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TRIM28 and Interacting KRAB-ZNFs Control Self-Renewal of Human Pluripotent Stem Cells through Epigenetic Repression of Pro-differentiation Genes

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SUMMARY

Reprogramming to induced pluripotent stem cells (iPSCs) and differentiation of pluripotent stem cells (PSCs) are regulated by epigenetic machinery. Tripartite motif protein 28 (TRIM28), a universal mediator of Krüppel-associated box domain zinc fingers (KRAB-ZNFs), is known to regulate both processes; however, the exact mechanism and identity of participating KRAB-ZNF genes remain unknown. Here, using a reporter system, we show that TRIM28/KRAB-ZNFs alter DNA methylation patterns in addition to H3K9me3 to cause stable gene repression during reprogramming. Using several expression datasets, we identified KRAB-ZNFs (ZNF114, ZNF483, ZNF589) in the human genome that maintain pluripotency. Moreover, we identified target genes repressed by these KRAB-ZNFs. Mechanistically, we demonstrated that these KRAB-ZNFs directly alter gene expression of important developmental genes by modulating H3K9me3 and DNA methylation of their promoters. In summary, TRIM28 employs KRAB-ZNFs to evoke epigenetic silencing of its target differentiation genes via H3K9me3 and DNA methylation.

INTRODUCTION

Krüppel-associated box domain zinc finger (KRAB-ZNF) proteins comprise the largest family of transcriptional repressors in mammals. The human genome contains at least 381 KRAB genes, which produce over 800 transcripts, including splicing isoforms and pseudogenes (Corsinotti et al., 2013; Huntley et al., 2006) involved in morphogenesis, differentiation, and cell growth (Ecco et al., 2017; Lupo et al., 2013). The majority of KRAB-ZNFs consist of two functional components: zinc finger modules that bind to specific DNA sequences and a KRAB domain that recruits KRAB-associated protein 1 (KAP1), also known as tripartite motif protein 28 (TRIM28).

TRIM28, the universal mediator of KRAB-ZNF function, is a scaffold protein that mediates interactions with various epigenetic modifiers (Emerson and Thomas, 2009). Upon binding to the KRAB domain of KRAB-ZNFs, TRIM28 recruits multiple histone-modifying genes and complexes, including the NuRD complex, which harbors histone deacetylase and nucleosome remodeling activity, SETDB1 histone H3K9 methyltransferase, and HP1 H3K9 methyl reader, which propagates heterochromatin (Czerwinska et al., 2017). In fact, TRIM28/KRAB-ZNF complexes mediate long-range heterochromatin formation that spreads within several tens of kilobases from the DNA-binding site, resulting in the epigenetic silencing of gene transcription and/or protection of homologous sequences

from recombination (Groner et al., 2010). Moreover, TRIM28 may induce DNA methylation through interaction with DNMTs (DNA methyltransferases), which further stabilizes closed chromatin structures (Quenneville et al., 2011, 2012; Wiznerowicz et al., 2007).

Differentiation of pluripotent stem cells (PSCs) and somatic cell reprogramming to generate induced PSCs (iPSCs) are dynamically regulated epigenetic processes, and chromatin modifiers are crucial for the acquisition of an epigenetic state that allows for the adequate execution of cell-type-specific gene expression programs (Apostolou and Hochedlinger, 2013). Indeed, TRIM28 has been shown to play an important role in the maintenance of PSC identity. Knockout of *Trim28* is lethal during early embryogenesis in mice (Cammas et al., 2000). Consistently, *Trim28* depletion in embryonic stem cells (ESCs) leads to the loss of pluripotency (Fazzio et al., 2008). TRIM28 has been shown to mediate repression of endogenous retroelements in ESCs (Rowe et al., 2010; Turelli et al., 2014) via interactions with murine Zfp809 (Wolf et al., 2015) and human ZNF91/93 (Jacobs et al., 2014). Trim28's interaction with another KRAB-ZNF protein, Zfp57, was shown to maintain monoallelic DNA and H3K9 methylation patterns of imprinted genes during early embryogenesis (Li et al., 2008; Quenneville et al., 2011). In addition, KRAB/TRIM28-mediated methylation was demonstrated to be site specific and essential for the genome-wide establishment of epigenetic marks maintained during development (Quenneville et al., 2012). Interestingly, whether such repression by KRAB/TRIM28 is reversible may be dependent on the specific developmental context. In somatic cells, KRAB/TRIM28-mediated transcriptional repression is reversible (Wiznerowicz and Trono, 2003), whereas, during early embryogenesis, the Krab/Trim28 complex evokes irreversible gene repression that is stabilized through DNA methylation (Wiznerowicz et al., 2007; Ying et al., 2015). Importantly, although several KRAB-ZNFs have been demonstrated to be involved in development (Ecco et al., 2017; Lupo et al., 2013), the molecular function and target genes of the majority of KRAB-ZNFs remain unknown. Results from our lab indicate that TRIM28 knockdown facilitates reprogramming of mouse and human somatic cells to iPSCs (Klimczak et al., 2017) (U.O., M.G., J.P.W., K.K., K.T., P.B., P.K.S., M.W., and S. Mazurek, unpublished data). However, the exact molecular mechanism underlying the role of TRIM28 in iPSC reprogramming and maintenance of their pluripotency is unknown.

While TRIM28 affects both differentiation and dedifferentiation processes, we hypothesize that TRIM28 function in PSCs may be dependent on certain pluripotency-specific KRAB-ZNFs. We hypothesize that these KRAB-ZNFs may act not only through regulation of retroelements but also through repression of differentiation genes. In this scenario, changes in KRAB-ZNF expression profiles may allow a switch that shifts cellular fates. Indeed, our data show that TRIM28 function in PSCs relies on the pluripotency-specific KRAB-ZNFs, and together they mediate gene repression of critical differentiation genes. We provide evidence that, during somatic cell reprogramming to iPSCs, an exogenous KRAB-domain-containing repressor evokes silencing of its target regions not only through H3K9me3 deposition but also through stabilizing, irreversible DNA methylation. We identify three KRAB-ZNF factors, ZNF114, ZNF483, and ZNF589, that bind to TRIM28, display increased expression in human PSCs compared with differentiated cells and are critical for maintaining a pluripotent state. Finally, we identify the targets of these KRAB-ZNFs by chromatin immunoprecipitation sequencing (ChIP-seq) analysis and demonstrate that these KRAB-ZNFs utilize DNA methylation to suppress the expression of genes involved in developmental processes. Taken together, our results suggest a model wherein TRIM28 mediates reprogramming and maintenance of pluripotency via its interactions with specific KRAB-ZNF proteins and by modulating DNA methylation of crucial differentiation genes.

RESULTS

KRAB/TRIM28-Dependent Epigenetic Inactivation of the *PGK* Promoter Is Reversible in Human Somatic Cells but Permanent in iPSCs due to DNA Methylation

The KRAB domain is one of the strongest epigenetic suppressors of transcription (Ma et al., 2014). The mode of action of the KRAB/TRIM28 protein complex differs across cell developmental stages. KRAB/TRIM28-mediated transcriptional repression has been shown to be reversible in somatic cells (Wiznerowicz and Trono, 2003). In contrast, promoter inactivation has been shown to be permanent if it occurs during the first few days of embryogenesis (Wiznerowicz et al., 2007; Ying et al., 2015). Based on these findings, we sought to compare the role of KRAB-domain-containing repressors in human somatic and iPSCs. We used an inducible system (Figures 1A and 1B) that relies on the conditional doxycycline (dox)-dependent binding of a chimeric tTRKRAB transrepressor to the *tetO* element from the *Escherichia coli* tetracycline operator (Wiznerowicz and Trono, 2003). To engineer reporter somatic cell lines, primary human dermal fibroblasts (PHDFs) were transduced with two lentiviral vectors: the first vector contained cDNA encoding *tTRKRAB* (pLV-HK), and the second vector either carried the *PGK-gfp* expression cassette flanked by *tetO* sequences (pLV-PGK-tetO) or was a control vector without the *tetO* sequence (pLV-PGK) (Figure 1A).

In the absence of dox, tTRKRAB binds to the *tetO* sequences in proximity to the *PGK* promoter. It then recruits

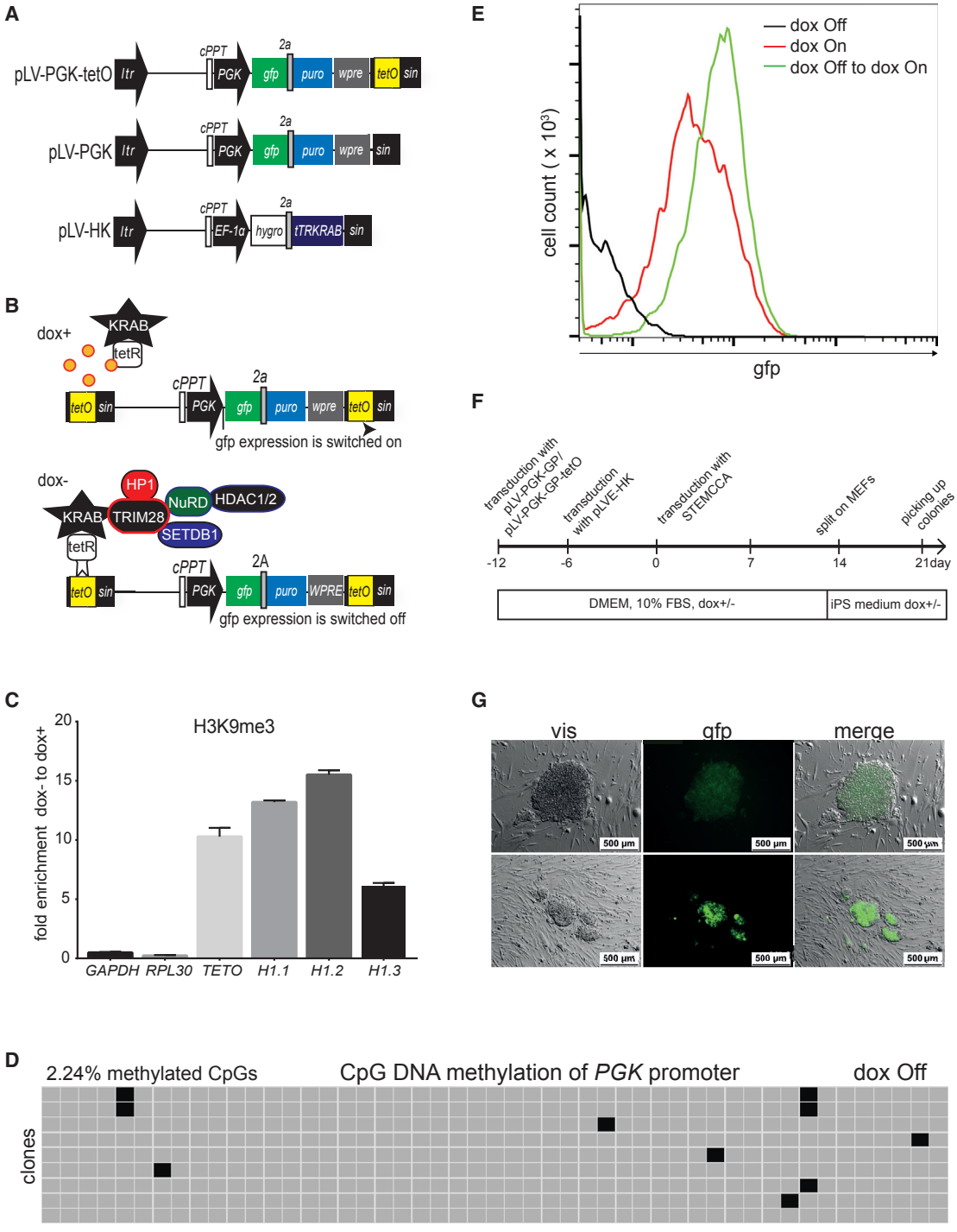

Figure 1. Generation of hiPSCs Expressing Chimeric KRAB-Transrepressor

(A) Lentiviral vectors carrying gfp regulated by the *PGK* promoter (with or without the *tet0* sequence in the long terminal repeat [*ltr*]) and a vector with a *KRAB* domain fused with the DNA-binding domain of the tetracycline repressor (*tetR*).

(B) In the absence of dox, TRIM28 is recruited to a specific DNA sequence by a KRAB-domain-containing repressor and acts as a scaffold for a multimolecular entity that silences exogenous *PGK* promoter transcription by triggering heterochromatin formation.

(C) ChIP-qPCR analysis confirmed enrichment of the chromatin repressive marker H3K9me3 adjacent to the KRAB/TRIM28 binding site in cells cultured without dox. Data are represented as means (N = 3) ± SD. The graph depicts representative results for one of three independent experimental replicates.

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TRIM28 to a specific DNA sequence, and exogenous *PGK* promoter transcription is silenced due to heterochromatin formation. Conversely, in the presence of dox, tTRKRAB is sequestered from the *PGK* promoter, and gfp is expressed (Figure 1B). KRAB/TRIM28-mediated repression is established by the long-range spreading of H3K9me3 (Groner et al., 2010). Therefore, we examined the deposition of the chromatin repressive marker H3K9me3 adjacent to the KRAB/TRIM28 binding site. Using primers specific for the several exogenous lentiviral vector fragments located in the vicinity of the tTRKRAB binding site, we observed significant enrichment of H3K9me3 in somatic cells cultured without dox (Figure 1C). An additional mechanism of transcription repression is promoter DNA methylation. Therefore, we performed bisulfite sequencing of these regions. In the absence of dox, we found the *PGK* promoter to be hypomethylated (2.24% of CpG methylation) (Figure 1D). After 10 days of dox administration in PHDFs (Figure 1E), gfp expression was restored, suggesting that the current experimental system is reversible in somatic cells. This is in agreement with previous findings (Szulc et al., 2006). Collectively, these findings suggest that the KRAB/TRIM28-mediated epigenetic inactivation of cellular promoters in somatic cells is caused by H3K9me3 deposition only, without the engagement of DNA methylation machinery.

Next, we sought to determine the impact of KRAB/TRIM28 on the chromatin status of the promoter and subsequent gene expression in human iPSCs (hiPSCs) generated from reporter PHDF cells with reprogramming (Takahashi and Yamanaka, 2006) (see Supplemental Experimental Procedures). These cells were cultured in the presence or absence of dox (1 μg/mL; Figure 1F), and individual hiPSC colonies were isolated and propagated for further analysis. The pluripotency of the hiPSCs was confirmed by immunofluorescence and alkaline phosphatase staining (Figures S1A and S1B). As expected, the cells cultured in the presence of dox showed gfp expression (Figures 1G, 2A, and 2B).

iPSCs carrying *gfp* under the *PGK* promoter without the *tetO* element showed high gfp expression levels and low *PGK* promoter methylation levels (Table S1), irrespective of drug administration (Figure 2A). In contrast, the *PGK* promoter that was subjected to tTRKRAB-mediated epigenetic repression during reprogramming in the absence of dox was seen to be significantly hypermethylated by bisulfite sequencing (Figure 2B; Table S1). Consistent with this observation, flow cytometric analysis of these reprogrammed cells cultured in the absence of dox showed undetectable levels of gfp fluorescence (Figure 2B). In hiPSCs generated in the presence of dox that sequestered tTRKRAB from the *tetO* cassette, the *PGK* promoter remained largely unmethylated (Figure 2A, Table S1), and gfp expression was high (Figure 2A). The difference in methylation was highly significant ($p < 0.0001$).

KRAB/TRIM28-mediated repression is permanent when triggered within the first few days of embryogenesis (Wiznerowicz et al., 2007; Ying et al., 2015). We hypothesized that this phenomenon also occurs during the dedifferentiation of human primary fibroblasts into hiPSCs. We administered dox to stable hiPSC lines that were generated in the absence of antibiotic and that displayed high levels of *PGK* promoter methylation and low levels of gfp fluorescence. We analyzed the *PGK* promoter status and gfp fluorescence levels after 10 days of dox treatment. The CpG methylation pattern remained unchanged, and gfp fluorescence was not restored (Figure 2C). These data suggest that transcriptional repressors containing a KRAB domain may be specifically involved in changing the landscape of DNA methylation during cellular reprogramming, thus affecting the transcriptome and phenotype of PSCs.

Identification and Validation of KRAB-ZNFs Important for the Phenotype of Pluripotent Stem Cells

Next, we turned our attention to endogenous KRAB-ZNFs to test which of them might play a role in PSCs. We sought to identify and validate KRAB-ZNFs specific for PSCs in a work flow that combined genome-wide expression profiles with experimental analysis. To this end, we analyzed the expression of 381 human KRAB-ZNFs extracted from Corsinotti et al. (2013) in the Progenitor Cell Biology Consortium (PCBC) dataset (http://www.synapse.org/pcbc) (Daily et al., 2017; Salomonis et al., 2016). The PCBC dataset includes stem cells (N = 93); embryonic bodies (N = 66); and cells induced to ectodermal (N = 29), endodermal (N = 36), and 5- (N = 29), 15- (N = 5), and 30-day (N = 6) mesodermal differentiation (total N = 264). Differential expression analysis and unsupervised clustering suggest that these KRAB-ZNFs show distinct tissue-type specificity (Figure 3A). This analysis also confirmed that the expression of the majority of KRAB-ZNFs is dependent on the differentiation status of the cell.

(D) The *PGK* promoter in somatic cells subjected to KRAB-mediated repression remains hypomethylated.
(E) Gfp expression is fully restored in somatic cells after 10 days of dox administration. Black line, dox−; red line, dox+; green line, cells after 10 days of dox treatment. Experiments for (D) and (E) were performed in three independent replicates. The images show results from a representative experiment.
(F) Timeline of generation of hiPSCs from PHDFs using a control or reporter system.
(G) hiPSCs cultured in the presence of dox show gfp expression by fluorescence microscopy.
See also Figure S1.

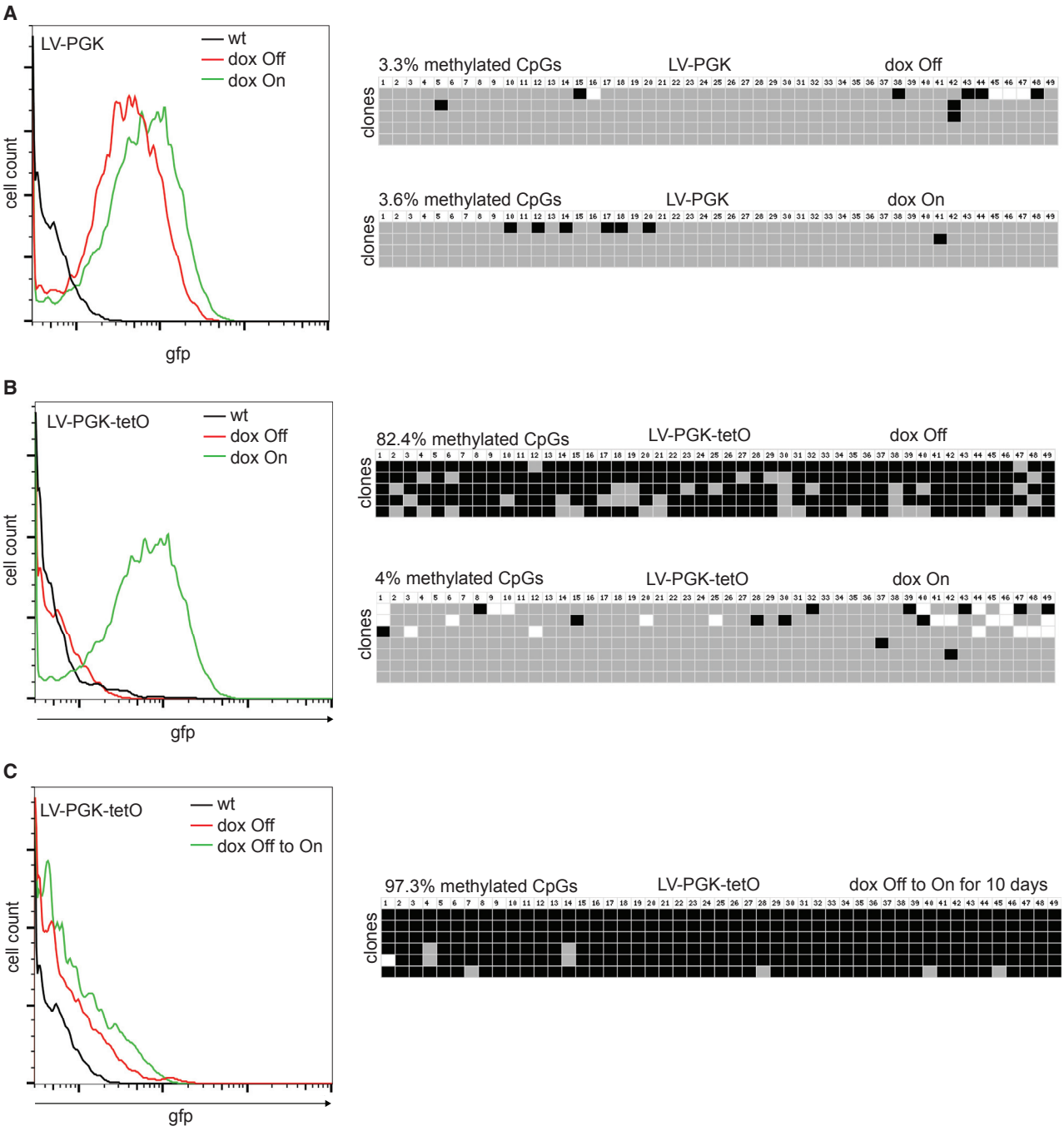

Figure 2. KRAB/TRIM28 Triggers Exogenous *PGK* Promoter Methylation

(A) hiPSCs transduced with control lentivirus (LV) carrying the *PGK* promoter without the *tetO* element showed high levels of gfp fluorescence. The *PGK* promoter was hypomethylated irrespective of drug treatment.

(B) hiPSCs transduced with a reporter LV carrying *PGK-tetO* showed decreased gfp fluorescence when cultured without dox. The exogenous *PGK* promoter was hypermethylated. Black line, isotype control; red line, dox−; green line, dox+.

(C) In contrast to somatic cells, gfp expression was not restored after 10 days of dox administration in hiPSCs, and the *PGK* promoter remained hypermethylated. Black line, isotype control; red line, dox−; green line, cells after 10 days of dox treatment. Methylation data: black square, 100% methylation; gray square, 0% methylation; white square, no data. All experiments were performed in independent triplicates on different fibroblast cell lines. Displayed results are from a representative experiment.

See also Table S1.

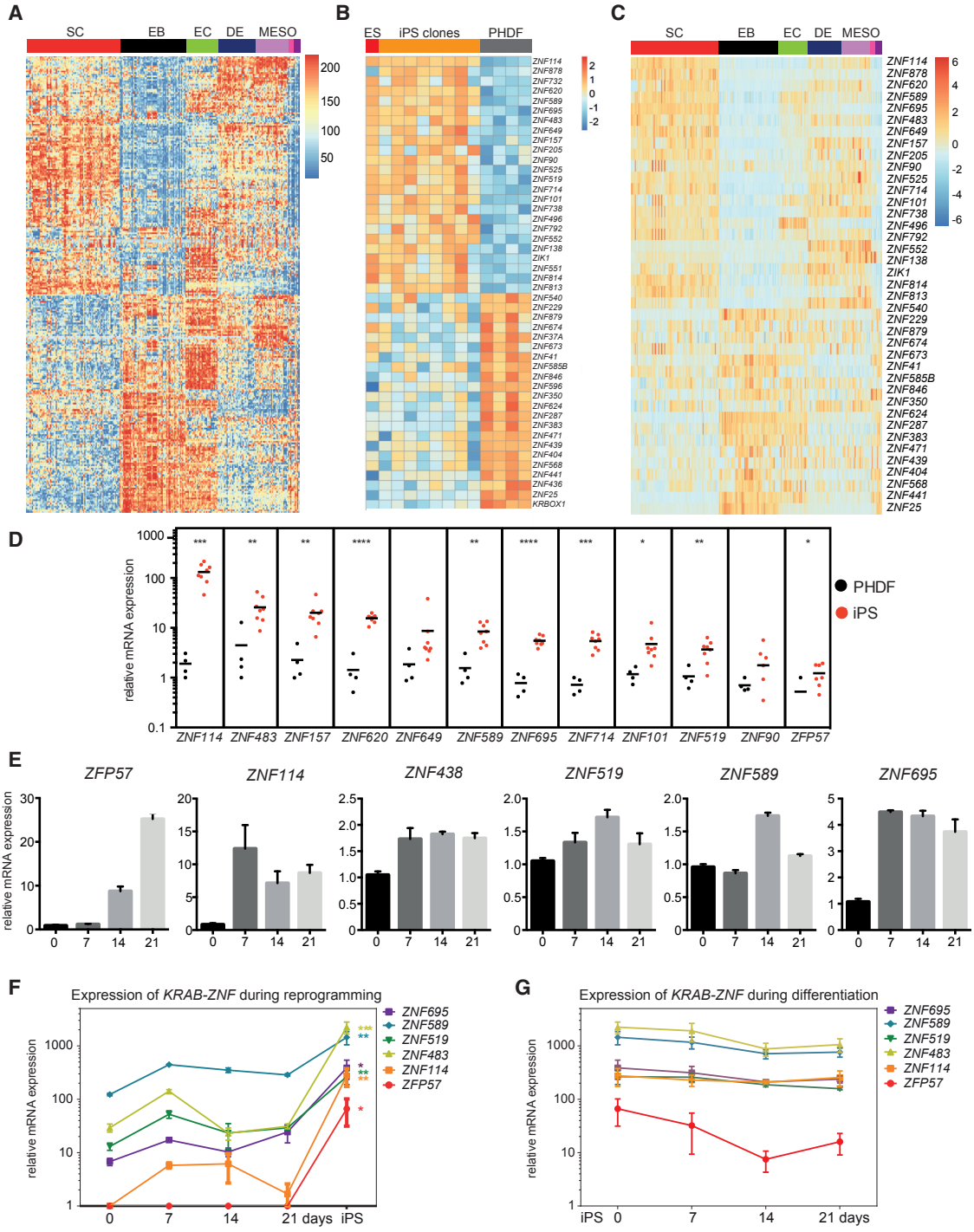

Figure 3. A Subset of Endogenous KRAB-ZNFs Is Upregulated in Pluripotent Stem Cells

(A) Heatmap and hierarchical clustering of the PCBC dataset shows tissue-specific expression of KRAB-ZNFs in stem cells (SC, N = 93), embryonic bodies (EB, N = 66), ectodermal (EC, N = 29), endodermal (DE, N = 36), and mesodermal (MESO) lineages (5, 15, and 30 days of mesodermal differentiation; N = 29, 5, and 6, respectively). Red indicates high and blue indicates low KRAB-ZNF expression.

(B) Heatmap and hierarchical clustering shows KRAB-ZNF expression signatures distinguishing fibroblasts (N = 4) from iPSCs (iPS; N = 8). The heatmap represents significantly (FDR < 0.1) altered KRAB-ZNF expression levels, as observed in our RNA-seq data.

(C) Heatmap and hierarchical clustering of the PCBC dataset confirms similar expression patterns of pluripotency-associated KRAB-ZNFs in stem cells (SC; N = 93) compared with embryonic bodies (EB; N = 66).

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We further characterized the differential expression of KRAB-ZNFs in our RNA sequencing (RNA-seq) dataset, comparing the expression profiles of PHDFs (N = 4) and iPSCs (N = 8) derived from these fibroblasts by reprogramming (U.O., M.G., J.P.W., K.K., K.T., P.B., P.K.S., M.W., and S. Mazurek, unpublished data). A total of 24 KRAB-ZNFs showed significantly increased expression and 22 showed significantly decreased expression in iPSCs compared with PHDFs (10% false discovery rate [FDR]; Figure 3B). To validate this observation, we compared KRAB-ZNF levels in stem cells and embryonic bodies using PCBC expression profiles. A total of 38 of 40 (95%) KRAB-ZNFs differentially expressed in iPSCs and PHDFs showed concordant differential expression patterns between stem cells and embryonic bodies (Figure 3C). This differential expression was statistically significant in 34 of 40 KRAB-ZNFs analyzed.

To further validate the differential expression status of KRAB-ZNFs during reprogramming, we performed single-gene RT-qPCR assays for 17 KRAB-ZNFs upregulated in iPSCs. In addition, we included *ZFP57*, which has been previously implicated in stem cell biology (Li et al., 2008; Quenneville et al., 2011). *ZFP57* is significantly differentially expressed in the PCBC dataset but fails to reach statistical significance in our RNA-seq data despite high-level expression in iPSCs. For the single-gene assays, we used the same cell line panel as indicated in Figure 3B. The RT-qPCR results confirmed significant upregulation of *ZNF114*, *ZNF483*, *ZNF157*, *ZNF620*, *ZNF589*, *ZNF695*, *ZNF714*, *ZNF101*, *ZNF519*, and *ZFP57*. They also confirmed a trend toward upregulation of *ZNF649* and *ZNF90* in iPSC lines compared with fibroblasts (Figure 3D). Other tested KRAB-ZNFs (*ZNF205*, *ZNF525*, *ZNF496*, and *ZNF732*) did not show upregulation in iPSCs, while *ZNF878* expression fell below the detection range (data not shown). The overall rate of validation of elevated KRAB-ZNFs observed in expression profiles by qPCR was 70%. Altogether, these analyses identified a subset of pluripotency-specific KRAB-ZNFs that may play a role in stem cell biology in the human genome.

The Expression of KRAB-ZNF Factors Changes during Reprogramming and Differentiation

Next, we assessed the kinetics of KRAB-ZNF expression during reprogramming of somatic cells to PSCs. For the reprogramming experiments, the cells were collected every 7 days for RT-qPCR and RNA-seq analyses. We selected top pluripotency-specific KRAB-ZNFs that were upregulated in iPSCs (in RNA-seq and RT-qPCR assays) and had the lowest FDRs (<5%) in RNA-seq analysis. Single-gene RT-qPCR assays indicated that their expression increased gradually but with variable kinetics during dedifferentiation (Figure 3E). Similar profiles of increasing KRAB-ZNF levels, with the highest expression reached in iPSCs, were also observed in RNA-seq data (Figure 3F). Furthermore, we induced differentiation of iPSCs by removing fibroblast growth factor (FGF) from the culture media, and we subsequently collected cells every 7 days for further analysis. As expected, RNA-seq data demonstrated a progressive decrease in the expression of pluripotency-specific KRAB-ZNFs in parallel with the loss of stemness properties of these cells (Figure 3G).

Depletion of Pluripotency-Specific KRAB-ZNFs Results in the Loss of Pluripotency of Human ESCs

To assess the effect of the identified KRAB-ZNFs on pluripotency, we performed RNAi-mediated knockdown of the six previously selected KRAB-ZNFs in a human ESC (hESC) line. Moreover, in this analysis, we also included *TRIM28*, which is known to induce differentiation in stem cells (Fazzio et al., 2008). The hESCs were transduced twice every 72 hr with a cocktail of three siRNA duplexes specific for each selected KRAB-ZNF and analyzed on the sixth day of silencing. The knockdown efficiency of each investigated gene was observed to be between 42% and 80% (Figure 4A). The untreated (WT) and non-specific siRNA-treated cells (MOCK) maintained morphologies characteristic of hESC colonies (Figure 4B). This was not the case for the cells with decreased expression of *TRIM28*, *ZFP57*, *ZNF483*, *ZNF519*, *ZNF589*, and *ZNF695*. In these cells, hESC colonies lost their distinct boundaries, and the cells became dispersed and acquired a spindle shape, indicating differentiation. Similar effects, albeit less pronounced and evident only at the colony borders, were observed in cells with *ZNF114* knockdown. To confirm loss of pluripotency, we evaluated the expression level of the pluripotency markers *NANOG* and *OCT3/4* in the siRNA-transfected cells. *NANOG* was significantly downregulated (67%–79%) in the cells with reduced

(D–F) The upregulation of selected KRAB-ZNFs in iPSCs (N = 8) compared with PHDFs (N = 4) was confirmed with single-gene RT-qPCR assays (D). KRAB-ZNF expression gradually increases during OSKM-induced reprogramming, as measured with RT-qPCR (E) and RNA-seq assays (F). After OSKM transduction at day 0, the cells were collected every 7 days. RT-qPCR analysis (E) was performed in three independent replicates. Displayed results are from a representative experiment. The bars represent means of three technical replicates ± SD.
(G) Differentiation of iPSCs resulted in progressive downregulation of KRAB-ZNF expression. For differentiation, the iPSCs were incubated without FGF and collected every 7 days. For RT-qPCR assays, expression of KRAB-ZNFs was normalized to *GAPDH* and calibrated to untreated control.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ as assessed by t test (D) or one-way ANOVA (F and G). Points (F and G) represent means of three independent experimental replicates ± SD.

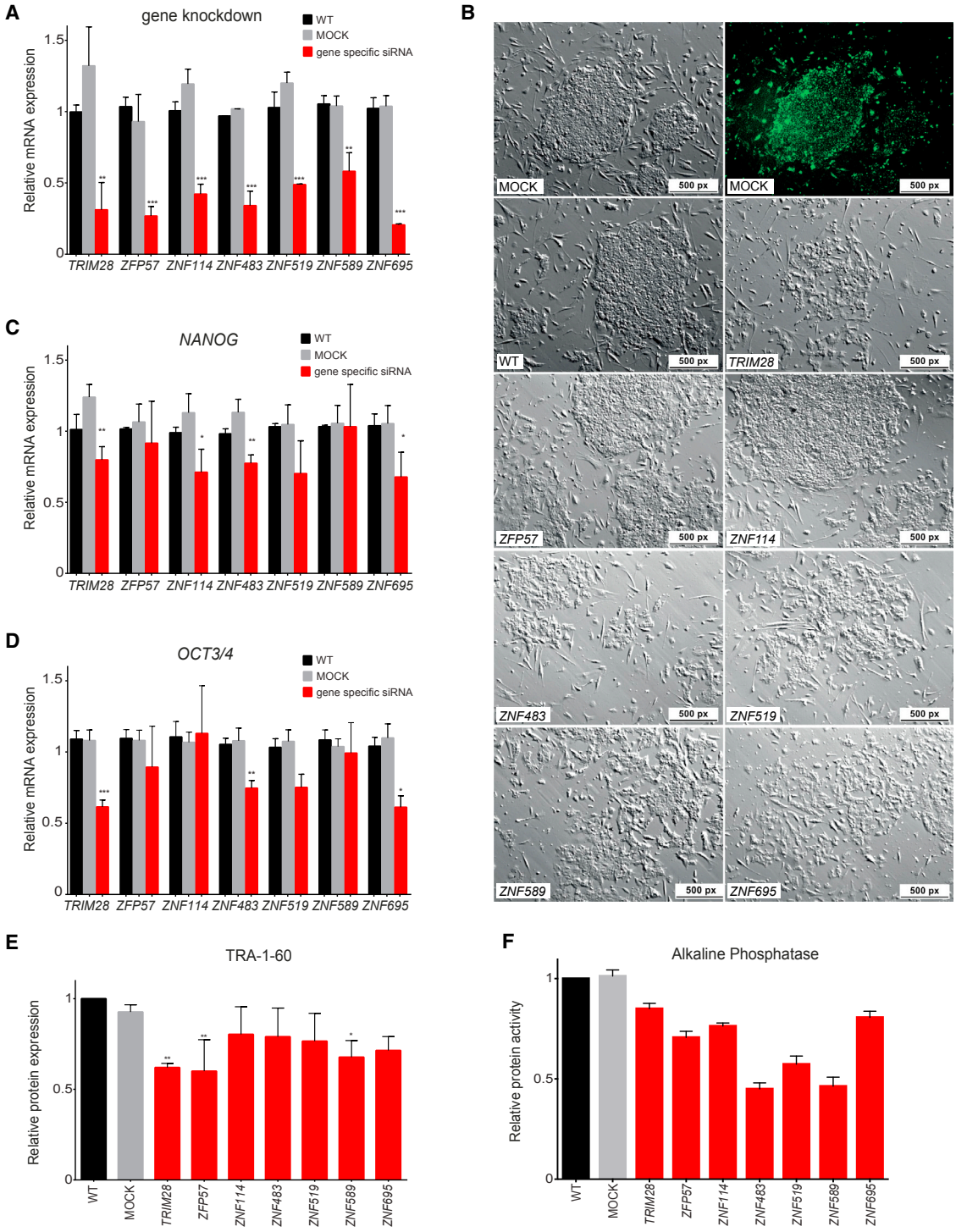

Figure 4. Depletion of Pluripotency-Associated KRAB-ZNFs Results in a Loss of Stemness in hPSCs

Human pluripotent stem cells were treated with non-specific fluorescein-isothiocyanate-conjugated scramble control (MOCK) or siRNA cocktails targeting *TRIM28* or each of the analyzed KRAB-ZNFs.

(A) Expression of each siRNA-targeted gene as measured by RT-qPCR.

(B) RNAi-mediated silencing of selected pluripotency-associated KRAB-ZNFs induced morphological changes in hESC colonies as shown on the microscopic images.

(legend continued on next page)

TRIM28, *ZNF114*, *ZNF483*, and *ZNF695* levels (Figure 4C). In addition, *OCT3/4* expression was significantly decreased (58%–75%) in cells with reduced *TRIM28*, *ZNF483*, *ZNF519*, and *ZNF695* levels (Figure 4D). We extended our KRAB-ZNF knockdown experiments using the commercially available ND41658*H iPSC line. We observed a marked decrease in TRA-1-60 expression (Figure 4E), as well as alkaline phosphatase activity (Figure 4F), in iPSCs with reduced KRAB-ZNF expression. These data suggest that the analyzed KRAB-ZNFs participate in the maintenance of pluripotency in stem cells.

Candidate KRAB-ZNFs Physically Interact with TRIM28

The canonical function of KRAB-ZNFs is gene repression through interaction with TRIM28, which recruits chromatin modifiers to induce H3K9 trimethylation, heterochromatization, and occasionally DNA methylation. Therefore, we confirmed that a similar mechanism occurred with the pluripotency-specific KRAB-ZNFs. This proof-of-principle analysis was performed for ZFP57 and the top three KRAB-ZNFs (ZNF114, ZNF483, and ZNF589) that fulfilled the previously mentioned criteria (i.e., showed the highest upregulation in iPSCs and the lowest FDR in the RNA-seq analysis comparing iPSCs and PHDFs). All of the selected proteins contain a C-terminal KRAB domain responsible for the interaction with TRIM28 and multiple N-terminal zinc finger domains that recognize and bind to specific DNA sequences (Figure 5A). As KRAB-ZNFs may be transcribed to various isoforms through alternative splicing mechanisms, we investigated which isoforms are present in PSCs using our RNA-seq data. For *ZFP57*, *ZNF483*, and *ZNF589*, the main isoform expressed in pluripotent cells constituted the variant coding the longest protein (Figure 5B). Interestingly, in the case of *ZNF114*, we identified two transcripts expressed with similar abundance. The one with slightly higher incidence rate (isoform 2) was devoid of its *KRAB* domain. Thus, in further studies, we decided to analyze only the isoforms encoding the longest proteins.

In order to confirm the possibility of a repressive function of the selected KRAB-ZNFs, we first investigated whether they localize to the nuclear compartment and physically interact with TRIM28. Indeed, fractionation analysis of cells expressing Flag-tagged derivatives of KRAB-ZNFs confirmed that all selected KRAB-ZNFs localized to the nucleus (Figure 5C). Furthermore, immunoprecipitation studies with an anti-flag antibody confirmed that the pluripotency-specific KRAB-ZNFs bind to TRIM28 (Figures 5D and 5E). Finally, as previous studies have indicated that the KRAB-ZNF/TRIM28 module may have an auto-regulatory function (O'Geen et al., 2007), we assessed the potential influence of *TRIM28* knockdown on the expression levels of the selected KRAB-ZNFs. *TRIM28* was silenced in hESC (Figure 5F) and PHDF (Figure 5G) cell lines to a level of 0.1 (±0.01) and 0.22 (±0.07), respectively. However, reduced *TRIM28* levels had no major effect on the expression of most of the tested KRAB-ZNFs in both cell lines, as only *ZNF483* and *ZNF589* expression in the PHDF cell line increased, to 1.5 (±0.01) and 1.6 (±0.01), respectively.

Genome-wide Analysis of KRAB-ZNF Binding

Next, we used an *in vivo* biotinylation system (Figure 6A) to tag selected KRAB-ZNFs in hESCs. The system was used for the generation of genome-wide binding profiles for ZFP57, ZNF114, ZNF483, and ZNF589 in hESCs overexpressing biotinylated factors (Figure 6B) via next-generation sequencing. We utilized the MACS algorithm (Zhang et al., 2008) to identify high-confidence peaks ($p < 1 \times 10^{-8}$) from the binding profiles. The peak distribution within the genomic regions demonstrated a gradual binding enrichment toward the 3′ region of the gene body (Figure 6C). Moreover, ZFP57, ZNF114, and ZNF589 showed a narrow peak in the vicinity of the transcription start site (TSS). Previous reports have proposed that the presence of the KRAB-ZNF/TRIM28 module at the 3′ end of the gene may protect highly homologous sequences from adverse recombination (Groner et al., 2010). Since we were interested in understanding the impact of gene regulation, we focused on the relatively low number of peaks that may occupy regulatory sequences near promoter regions.

We used DREME analysis (Bailey, 2011) to identify DNA motifs bound by each analyzed KRAB-ZNF (Figure 6D) from the list of high-confidence binding sites identified by MACS. DREME analysis confirmed the core DNA-binding motif for ZFP57 (TGCCGC) reported previously (Quenneville et al., 2011). We discovered binding sites for ZNF114 (CCATTCCA), ZNF483 (ACTCCACT), and

(C and D) Knockdown of the selected KRAB-ZNFs resulted in downregulation of the pluripotency markers *NANOG* (C) and *OCT3/4* (D).
(E) Flow cytometry analysis showed reduction in TRA-1-60 expression level in iPSCs after KRAB-ZNF knockdown.
(F) Alkaline phosphatase activity was significantly reduced upon KRAB-ZNF knockdown in iPSCs. The alkaline phosphatase assay was performed in three independent replicates. Displayed results are from a representative experiment. The bars represent means of three technical replicates ± SD.
(A, C, and D) The relative mRNA expression levels were measure by RT-qPCR assays. Gene expression was normalized to *GAPDH* and calibrated to non-treated, wild-type (WT) cells. (A and C–E) Bars represent means of three independent experimental replicates ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, as assessed by t-test.

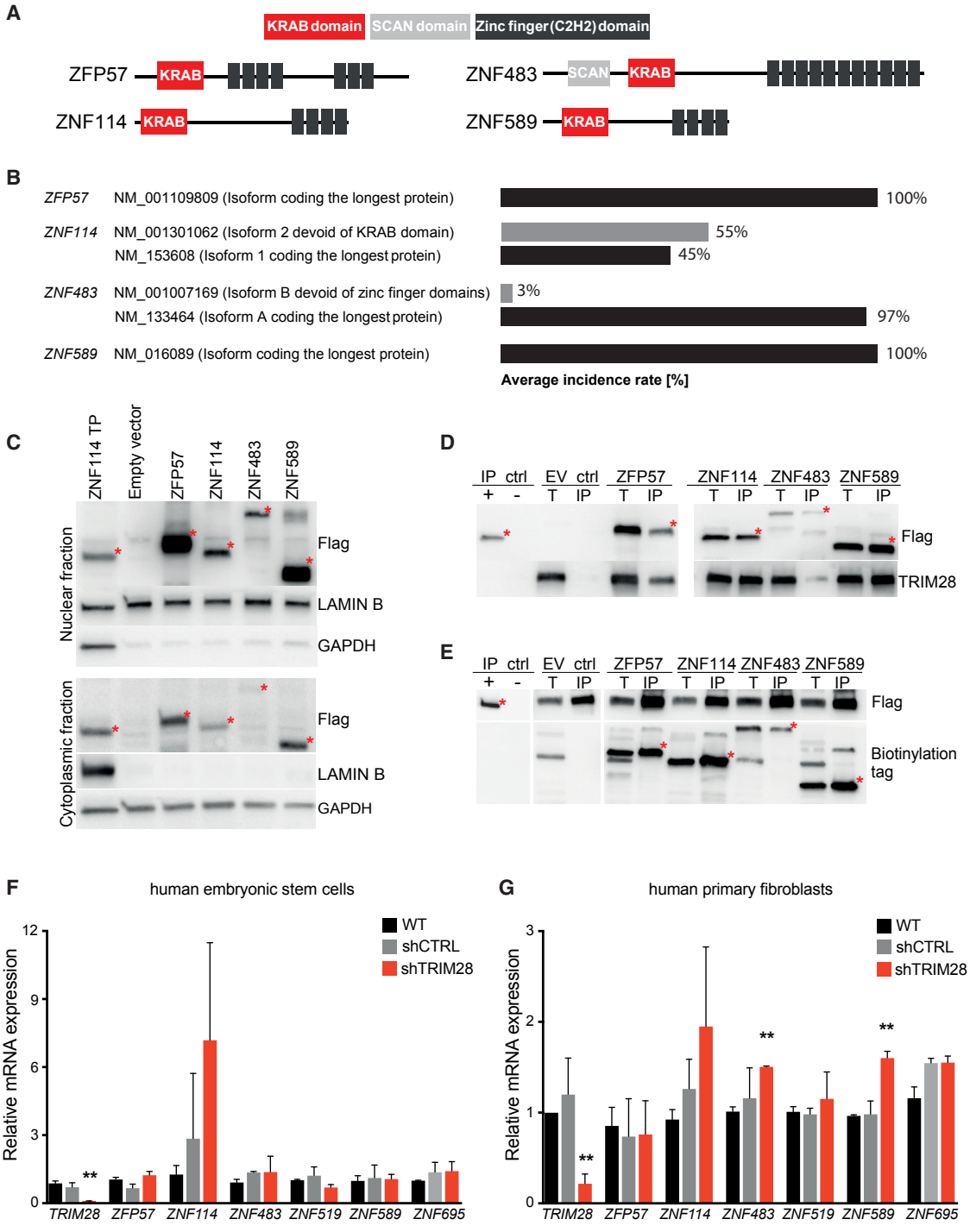

Figure 5. Pluripotency-Associated KRAB-ZNFs Localize to the Nucleus and Physically Interact with TRIM28

(A) Schematic representation of the structure of the pluripotency-associated KRAB-ZNFs: ZFP57, ZNF114, ZNF483, and ZNF589.

(B) Isoforms of selected KRAB-ZNFs transcribed in pluripotent stem cells as identified through splicing analysis of the RNA-seq data.

(C) All analyzed flag-tagged KRAB-ZNFs localized to the nucleus. After cellular fractionation, the fractions were immunostained with antibodies against Flag, LAMIN B (specific to the nucleus), and GAPDH (specific to the cytoplasm). Total protein extracted from the cells overexpressing ZNF114 (ZNF114TP) was used as a control. (D and E) Pluripotency-associated KRAB-ZNFs physically interact with the TRIM28 protein. Flag-tagged KRAB-ZNFs (D) or flag-tagged TRIM28 (E) was immunoprecipitated with the flag resins. Western blot images

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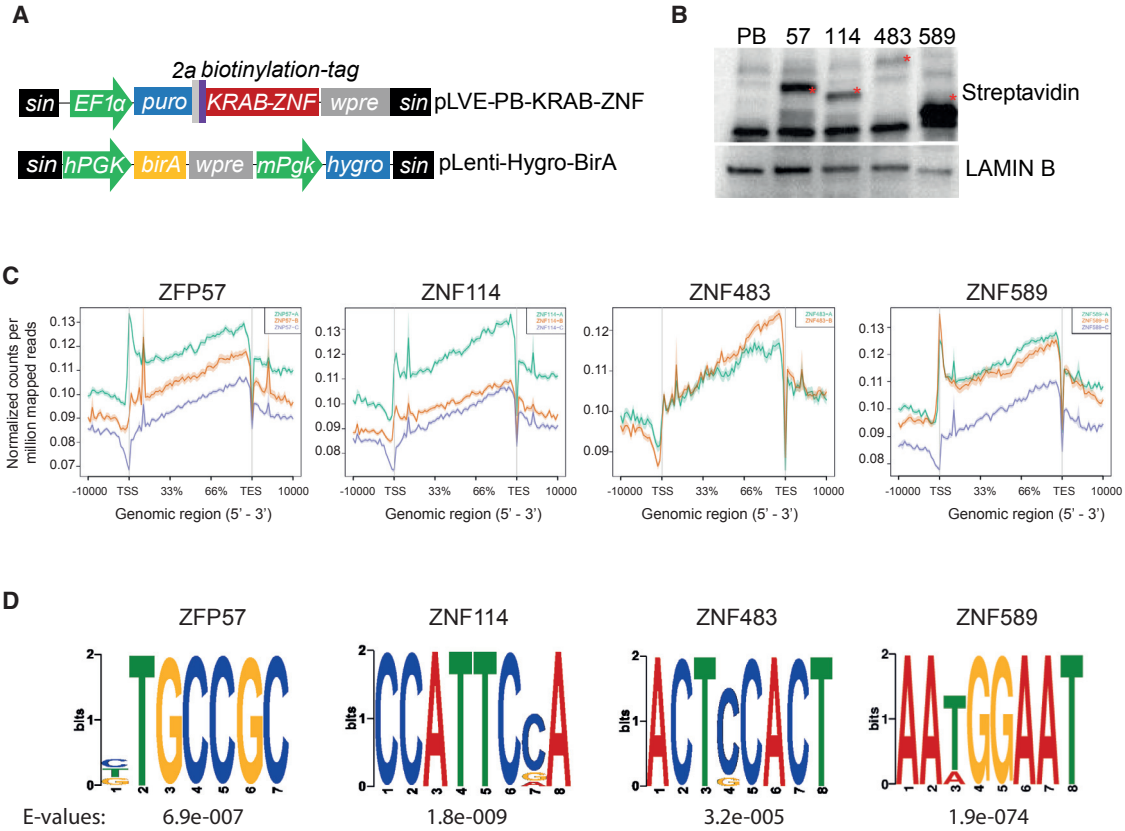


Figure 6. Genome-wide Analysis of KRAB-ZNF Deposition

(A) Schematic representation of the vectors used for *in vivo* biotinylation and subsequent ChIP-seq analyses.

(B) Western blot analysis confirmed the overexpression and biotinylation of KRAB-ZNFs in hESCs (asterisks mark the band specific for each KRAB-ZNF).

(C) Pluripotency-associated KRAB-ZNFs bind frequently within the transcription start site (TSS) and the 3′ end of the gene body. The graphs show patterns of binding for two or three biological replicates.

(D) Top motif hits of KRAB-ZNF DNA-binding sites identified via DREME analysis.

ZNF589 (AATGGAAT) with high confidence (Figure 6D). DREME analysis for a specific factor did not identify motifs for other factors in this study, suggesting that, despite similar peak distributions, these factors may function independently of each other.

Pluripotency-Specific KRAB-ZNFs Repress the Expression of Genes Involved in Differentiation

We hypothesized that the genes repressed by pluripotency-specific KRAB-ZNFs may be required during differentiation. To understand targets repressed at the gene expression level by KRAB/TRIM28, we overlaid their binding peaks close to promoter regions with promoter methylation information derived from Illumina arrays and multiple RNA-seq datasets (Figure 7A, Table S2). The RNA-seq profiles included comparisons of iPSCs to fibroblasts, genes upregulated upon TRIM28 knockdown in hESCs and iPSCs, as well as in PHDFs undergoing reprogramming (U.O., M.G., J.P.W., K.K., K.T., P.B., P.K.S., M.W., and S. Mazurek, unpublished data), and/or genes downregulated in samples with high

show immunostaining of flag-KRAB-ZNFs and TRIM28 (D) or flag-TRIM28 and KRAB-ZNFs tagged with the biotinylation peptide (E). Empty vector-transfected cells (EV ctrl) were used as a negative control. T, total protein; IP, immunoprecipitated fraction.

(F and G) *TRIM28* knockdown in hESCs (F) and PHDFs (G) had little effect on the expression of the pluripotency-associated KRAB-ZNFs. Upon *TRIM28* knockdown, relative mRNA levels of the selected KRAB-ZNFs were measured with RT-qPCR, normalized to *GAPDH* and calibrated to non-treated, wild-type (WT) cells. Bars indicate means of three independent experimental replicates ± SD. $^{**}p < 0.01$ as assessed by t-test. All proteomic analyses were performed in three independent replicates. The images show results from a representative experiment. Asterisks at the western blot images mark the bands specific to each KRAB-ZNF.

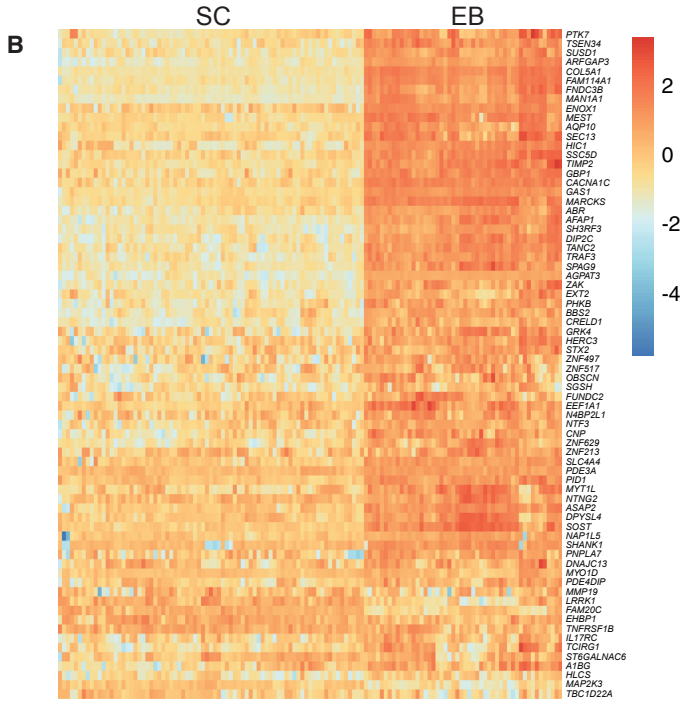
A

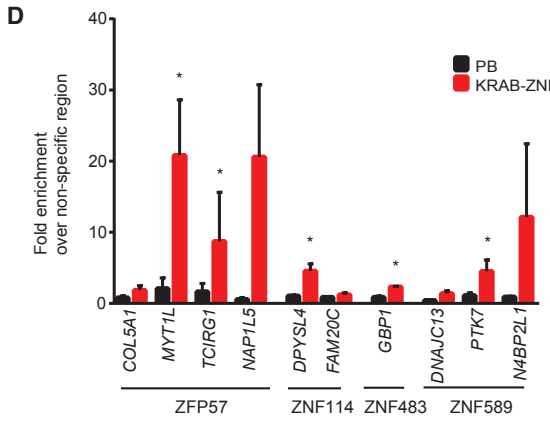
Datasets used for integration with ChIP-seq data
(*datasets used for identification of pluripotency-associated KRAB-ZNFs)

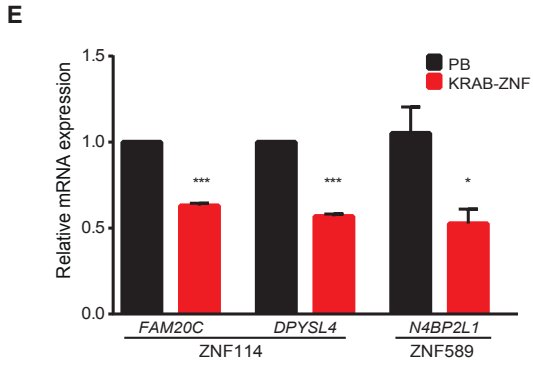
iPSCs vs PHDFs (RNA-seq* & methylation microarrays)	PCBC (RNA-seq* & methylation microarrays)
TRIM28^{KD} PHDFs undergoing reprogramming (RNA-seq & methylation microarrays)	TRIM28^{KD} hESCs undergoing TRIM28^{KD}-mediated differentiation (RNA-seq)

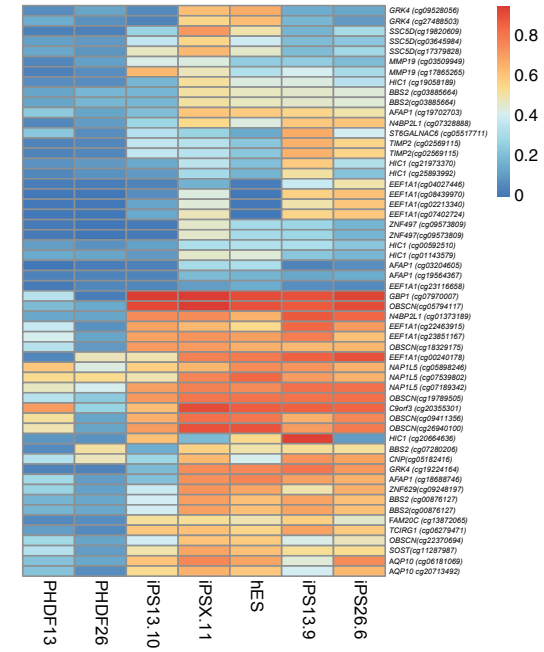
C

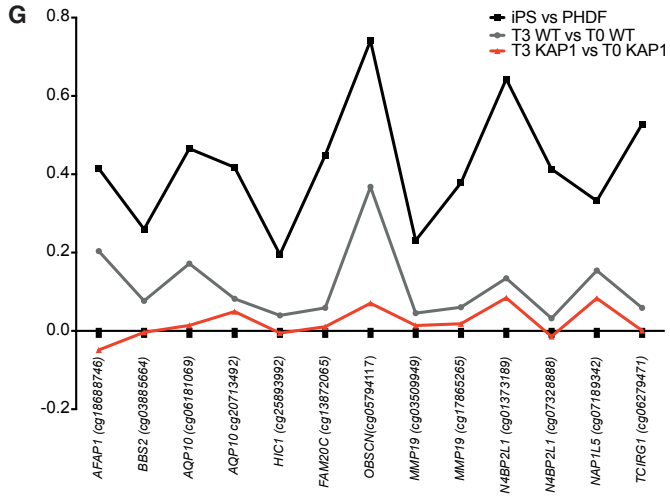
PATHWAY	GENE NAME
Developmental Biology	*DPYSL4, CACNA1C, SPAG9, COL5A1*
Organelle biogenesis and maintenance	*BBS2*
Immune System	*SEC13, MAP2K3, TRAF3, GBP1, TNFRSF1B, TCIRG1*
Hemostasis	*PDE3A*
Cell Cycle	*SEC13*
Extracellular matrix organization	*TIMP2, COL5A1, MMP19*
Gene Expression	*ZNF517, EEF1A1, TSEN34, ZNF213, C12orf5*
Metabolism	*MARCKS, SGSH, AGPAT3, EXT2, CACNA1C, PHKB, HLCS*
Metabolism of proteins	*MAN1A1, ST6GALNAC6, ARFGAP3*
Signal Transduction	*OBSCN, SOST, GAS1, SEC13, GRK4, ABR, PDE3A, COL5A1, TCIRG1*
Cellular responses to stress	*EEF1A1, MAP2K3*
Transmembrane transport of small molecules	*AQP10, TANC2, SLC4A4, TCIRG1*
Vesicle-mediated transport	*SEC13, ARFGAP3*











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expression of a given KRAB-ZNF (PCBC dataset) (Figure 7B). Integration of ChIP-seq profiles with four of the RNA-seq datasets (described above) resulted in 111 potential direct target genes. A total of 72 genes of these 111 showed reduced expression in these datasets. Gene Ontology enrichment analysis showed that several of the potential targets are involved in developmental biology, gene expression, signal transduction, or extracellular matrix organization (Figure 7C, Table S3). Single-gene ChIP-qPCR analysis (Figure 7D) confirmed binding of ZFP57 to *COL5A1*, *TCIRG1*, *MYT1L*, and *NAP1L5*; ZNF114 to *DPYSL4*; ZNF483 to *GBP1*; and ZNF589 to *DNAJC13*, *PTK7*, and *N4BP2L1*. Moreover, we overexpressed these KRAB-ZNF factors in the PHDF cell line, and 10 days post-transduction, we observed that several of the target genes (*DPYSL4*, *FAM20C*, and *N4BP2L1*) were downregulated (Figure 7E). These observations further confirm the repressive function of the pluripotency-specific KRAB-ZNFs on genes implicated in development.

Since the KRAB/TRIM28 module can induce promoter hypermethylation, we generated Illumina 450K methylation microarray data (Figure 7A) and identified genes whose promoter region (TSS ± 20 kb) is hypermethylated in iPSCs. We found that of the 72 target genes identified through integrating KRAB-ZNF binding-site data with the RNA-seq datasets (Figure 7B), 21 gene promoters (29%) became hypermethylated during reprogramming (Figure 7F). This is consistent with the *PGK* promoter methylation results observed with the dox-inducible system. Interestingly, a subset of these promoters (N = 10) remained hypomethylated during reprogramming upon *TRIM28* depletion (Figure 7G). Overall, integration of the three different types of information suggests a mechanism of KRAB-ZNF binding resulting in promoter methylation to suppress gene expression in PSCs. Since the canonical KRAB-ZNF mechanism of repression is mediated by H3K9me3 deposition, we overlapped identified target genes with the H3K9me3 ChIP-seq peaks from the DF19.11 iPSC line available from ENCODE. We found that 43 of the 71 target genes (60%) had H3K9me3 in the analyzed cell line (Figure S2). Finally, we determined whether the TRIM28/KRAB-ZNF module may cooperate with other factors involved in the suppression of differentiation genes (e.g., OCT4/SOX2/NANOG). We used TRANSFAC and JASPAR PWM tools to search for common transcription factor binding sites within the promoters of identified target genes. We did not find enrichment in factors that suppress differentiation; instead, we found a significant enrichment in transcription factors participating in developmental processes (Table S4). Interestingly, among those factors was KLF4, which is known not only for its impact on pluripotency and reprogramming to iPSCs but also for its involvement in various differentiation pathways. In summary, we have identified KRAB-ZNF proteins that are critical for maintaining pluripotency and repressing important differentiation genes in conjunction with TRIM28 by modulating H3K9me3 and DNA methylation levels of crucial differentiation genes.

DISCUSSION

The transition from a somatic to a pluripotent state is tightly regulated by reprogramming factors and is followed by large-scale changes in the cellular gene expression profile and chromatin environment (Onder et al., 2012; Plath and Lowry, 2011). Integrative genomic analyses have provided strong evidence that DNA methylation is critical in the reestablishment of

Figure 7. Validation of ChIP-Seq Data

(A) Expression and DNA methylation datasets used for integration with ChIP-seq data.

(B) Many genes bound by KRAB-ZNFs within ±20 kb of the TSS are downregulated in stem cells (SC, N = 93) compared with embryonic bodies (EB, N = 66) in the PCBC dataset, as shown by hierarchical clustering. Red indicates upregulated and blue indicates downregulated genes.

(C) Ontology of the genes targeted by pluripotency-associated KRAB-ZNF factors, as analyzed with REACTOME.

(D) Single-gene ChIP-qPCR confirms KRAB-ZNF binding to their target regions identified through the ChIP-seq approach. Overall fold enrichment levels over non-specific regions are shown for the empty vector control (PB) and specific KRAB-ZNFs. Bars indicate means of independent experimental replicates (N = 3–4) ± SEM, *p < 0.05 as assessed by t-test.

(E) Expression of KRAB-ZNF target genes decreases upon overexpression of their KRAB-ZNF repressor in a fibroblast cell line. Expression of the genes was measured with RT-qPCR and calibrated against *GAPDH*. Bars indicate means of independent experimental replicates (N = 3–4) ± SEM, *p < 0.05, ***p < 0.001 as assessed by t-test.

(F) 450K methylation microarray data analysis indicates hypermethylated CpGs (N = 56) within 21 KRAB-ZNF target gene promoters in iPSCs.

(G) *TRIM28* knockdown during reprogramming impairs methylation of 13 CpGs within the promoter region of 10 KRAB-ZNF target genes. Differences in methylation level were calculated as differences in beta values between iPSCs and PHDFs and between reprogramming time points T3 and T0 for wild-type (WT) cells and cells with *TRIM28* knockdown.

See also Tables S2–S4.

pluripotency (Mikkelsen et al., 2008). De novo DNA methylation is an essential aspect of this epigenetic reprogramming; however, the factors responsible for its establishment at specific genomic loci are poorly defined. TRIM28/KRAB-ZNFs have been previously shown to repress genes in many contexts; however, the exact identities of human KRAB factors associated with pluripotency and reprogramming, as well as their target genes, have remained elusive. Our results identify TRIM28 and a subset of KRAB-ZNFs as important regulators of de novo methylation in the context of reprogramming. Prior evidence supports this observation. The KRAB-ZNF-mediated recruitment of TRIM28 to DNA in ESCs induces H3K9me3 deposition, followed by cytosine methylation affecting CpG sites up to 5 kb from the binding site (Quenneville et al., 2012). Knockout of maternal *Trim28* early in murine development at the eight-cell stage causes defective demethylation of imprinted loci, leading to epigenetic chimerism in embryos (Messerschmidt et al., 2012).

A specific role for only a few KRAB-ZNFs has been identified in PSCs. Several of these KRAB-ZNFs (e.g., Zfp80 and Zfp819) are specific to the mouse genome and regulate retroelements (Tan et al., 2013; Wolf and Goff, 2007, 2009; Wolf et al., 2015). Starting with a genome-wide list of 381 human KRAB-ZNFs, we identified a subset of KRAB-ZNFs that showed increased expression in human iPSCs. Utilizing PCBC profiles comparing stem cells with more specialized cell types allowed us to refine this list further for functional validation. Here, we provide a list of validated human pluripotency-associated KRAB-ZNFs in the human genome. Importantly, we show functional evidence that these KRAB-ZNFs maintain stemness properties in PSCs.

Expression of KRAB-ZNFs has been shown to be tissue-type specific only in a few studies (Bellefroid et al., 1991; Lorenz et al., 2010). In the present study, we provide a map of differentially expressed KRAB-ZNFs with respect to various cell lineages by analyzing PCBC profiles. The expression profile of all annotated KRAB-ZNFs was compared between stem cells and cells differentiated toward embryonic bodies, the ectoderm, the endoderm, and the mesoderm. Our results demonstrate that the expression of KRAB-ZNFs dynamically changes during cellular differentiation. Moreover, the factors segregate separately, demonstrating clusters with expression profiles specific to certain cell lineages. This observation emphasizes the tissue specificity of the majority of KRAB-ZNF factors and corroborates the hypothesis that KRAB-ZNFs participate in developmental processes.

We characterized the subset of KRAB-ZNFs associated with pluripotency through loss of function experiments, nuclear localization studies, and physical interaction with TRIM28. Then, we characterized binding sites and identified binding motifs for these factors. From this genome-wide list, only Zfp57 had previously been shown to be specific to PSCs through its participation in repressive mechanisms controlling genomic imprinting (Li et al., 2008; Quenneville et al., 2011). However, Zfp57 binding to non-imprinted regions has also been reported in a mouse model (Strogantsev et al., 2015). The targets identified for the human ZFP57 in the current study fell similarly into the categories of imprinted and non-imprinted genes. Moreover, the ZFP57 binding motif identified in our analysis confirmed the previously reported consensus sequence: TGCCGC (Quenneville et al., 2011; Strogantsev et al., 2015). Another factor, ZNF589/SZF1, was previously linked to CD34+ stem cells (Liu et al., 1999). As PSCs were not included in their study, our findings provide additional observations of the ZNF589 expression pattern in pluripotent and differentiated cells.

Taking advantage of our dox-induced system, we demonstrated that KRAB/TRIM28-mediated transcriptional repression is reversible in somatic cells but becomes irreversible during somatic cell dedifferentiation into hiPSCs. Our data agree with previous findings that the same mechanism is responsible for permanent methylation in the early stages of murine embryogenesis (Wiznerowicz et al., 2007; Ying et al., 2015). We showed that KRAB-domain-containing transrepressor evokes permanent silencing of its target region through deposition of H3K9me3 and DNA methylation. Consistent with this observation, we identified a set of genes that are targets of endogenous KRAB-ZNFs and that become hypermethylated and, thus, downregulated during reprogramming. These genes are enriched in developmental biology and other relevant processes. Thus, it is tempting to speculate that endogenous KRAB-ZNFs may also mediate stable gene repression during reprogramming, thus protecting self-renewal and pluripotency. Nevertheless, only 30% of the KRAB-ZNF targets showed DNA hypermethylation in their promoters in iPSC, arguing against a major role in gene repression during reprogramming. However, the repression of KRAB-ZNF target genes through stabilization of DNA methylation may be essential for the maintenance of pluripotency and self-renewal in stem cells.

In summary, we showed in the present study that KRAB-domain-containing genes are capable of mediating irreversible gene repression stabilized by DNA methylation during human somatic cell reprogramming to iPSCs. Moreover, we demonstrated how human KRAB-ZNF expression changes between stem cells and other cell lineages, and we validated a subset of KRAB-ZNFs associated with pluripotency. Finally, we provided functional and molecular evidence for the involvement of a subset of KRAB-ZNF factors

in the maintenance of pluripotency through repression of differentiation-associated genes.

EXPERIMENTAL PROCEDURES

hESCs BG01V (ATCC), human iPSC line ND41658*H (NINDS Human Genetics DNA and Cell Line Repository), human iPSC lines (home generated with an OSKM-lentiviral vector), HEK293, mouse embryonic fibroblasts, and PHDFs were cultured in standard conditions. All lentiviral particles were produced in HEK293T cells transfected with the calcium phosphate method using a second-generation packaging system (Wiznerowicz and Trono, 2003). KRAB-ZNF and *TRIM28* RNAi-mediated silencing was performed using a cocktail of two or three specific siRNA duplexes (100 μM each). ChIP was performed based on biotin-streptavidin interaction. The libraries for RNA-seq (Illumina), ChIP-seq (NEB), and Infinium HumanMethylation450 BeadChip (Illumina) were prepared according to the manufacturer's protocols. All experimental details are provided in the Supplemental Information.

ACCESSION NUMBERS

The accession numbers for the ChIP-seq and methylation microarray data reported in this paper are GEO: GSE97403 and GSE95096.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, two figures, and four tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.10.031.

AUTHOR CONTRIBUTIONS

Conceptualization, M.W., U.O., M.G., K.R., and P.K.S.; Methodology and Investigation, U.O., M.G., K.R., H.H., P.C., A.A., J.P.W., K.T., K.K., N.S., R.P., and M.W.; Software, Validation, and Formal Analysis, U.O., M.G., A.T.R., P.K.S., B.S., K.F., M.K., P.A., H.H., and E.M.; Data Curation, A.T.R. and H.H.; Writing – Original Draft, U.O. and M.G.; Writing – Review & Editing, A.T.R., K.R., M.W., and P.K.S.; Visualization, U.O., M.G., A.T.R., P.A., and M.W.; Supervision, K.R., P.K.S., P.B., and M.W.; Funding Acquisition, M.E., K.R., and M.W.

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