 500mM EGTA) and 110µl of 10x ChIP buffer (167mM Tris pH 8.0, 1.67M NaCl, 12mM EDTA, 10% TritonX, and 0.1% SDS) with a further incubation for 5min on ice. Nuclei were gently disrupted by sonication in Diagenode 15ml Falcon tubes for 8 cycles (5 seconds ON, 5 seconds OFF) in ice cold water using a Bioruptor Plus. Lysates were centrifuged at 14000g for 5min at 4°C, 5% of the supernatant was kept as input sample, and the remaining supernatant precleared for 2h over 10µl Dynabeads at 4°C on a rotating wheel. Chromatin was incubated for 1h with M-280 Streptavidin coupled Dynabeads (Invitrogen) at 4°C on a rotating wheel, and washed with 1ml of the following buffers for 5min each at 4°C: twice with TE buffer (10mM Tris pH 8.0, and 1mM EDTA) supplemented with 2% SDS, once with high salt buffer (50mM HEPES pH 7.5, 500mM NaCl, 1mM EDTA, 1% TritonX, and 0.1% Na-Deoxycholate), once with wash buffer 3 (see above) and twice with TE buffer. Beads were eluted in 60µl elution buffer (see above) supplemented with 2µl RNaseA (10mg/ml stock) and incubated for 30min at 37°C while mixing. After supplementation to 10mM EDTA, 10mM Tris pH 8.0 and 2µl ProteinaseK (10mg/ml, Promega), the beads suspension was further incubated for 3h at 55°C and overnight at 65°C while shaking. The same procedure was followed for input samples, including RNaseA and ProteinaseK digestion. DNA was purified using AMPure XP beads (Beckman Coulter).

Sequencing libraries

RNA from ESCs grown in 2i and 16h and 32h after 2i withdrawal was isolated using RNAeasy kit (Qiagen). For *Zfp281* KO and corresponding *WT* cells, total RNA was subjected to ribosomal RNA depletion using Ribozero removal kit (Illumina) followed by library construction using ScriptSeq V2 library preparation kit (Illumina). For *Ehmt1*, *Zic2*, *Ehmt1/Zic2* KO and corresponding *WT* cells, RNA-seq libraries were prepared using TruSeq mRNA Library preparation kit (Illumina). ChIP-seq libraries were prepared using NEBNext Ultra kit (New England BioLabs) following the manufacturer's recommendations. Sequencing was performed on an Illumina HiSeq2500 machine (50bp single-end reads).

Quantification and statistical analysis

Screen analysis

For the EpiSC reprogramming screen Z scores were calculated for each plate, excluding the two outer most columns and rows (Table **EV1**). Screen replicates are presented in Figure **EV1B**. Average Z scores >2 were considered as screen hits, identifying 146 genes (Figure **1B**). We quantified their role in exit from the ESC state by extracting primary data for these 146 genes from two previous ESC differentiation studies (Betschinger *et al*, 2013; Li *et al*, 2018), and computing Z scores on this subset (Table **EV1**). 67 and 129 of the 146 genes were mapped on results from (Betschinger *et al*, 2013) and (Li *et al*, 2018), respectively, resulting in mapping of 130 genes in total. Average Z scores from both ESC differentiation screens are presented in Figure **1C**.

Quantification of immunostaining

Quantification of H3K9me2 was performed in CellProfiler3 (Broad Institute). Nuclei were identified using Hoechst33342 staining and average H3K9me2 and DNA fluorescence intensity per nucleus determined: WT_{2i} (453 cells), $Zfp281_{2i}$ (574 cells), WT_{32h} (465 cells), $Zfp281_{32h}$ (792 cells).

For quantification of d3 and d4 spheroids from two biological replicates, images were stitched to generate a single image per channel and per well, and used for object segmentation with Matlab (MathWorks). Segmented object outlines were exported and used for feature extraction with software package CellProfiler3. Extracted features (84 features) describing object area, shape and intensity were normalized within corresponding assay plates using Z score transformation and unified into a cross-comparable dataset. Normalized features were used to extract 10 principal components (PC), which were further used for unsupervised clustering with the software package PhenoGraph (Levine *et al*, 2015). Unsupervised clustering returned 13 clusters, objects belonging to the 2 sparsest clusters were discarded as outliers based on extreme variance in measured features in the respective classes. To describe radial distribution of F-actin signal intensity, segmented objects were divided into 20 concentric regions of same width. Distribution of F-actin staining intensity was described as mean fraction of intensity per region (MeanFrac) whereby the total intensity in the given region was normalized to the fraction of object area corresponding to the region. Mean fractions of intensity per region were not Z scored as these values were normalized per object *ad initio*. Visualization in Figure **EV5G** shows the mean of F-actin signal in a given concentric ring of all d4 spheroids per genotype and medium condition: WT_{2i} (559 spheroids), $Ehmt1_{2i}$ (349 spheroids), $Zic2_{2i}$ (292 spheroids), $Ehmt1/Zic2_{2i}$ (164 spheroids), $Zfp281_{2i}$ (324 spheroids), WT_{96h} (437 spheroids), $Ehmt1_{96h}$ (228 spheroids), $Zic2_{96h}$ (202 spheroids), $Ehmt1/Zic2_{96h}$ (233 spheroids), $Zfp281_{96h}$ (547 spheroids). Heatmap was generated using aheatmap function from the Bioconductor package NMF (Gaujoux & Seoighe, 2010) (package version 0.21.0).

Protein identification and quantification

Relative quantification of mass-spec data from three biological replicates (Table **EV3**) was performed with MaxQuant (version 1.5.3.8) using Andromeda as search engine and label free quantification as described (Villegas *et al*, 2019). Briefly, the mouse subset of the UniProt version 2015_01 combined with the contaminant DB from MaxQuant was searched and the protein and peptide FDR were set to 0.01. For Figure **4A**, **B**, proteins were considered which passed an interaction threshold of an at least twofold enrichment in IPs from WT_{2i} or WT_{40h} lysates compared to matched Zfp281 KO lysates with a significant p-value<0.1, and were quantifiable with at least two unique razor peptides.

RNA-seq and Gro-seq analysis

RNA-seq reads from wildtype and mutant cells in 2i, and 16h and 32h after 2i withdrawal were aligned to the mouse GRCm38/mm10 genome using qAlign from the Bioconductor package QuasR (Gaidatzis *et al*, 2015) (package version 1.22.0) with default parameters except for splicedAlignment=TRUE. Published RNA-seq from ESCs cultured in 2i/Lif and EpiLC (Buecker *et al*, 2014) were 36bp reads, and therefore no spliced alignment could be performed. RNA-seq from ESCs cultured in 2i/Lif and EpiSCs (Factor *et al*, 2014; Bao *et al*, 2018), and global run-on sequencing (GRO-seq) data from 2i/Lif cultured ESCs (Dorighi *et al*, 2017) were 100bp and 50bp paired-end reads, respectively, and therefore paired="fr" was used. For *in vivo* embryo data (Boroviak *et al*, 2015), pre-existing alignments to mouse GRCm38/mm10 genome were downloaded from ArrayExpress (E-MTAB-2958) and used. Alignments were quantified for known UCSC genes obtained from the TxDb.Mmusculus.UCSC.mm10.knownGene package (package version 3.4.4) using qCount from the Bioconductor package QuasR with default parameters (Table **EV2**).

Only transcripts with at least 3 counts per million in at least two biological samples from this study were considered as expressed genes (total: 13,096 genes). For identification of significantly deregulated genes, edgeR (Robinson & Oshlack, 2010) (package version 3.24.0) was used and detected genes were fitted to two generalized linear models:

(a) ~ *time* + *genotype* + *time:genotype*: This model uses WT_{2i}, WT_{16h}, WT_{32h}, $Zfp281_{2i}$, $Zfp281_{16h}$ and $Zfp281_{32h}$ expression datasets. Genes with a significant *time* coefficient are genes that change either between WT_{2i} and WT_{16h} or WT_{2i} and WT_{32h} cells, genes with a significant *genotype* coefficient are differentially expressed between $Zfp281_{2i}$ and WT_{2i} cells, and genes with a significant interaction term *time:genotype* are deregulated in *Zfp281* KO cells specifically during 16h or 32h differentiation.

(b) ~ *genotype*: This model identifies genes that differ between $Ehmt1_{2i}$ and WT_{2i} cells.

Raw P values were corrected for multiple testing by calculating false discovery rates (FDR). Significant genes were identified as genes with an absolute log2 fold-change greater than 1.0 and an FDR of less than 0.01.

For visualization of RNA-seq data, except principal component analysis (PCA) in Figure **6A** and **6B** and heatmap of selected markers in Figure **EV6A**, log2 fold-change values were used that were obtained from edgeR by fitting the indicated datasets to the following models:

(c) ~ *time* (EpiLCs or EpiSCs): EpiLCs (Buecker *et al*, 2014) or EpiSCs (Factor *et al*, 2014) compared to $WT_{2i/Lif}$ (Buecker *et al*, 2014) and EpiSC compared to $WT_{2i/Lif}$ (Bao *et al*, 2018) (used for Figure **3A**, **B**).

(d) ~ *genotime*: $Zfp281_{16h}$ or $Zfp281_{32h}$ compared to WT_{2i} (used for Figure **3A**, **B** and **EV3A**), where *genotime* is the combination of genotype and time.

(e) ~ *genotype* (cell state-specific): KO cells in 2i compared to WT_{2i}, or KO cells 16h or 32h after 2i withdrawal compared to WT_{16h} or WT_{32h}, respectively (used for Figure **3C** and **6C**, **D** and **EV6B**-**E**).

For heatmap visualization in Figure **3A** only significantly deregulated genes in at least one condition were considered (model (a): *time*, *genotype* or *time:genotype*; total: 2,495 genes) and the following log2 contrast were used for clustering: WT_{16h}-WT_{2i}, WT_{32h}-WT_{2i}, $Zfp281_{2i}$-WT_{2i}, $Zfp281_{16h}$-WT_{2i}, $Zfp281_{32h}$-WT_{2i}, $Zfp281_{16h}$-WT_{16h} and $Zfp281_{32h}$-WT_{32h}. For heatmap visualization in Figure **EV6A**, RNA-seq read counts were normalized (divided by the total number of aligned reads (library size), multiplied with minimal library size, and added with a pseudocount of 8) and log2 transformed, and the mean of biological replicates was plotted. For visualization in Figure **EV6B**, significantly deregulated genes in $Ehmt1_{2i}$ (model (b): *genotype*) but not $Zfp281_{2i}$ (model (a): *genotype*) are highlighted. In Figure **3B,** dots represent the median and shades the lower and upper quartile of indicated samples. Boxplots were generated using the boxplot function in R with default parameters except outline=FALSE. Correlation plots (Figure **EV6C**, **D**) were generated using corrplot function from the Bioconductor package corrplot (https://github.com/taiyun/corrplot) (package version 0.84). Heatmaps (Figure **3A**, **EV3A** and **EV6A**) were generated using aheatmap function from the Bioconductor package NMF. For PCA represented in Figure **6A**, normalized (see above) and log2 transformed read counts were centered by subtracting the average of *WT* ESCs within each of the following four RNA-seq data sets: 1) *Zfp281* KO and corresponding *WT* samples from this study; 2) *Ehmt1*, *Zic2, Ehmt1/Zic2* KO and corresponding *WT* samples from this study; 3) $WT_{2i/Lif}$ cells and EpiLCs (Buecker *et al*, 2014); and 4) $WT_{2i/Lif}$ cells and EpiSCs (Bao *et al*, 2018). For PCA represented in Figure **6B**, normalized (see above) and log2 transformed read counts were centered by subtracting the average over all samples within each of the following three RNA-seq data sets: 1) *Zfp281* KO and corresponding *WT* samples from this study; 2) *Ehmt1*, *Zic2, Ehmt1/Zic2* KO and corresponding *WT* samples from this study; 3) $WT_{2i/Lif}$ cells and embryo samples (Boroviak *et al*, 2015). Centered read counts for each PCA are provided in Table **EV2** and the detailed R code is provided in Appendix File **1**. PCA was performed using the prcomp function in R. Analyses of enriched gene sets (Figure **EV1C**, **EV3B** and **EV6B**) were performed using DAVID (Huang *et al*, 2009) (version 6.8) for GO terms of biological processes.

The linear model to estimate synergistic transcriptional effects of *Ehmt1* and *Zic2* (Figure **EV6E**) was fitted using lm function in R:

$$\Delta Ehmt1/Zic2_i \sim \beta_E \Delta Ehmt1_i + \beta_Z \Delta Zic2_i + \beta_{int} \Delta Ehmt1{:}Zic2_i$$

Δ: transcriptional difference (KO – WT) of gene $_i$

β: regression coefficient:

$_E$: Ehmt1

$_Z$: Zic2

$_{int}$: non-additive interaction

ChIP-seq and DHS-seq analysis

ChIP-seq data from this study, published datasets (Buecker *et al*, 2014; Huang *et al*, 2017; Ishiuchi *et al*, 2019) and DNaseI hypersensitive site sequencing (DHS-seq) (Encode; accession number: ENCSR000CMW) reads were aligned to mouse GRCm38/mm10 genome using qAlign from the Bioconductor package QuasR with default parameters. Published H3K4me3, H3K27me3 and H3K9me2 ChIP-seq data (Kurimoto *et al*, 2015) were aligned using Bowtie (Langmead *et al*, 2009)

(version 4.4.7) with parameter -C in colorspace. Alignments were sorted and indexed using SAMtools (package version 1.2), and all ChIP-seq data were quantified with qCount from the Bioconductor package QuasR. Read counts were normalized (divided by the total number of aligned reads (library size), multiplied with minimal library size and added with a pseudocount of 8) and log2 transformed. For DHS-seq, reads per million (RPM) were calculated by dividing the total number of aligned reads, multiplying with one million, adding a pseudocount of 8 and log2 transforming the data.

We observed a non-linear relationship in Zfp281 ChIP-seq data when comparing read counts in Zfp281 peaks between ChIP-seq replicates in WT_{2i}, and therefore performed loess regression using normalizeBetweenArrays function of the Bioconductor package limma (Ritchie *et al*, 2015) (package version 3.38.2) with method=cyclingloess.

In Ehmt1 ChIP-seq data we detected a variable dependency of read counts in genomic tiles on the tile's GC composition (GC bias) which was most pronounced in input samples. In order to reduce this bias, we used a loess-based normalization method: First, reads were counted in each sample in 10kb windows (either genome-tiling windows or windows centered on Zfp281 peaks and corresponding control windows, see below). The counts were then scaled (divided by the total number of aligned reads (library size)), multiplied with minimal library size, added with a pseudocount of 8, and log2 transformed. A loess curve was fit to the log2-transformed counts as a function of the fraction of G+C bases in the window using the R function loess with span = 0.3. This fit robustly captures the global signal dependency on the underlying GC composition. GC-corrected log2 read counts were then obtained by subtracting the values predicted by the loess fit from the observed log2 read counts (residuals of the fit).

Zfp281 peaks were called on Zfp281 ChIP-seq reads in WT_{2i} and WT_{32h} cells using Macs2 (Zhang *et al*, 2008) (version 2.1.1.20160309) with default parameters. Peaks that were at least 2-fold enriched (IP over respective inputs) in at least one of the four Zfp281 ChIP samples were considered (total: 23,756 peaks) (Table **EV2**). For comparison of Zfp281 ChIP samples from this study to ChIP in EpiSCs (Huang *et al*, 2017) and TSCs (Ishiuchi *et al*, 2019), peaks were called on Zfp281 ChIP-seq reads in WT_{2i}, WT_{32h}, EpiSCs and TSCs. Peaks that were at least 2-fold enriched (IP over respective inputs) in at least one of the four Zfp281 ChIP samples from this study, in the one Zfp281 ChIP sample in EpiSCs or in one of the two Zfp281 ChIP samples in TSCs were considered (total: 27,435 peaks) and used for plotting (Figure **EV3E** and **F**). Zic2 peaks were called on Zic2 ChIP-seq reads in WT_{2i}, WT_{32h}, $Zfp281_{2i}$ and $Zfp281_{32h}$ cells. To quantitatively compare Zfp281 and Zic2 binding, both peak sets were combined and overlapping peak regions were merged using the function reduce from Bioconductor package GenomicRanges (Lawrence *et al*, 2013) (package version 1.34.0). Fused peaks were classified into single- or co-bound as follows: Peaks that were enriched at least 2-fold (IP over respective inputs) in at least one of the four Zfp281 ChIP samples and in at least one of the eight Zic2 ChIP samples were considered as co-bound (total: 8,312 peaks), while if detected only in Zfp281 ChIP or only in Zic2 ChIP samples were considered as Zfp281-only (total: 15,659 peaks) or Zic2-only (total: 20,183 peaks), respectively (Table **EV2**). Reads of H3K27ac ChIP-seq datasets were counted in Zfp281, Zic2 or Zfp281/Zic2 co-bound peaks and normalized as described above.

Zfp281 or Zfp281/Zic2 co-bound peaks were assigned to genes by calculating the distances of peak midpoint to the nearest transcriptional start site (TSS) using a set of non-redundant TSSs with a single start site randomly selected for each gene. Zfp281 peaks were classified as proximal if the distance to the nearest TSS was less than 2000bp (7,697 peaks) and as distal otherwise (16,059 peaks) (Figure **EV3G**). H3K27ac reads in WT_{2i} and WT_{32h} cells, and H3K4me1 (Buecker *et al*, 2014) and H3K4me3 (Kurimoto *et al*, 2015) reads in $WT_{2i/Lif}$ and EpiLCs were counted in Zfp281 peak regions and normalized as described above. Proximal Zfp281 binding sites with at least 1.5-fold enrichment of H3K27ac over respective inputs in either WT_{2i} or WT_{32h} cells and with at least 2-fold enrichment of H3K4me3 over respective inputs in either $WT_{2i/Lif}$ cells or EpiLCs were considered as associated with active promoters (54% of proximal peaks, total: 4,128). Distal Zfp281 binding sites with at least 1.5-fold enrichment of H3K4me1 over respective inputs in either $WT_{2i/Lif}$ cells or EpiLCs were considered as putative enhancers (62% of distal peaks, total: 9,990), of which sites additionally enriched at least 1.5-fold over respective inputs in H3K27ac in either WT_{2i} or WT_{32h} cells were classified as active enhancers (38% of putative enhancers, total: 3,818). For quantification at target sites differentially bound by Zfp281 during differentiation (Figure **3F**, **7C**, **D**), the 1000 binding sites with strongest increase (Zfp281 UP) and decrease (Zfp281 DOWN) in Zfp281 ChIP signal in WT_{32h} compared to WT_{2i} were considered.

Due to the broad chromatin distribution of Ehmt1 and H3K9me2, ChIP-seq reads were first quantified in genome-tiling windows of 10kb which were generated using tileGenome function from Bioconductor package GenomicRanges with tilewidth=10000 and cut.last.tile.in.chrom=TRUE. In Figure **EV7C**, all 10kb genome-tiling windows were separated in 5 bins with equal number of tiles but increasing Ehmt1 ChIP log2 enrichment over respective input in WT_{2i} cells. To quantify Ehmt1 and H3K9me2 enrichment at Zfp281 binding sites, Zfp281 peak regions were extended to 10kb centered on the peak midpoint using the function resize from the Bioconductor package GenomicRanges. As a control set, DHS-seq peaks (peak annotation downloaded from ENCODE; accession number: ENCSR000CMW) were extended to 10kb centered on the peak midpoint and only regions non-overlapping with 10kb extended Zfp281 peaks were considered. The final set of control regions was obtained by randomly sampling one DHS 10kb peak per Zfp281 10kb peak, such that the distributions of DHS-seq signal (log2 RPM) and GC-content between Zfp281 10kb extended peaks and the selected control regions matched closely.

Boxplots were generated using the boxplot function in R with default parameters except outline=FALSE. Genomic profiles for heatmaps centered on the Zfp281 peak midpoint (Figure **EV3G**) were generated with qProfile from the Bioconductor package QuasR, and visualized using ComplexHeatmap (package version 1.20.0) (Gu *et al*, 2016). Except for Zfp281 ChIP-seq and GRO-seq (Dorighi *et al*, 2017), the averages of two replicates are shown.

Details for quantification and statistical analysis in Figures

Details of experiments can be found in the figure legends, including number of biological or technical replicates and the statistical test used. All data quantification is represented as the mean and standard deviation (SD).

Data availability

Next-generation sequencing data reported in this study have been deposited at the Gene Expression Omnibus with accession number GSE131017. Reviewers can get access to the data by searching for the above GSE identifier and then using the token kbsncwsufzknpgz.

EXTENDED VIEW FIGURES

Mayer et al., Figure EV1

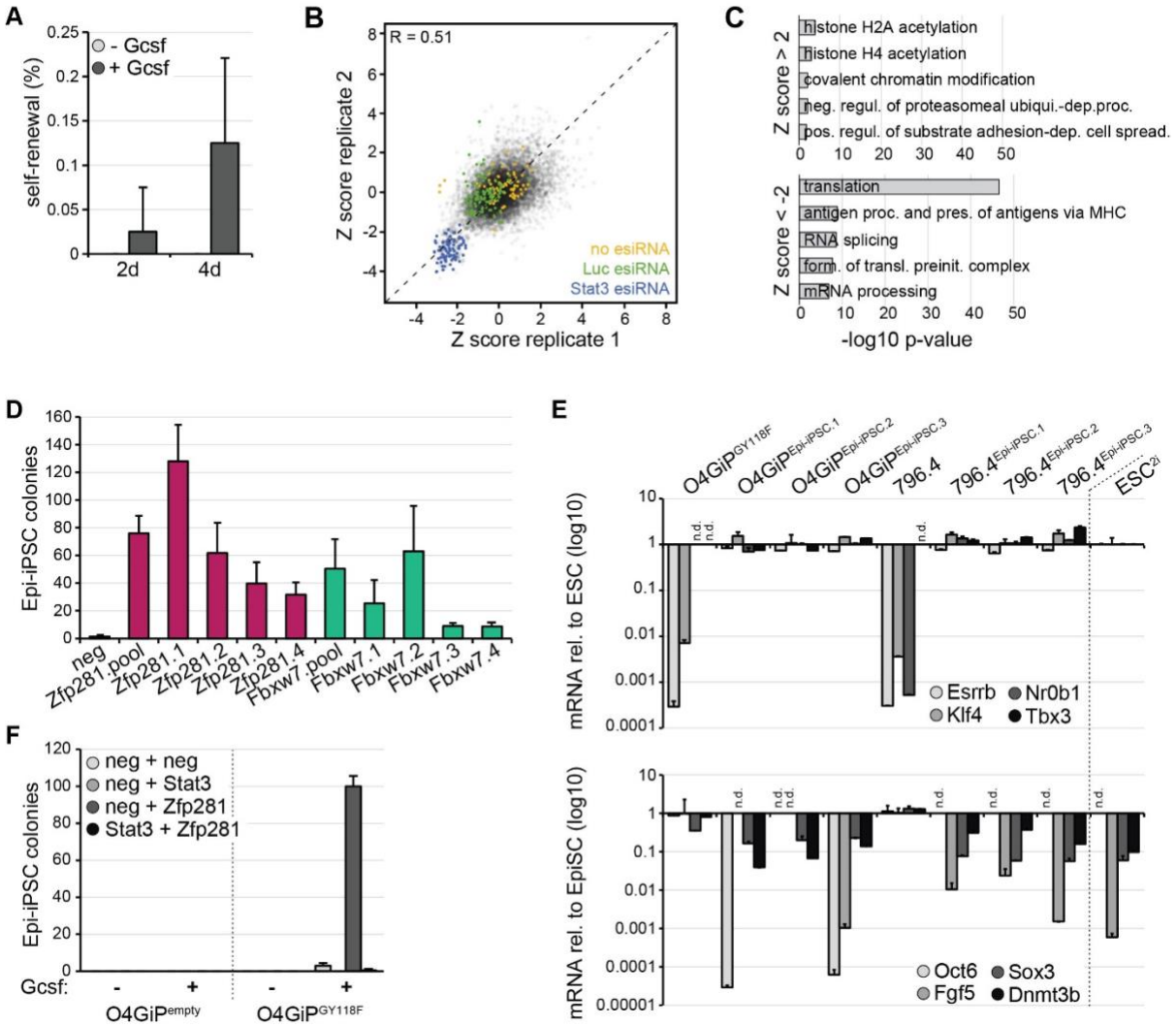

Figure EV1: Enhanced reprogramming of EpiSCs in the absence of Zfp281.

(**A**) Self-renewal of O4GIP$_{GY118F}$ reprogramming intermediates after 2 or 4d in 2i in the presence or absence of Gcsf. Average and SD of 2 experiments performed in duplicates.

(**B**) Scatter plot of Z scores between screen replicates. Negative controls (no esiRNA and non-targeting Luc esiRNA) are marked in yellow and green, respectively, and positive controls (Stat3 esiRNA) in blue. Pearson's correlation coefficient (R).

(**C**) Top 5 GO terms enriched in screen hits with Z scores > 2 (top) and < -2 (bottom).

(**D**) Deconvolution of siRNA pools: Epi-iPSC colonies derived from 796.4 EpiSCs transfected with indicated siRNAs (individual siRNAs or pools), stimulated for 4d with Gcsf and 2i, and selected with Puromycin. Average and SD of 3 experiments performed in duplicates.

(**E**) Induction of naïve (top) and repression of primed (bottom) pluripotency markers in Epi-iPSCs derived from Zfp281-depleted and Gcsf-stimulated O4GIP$_{GY118F}$ and 796.4 EpiSCs. mRNA fold changes

relative to ESCs (top) and EpiSCs (bottom) are shown on a log(10)-scaled axis. Average and SD of 2 technical replicates. Not detected (n.d.).

(**F**) Epi-iPSC colonies derived from O4GIP$_{empty}$ and O4GiP$_{GY118F}$ EpiSCs transfected with indicated siRNAs, incubated for 4d in 2i in the presence or absence of Gcsf, and selected with Puromycin. Average and SD of 2 technical replicates.

Mayer et al., Figure EV2

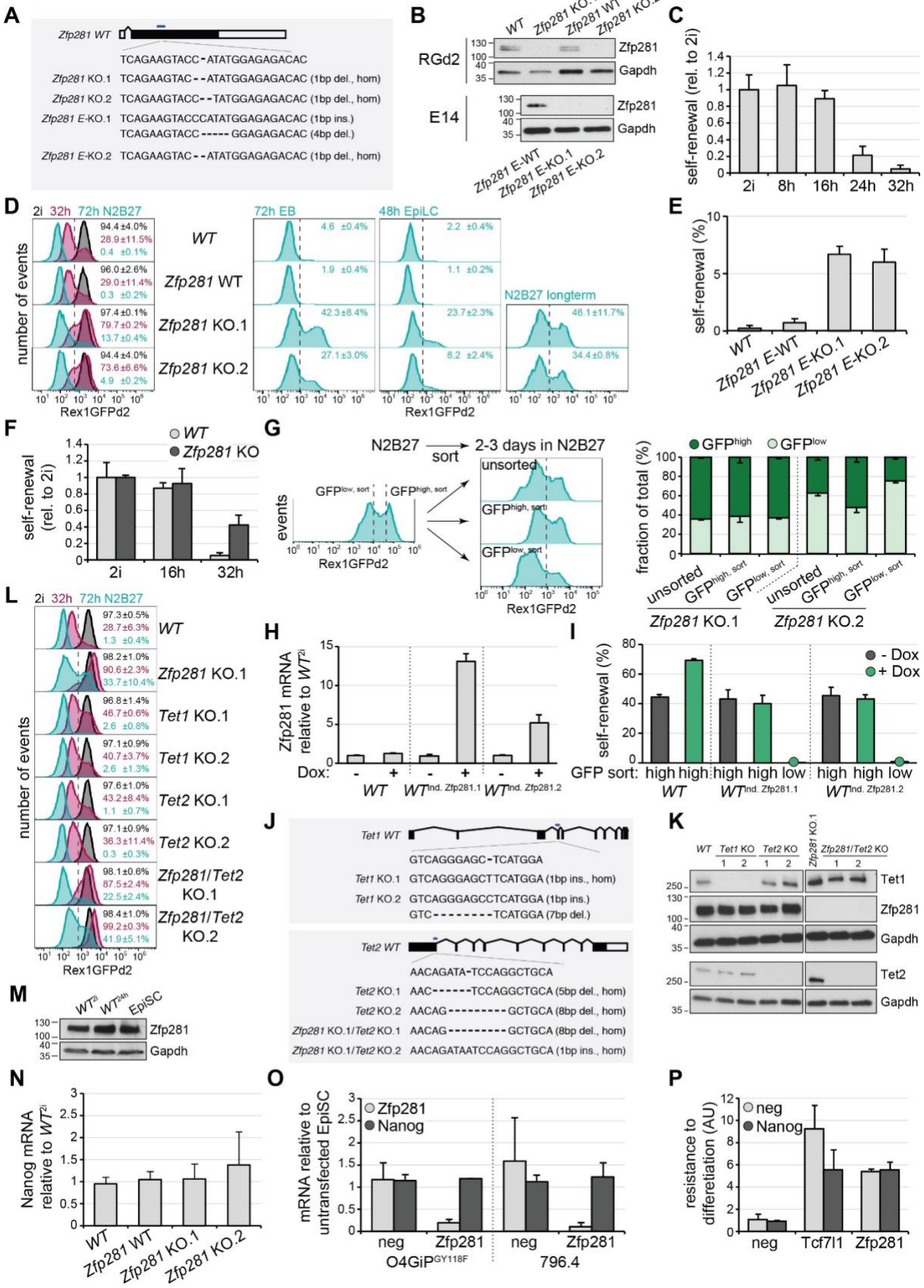

Figure EV2: Characterization of Zfp281 and Tet enzymes in ESC differentiation.

(**A**, **B**) Sequence of genome-edited *Zfp281* locus (**A**) and absence of protein (**B**) in KO cells. E denotes E14 parental cell line origin.

(**C**, **E**, **F**) Self-renewal in RGd2 cells (**C**) and of indicated genotypes (**E**, **F**) after 3d (**E**) or indicated timepoints (**C**, **F**) of 2i withdrawal. Average and SD of 2 experiments performed in duplicates.

(**D**, **L**) Representative flow cytometry profiles of RGd2 ESCs of specified genotypes, at indicated timepoints and in indicated conditions. Numbers are average and SD of GFP_{high} cells in 2 experiments.

(**G**) Flow cytometry profiles (left panel) of longterm-differentiated *Zfp281* KO.2 cells in N2B27 and indicating GFP sorting gates (left), and of unsorted or sorted $GFP_{low,sort}$ and $GFP_{high,sort}$ cells after an additional 2-3 days of culture in N2B27 and indicating gates used for quantification of GFP distribution (right). Please note that profiles shown on the right were recorded on a different instrument than the profile presented on the left. Quantification of GFP distribution (right panel) in N2B27 cultures derived from indicated sorted cells of specified genotypes. Average and SD of 2 experiments.

(**H**) *Zfp281* transcription relative to untreated WT_{2i} cells in Zfp281-inducible ESC clones after 48h in 2i and in the presence or absence of Dox. Average and SD of 2 technical replicates.

(**I**) Self-renewal of sorted $GFP_{high,sort}$ and $GFP_{low,sort}$ populations of indicated genotypes after exposure to Dox (green) or control conditions (black) for 48h. Green circles on x axis mark Dox-treated non-self-renewing samples. Average and SD of 2 technical replicates.

(**J**, **K**) Sequence of genome-edited *Tet1* and *Tet2* loci (**J**) and absence of proteins (**K**) in KO cells.

(**M**) Western blot showing Zfp281 protein levels during ESC progression.

(**N**,**O**) Nanog (**N**,**O**) and Zfp281 (**O**) mRNA levels relative to WT_{2i} cells in ESCs of specified genotypes (**N**) and in indicated EpiSCs 24h after transfection of neg and Zfp281 siRNAs (**O**). Average and SD of 5 (**N**) and 2 (**O**) experiments performed in duplicates.

(**P**) Resistance to differentiation in O4GIP ESCs transfected with indicated siRNA combinations after 3d of 2i withdrawal relative to untransfected cells. Average and SD of 2 experiments performed in duplicates.

Mayer et al., Figure EV3

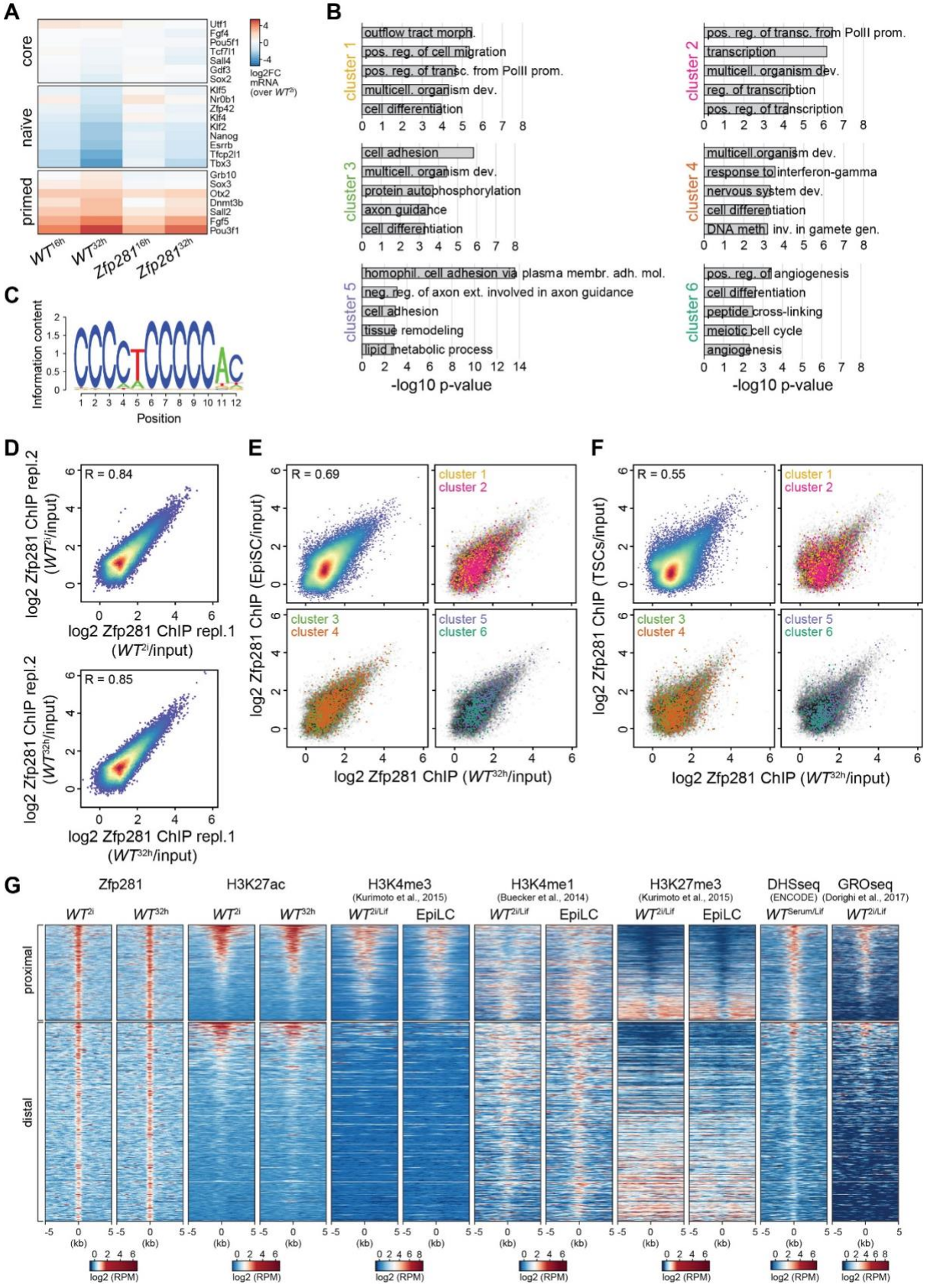

Figure EV3: Genomics of Zfp281.

(**A**) mRNA log2FC relative to WT_{2i} cells of selected core, naïve and primed pluripotency markers in differentiating *WT* and *Zfp281* KO cells at indicated timepoints.

(**B**) Top 5 enriched GO terms in clusters 1-6.

(**C**) Sequence logo from the *de novo* identified binding motif in 82.4% of 23756 Zfp281 peaks.

(**D**) Scatter plot comparing log2 Zfp281 peak ChIP enrichment over matched inputs between replicates in WT_{2i} (top) and WT_{32h} (bottom) cells.

(**E**, **F**) Scatter plot comparing log2 Zfp281 ChIP enrichment over matched inputs in WT_{32h} cells and EpiSCs (Huang *et al*, 2017) (**E**) and TSCs (Ishiuchi *et al*, 2019) (**F**). Peaks were assigned to closest transcriptional start sites (TSSs) and colored according to association with gene clusters 1-6.

(**G**) Heatmap of Zfp281, H3K27ac, histone H3 K4 tri-methylation (H3K4me3), H3K4 mono-methylation (H3K4me1) and histone H3K27 tri-methylation (H3K27me3) ChIPseq, DNAse-hypersensitive sites (DHS) and global run-on sequencing (GROseq) read densities across all proximal (+/- 2kb of TSS) Zfp281 (top) and distal Zfp281 (bottom) peaks. Each row represents a 10kb window centered on the peak mid of Zfp281. Rows are sorted for H3K27ac ChIP read densities in ESCs. Reads per million (RPM).

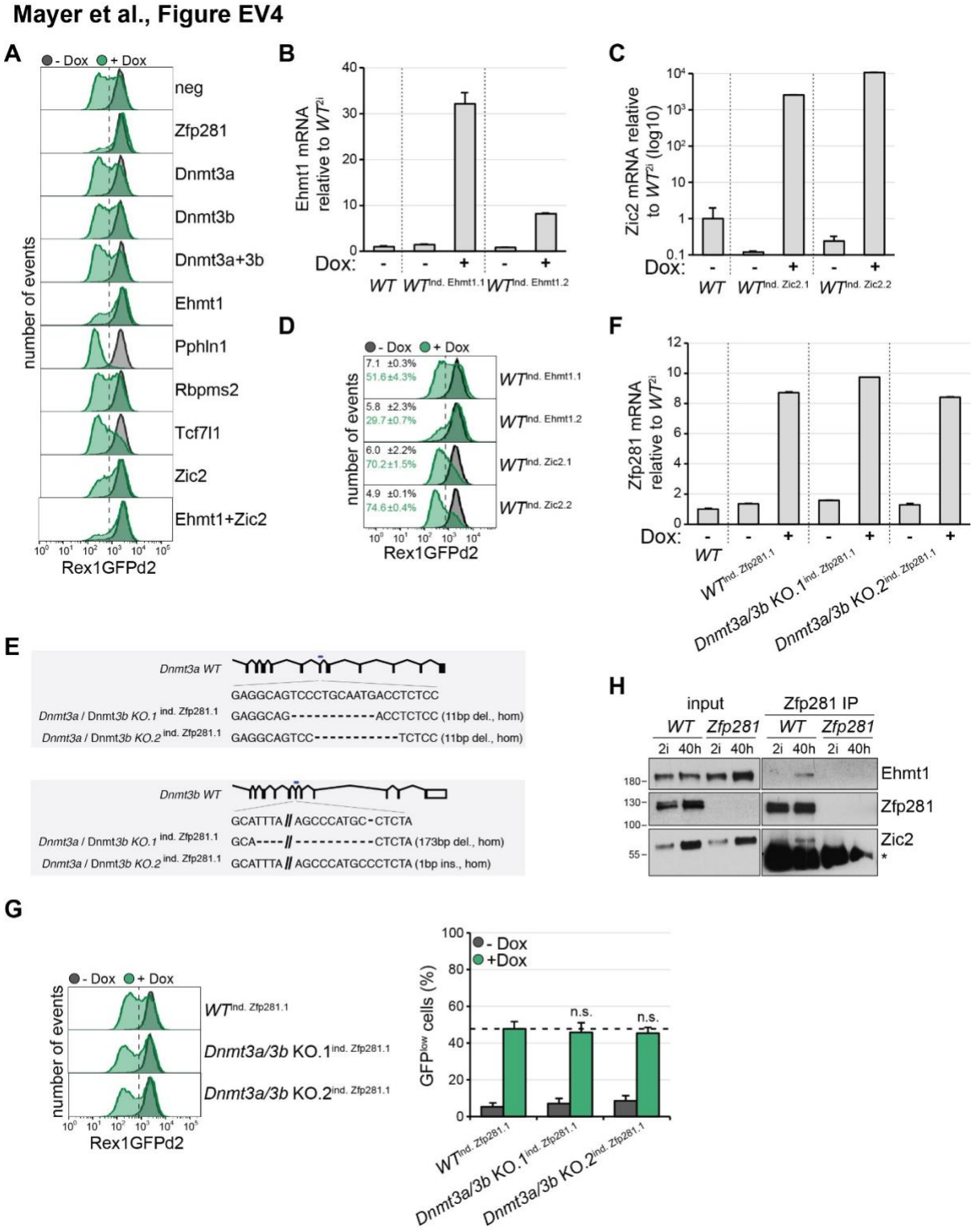

Figure EV4: Characterization of Ehmt1 and Zic2 downstream of Zfp281.

(**A**, **D**, **G**) Representative flow cytometry profiles of Zfp281-inducible RGd2 ESCs transfected with indicated siRNAs (**A**), of RGd2 ESCs with conditional Ehmt1 and Zic2 expression (**D**), and of *Dnmt3a*/*3b* compound KO RGd2 ESCs with conditional Zfp281 expression (**G**) after 32h in 2i and in the presence (green) or absence (black) of Dox. Significance (**G**) was determined using a Wilcoxon Mann-Whitney

rank sum test compared to $WT_{ind.\ Zfp281.1}$ Dox-treated cells. not significant (n.s.)>=0.05. Numbers are the average and SD of GFP_{low} cells in 2 (**D**) and 4 (**G**) experiments.

(**B**, **C**, **F**) FC Ehmt1 (**B**), Zic2 (**C**) and Zfp281 (**F**) transcription relative to untreated WT_{2i} cells in indicated ESC clones after 32h in 2i in the presence or absence of Dox. Average and SD of 2 technical replicates.

(**E**) Sequences of genome-edited *Dnmt3a* and *Dnmt3b* loci in $WT_{ind.\ Zfp281.1}$ cells.

(**H**) Whole cell lysate Zfp281 IPs in *WT* and *Zfp281* KO cells in 2i or 40h after 2i withdrawal, and probed for indicated proteins. Input (left) and Zfp281 IP (right). (*) Ig heavy chain.

Mayer et al., Figure EV5

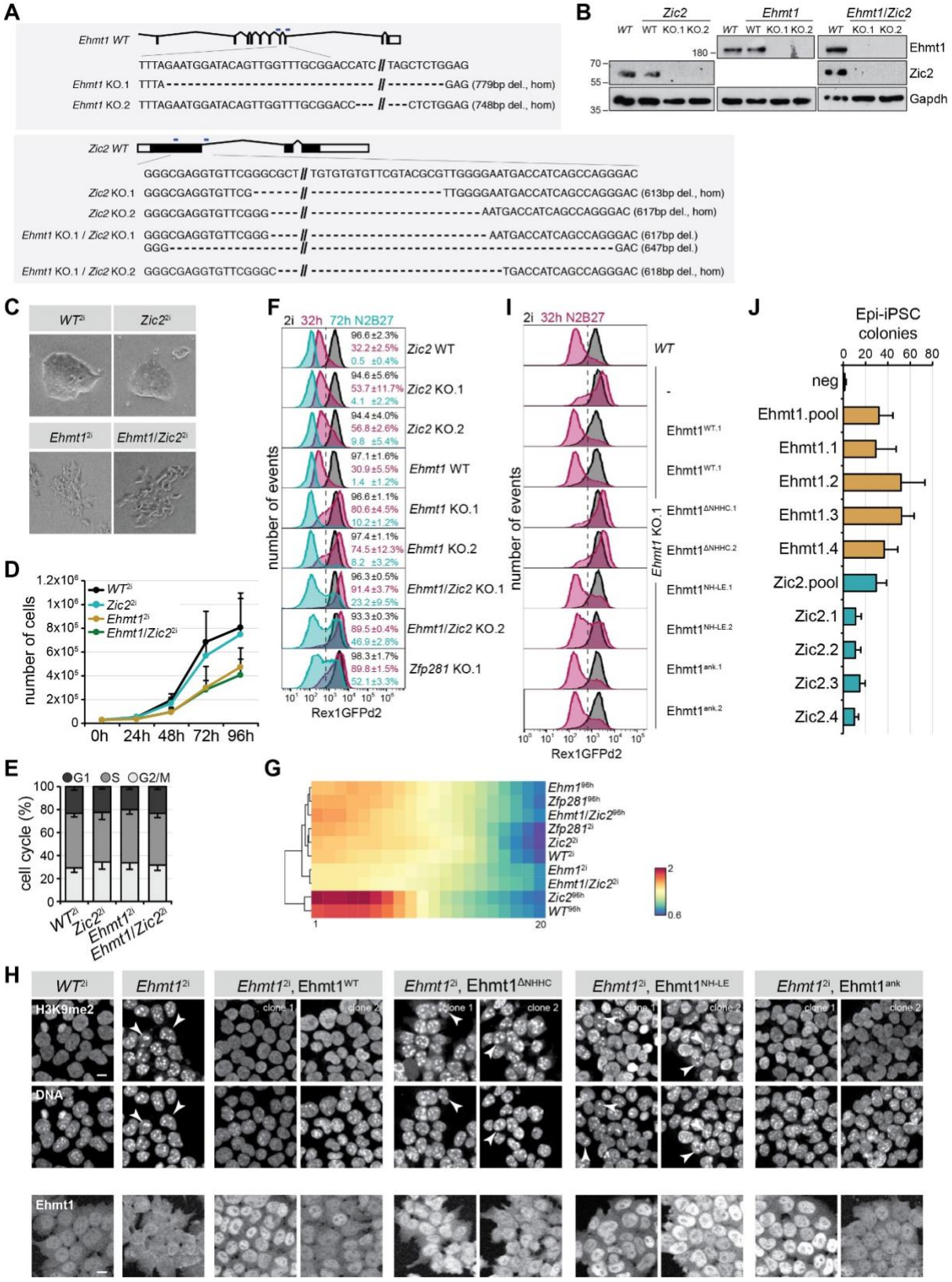

Figure EV5: Characterization of *Ehmt1*, *Zic2* and *Ehmt1*/*Zic2* KO cells.

(**A**, **B**) Sequence of genome-edited *Ehmt1* and *Zic2* loci (**A**) and absence of proteins (**B**) in KO cells.

(**C**-**E**) Cell morphologies (**C**), growth curves (**D**) and cell cycle analyses using propidium iodide staining (**E**) of indicated genotypes in 2i. Average and SD of 3 experiments (**D**, **E**).

(**F, I**) Representative flow cytometry profiles of indicated genotypes in 2i, and after 32h and 72h of 2i withdrawal (**F**), and in 2i and 32h after 2i withdrawal (**I**). Numbers (**F**) are the average and SD of GFP_{high} cells in 2 experiments.

(**G**) Quantification and hierarchical clustering of normalized F-actin intensity in 20 concentric rings (from center to circumference) in spheroids derived from ESCs with indicated genotypes in 2i or N2B27 for 4d. Intensity is color-coded and illustrates central F-actin accumulation and, hence, polarization of *WT* and *Zic2* KO cells during differentiation.

(**H**) Representative immunofluorescence staining of *WT* or *Ehmt1* KO ESCs expressing the indicated transgenes. Top: H3K9me2 and DAPI. Bottom: Ehmt1. Co-localization of H3K9me2 with DAPI-rich speckles in $Ehmt1_{2i}$ cells expressing no transgene, the ΔNHHC, or NH-LE alleles is indicated by arrowheads. Please note absence of nuclear Ehmt1 staining in $Ehmt1_{2i}$ cells and restoration by Ehmt1 transgenes. Scale bar is 10µm.

(**J**) Deconvolution of siRNA pools: Epi-iPSC colonies derived from 796.4 EpiSCs transfected with indicated siRNAs (individual siRNAs or pools), stimulated for 4d with Gcsf and 2i, and selected with Puromycin. Average and SD of 3 experiments performed in duplicates.

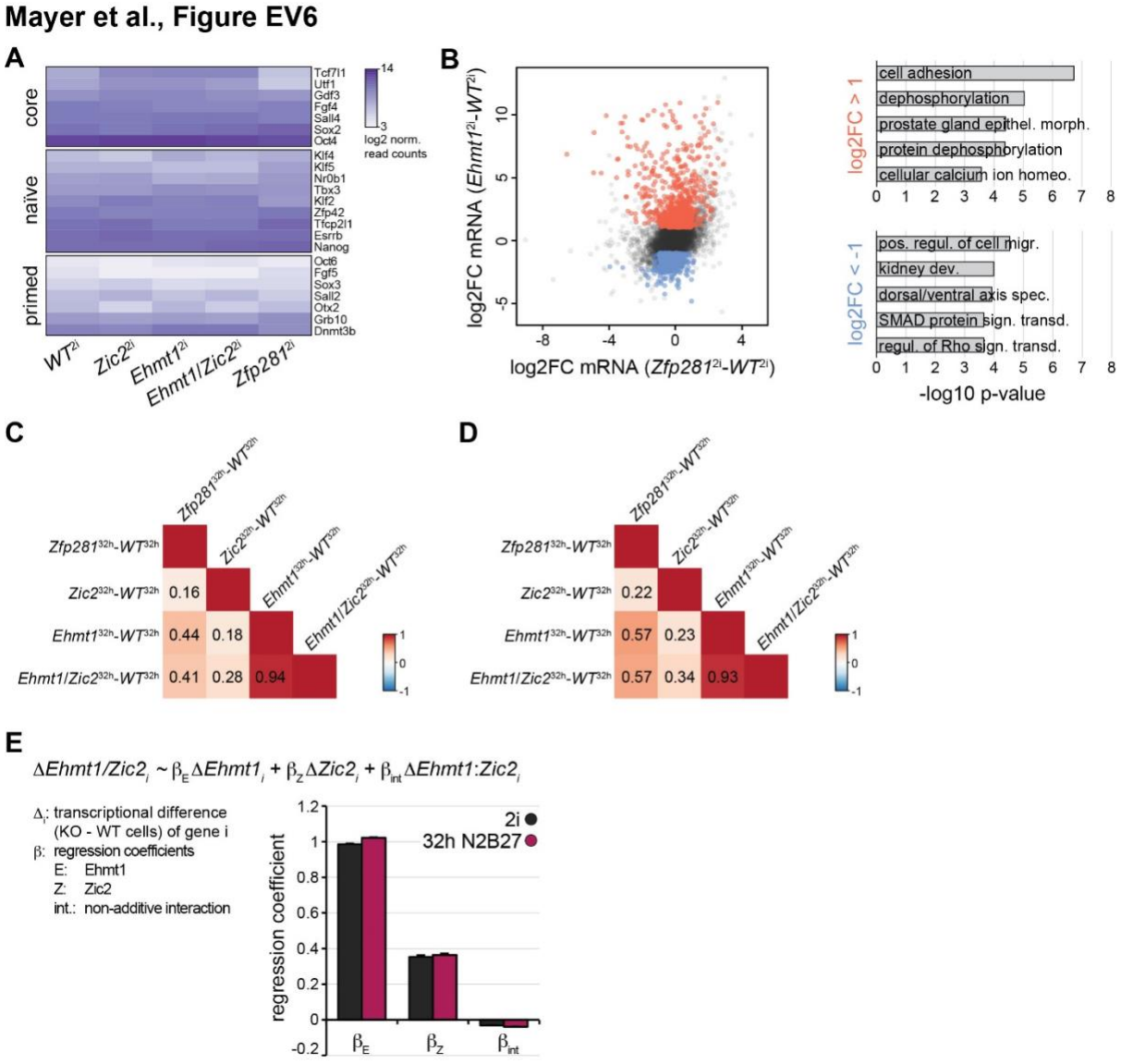

Figure EV6: Ehmt1 and Zic2 transcriptomics.

(**A**) log2 normalized read counts of selected core, naïve and primed pluripotency markers in ESCs of indicated genotypes.

(**B**) Scatter plot of mRNA log2FC in *Zfp281*$_{2i}$ and *Ehmt1*$_{2i}$ cells (left). Top 5 GO terms enriched in genes upregulated (red) and downregulated (blue) specifically in *Ehmt1*$_{2i}$ cells (right).

(**C, D**) Pairwise Pearson correlation coefficients of mRNA changes between indicated differentiated cells considering all detected transcripts (**C**) or gene cluster 1-6 transcripts (**D**).

(**E**) Estimated regression coefficients for the contribution of *Ehmt1* (Δ*Ehmt1*), *Zic2* (Δ*Zic2*) and their interaction (Δ*Ehmt1:Zic2*) to cell state-specific gene expression changes in *Ehmt1*/*Zic2* compound KO cells.

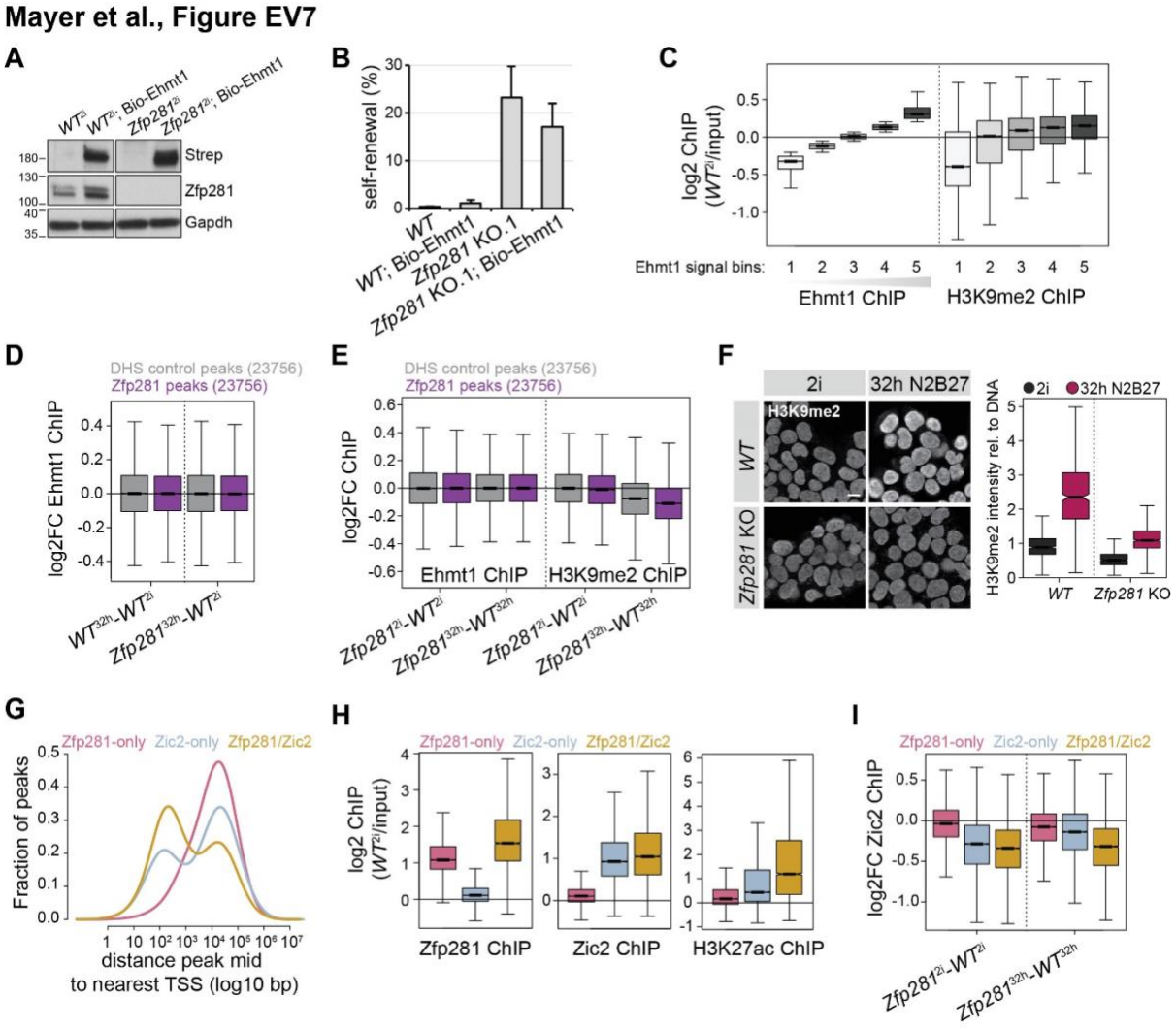

Figure EV7: DNA binding of Ehmt1 and Zic2.

(**A**) Western blot confirming Ehmt1 biotinylation (probed with Streptavidin (Strep)) in ESCs of indicated genotypes expressing the BirA ligase.

(**B**) ESC self-renewal of indicated genotypes after 3d of 2i withdrawal. Average and SD of 3 experiments performed in duplicates.

(**C**) Log2 Ehmt1 and H3K9me2 ChIP enrichment in ESCs over matched inputs at five classes of 10kb genome-wide windows binned by increasing Ehmt1 chromatin association.

(**D**, **E**) Ehmt1 (**D**, **E**) and H3K9me2 (**E**) ChIP log2FC between indicated cell states and genotypes at Zfp281 peaks (purple) or matching and non-overlapping DHS control peaks (grey) extended to 10kb windows.

(**F**) Representative immunofluorescence staining of H3K9me2 (left) and quantification relative to DNA (right) in indicated genotypes and conditions. Scale bar is 10µm.

(**G**) Density plot showing distance of Zfp281-only (pink), Zic2-only (blue) and Zfp281/Zic2 co-bound peaks (yellow) to nearest TSS.

(**H**) Zfp281 (left), Zic2 (middle) and H3K27ac (right) log2 ChIP enrichment over matched inputs in ESCs at Zfp281-only (pink), Zic2-only (blue) and Zfp281/Zic2 co-bound (yellow) peaks.

(**I**) Cell state-specific Zic2 ChIP log2FC between indicated genotypes and cell states at Zfp281-only (pink), Zic2-only (blue) and Zfp281/Zic2 co-bound (yellow) peaks.

EXTENDED VIEW AND APPENDIX FILES:

Table EV1: EpiSC reprogramming screen.

Table EV2: Zfp281, Ehmt1 and Zic2 genomics.

Table EV3: Zfp281 protein interactors.

Table EV4: Oligonucleotide sequences.

Appendix File 1: R code for PC analyses.