Mutation of TRIM28-Lys304 to Gln Attenuates its Interaction with KRAB-ZNFs and Promotes Differentiation of Erythroleukemic K562 Cells

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Abstract

Background

TRIM28/KAP1/TIF1 β is a key epigenetic modifier. Genetic ablation of *trim28* is embryonic lethal although RNAi-mediated knockdown in somatic cells yields viable cells. Reduction in TRIM28 abundance at the cellular or organismal level results in polyphenism. Posttranslational modifications such as phosphorylation and sumoylation have been shown to regulate TRIM28 activity. Moreover, the methylation of DNA, RNA and histones and acetylation of histones are key epigenetic modifications that regulate gene expression. A number of lysine residues of TRIM28 are subject to acetylation, but how acetylation of TRIM28 affects its functions remains poorly understood.

Results

Here we report that, compared with wild-type TRIM28, the acetylation-mimic mutant TRIM28-K304Q has an altered interaction with Krüppel-associated box zinc-finger proteins (KRAB-ZNFs), with consequent effects on the phenotype of the erythroleukemic cell line K562. TRIM28-K304Q was comparable with its wild-type counterpart with respect to intracellular level, homodimerization, phosphorylation at S473 and S824, and interactions with heterochromatin-binding protein HP1. The expression of embryonic-related and fetal globin genes was activated in TRIM28-K304Q mutant cells. Transcriptome analysis revealed that TRIM28-K304Q and *TRIM28* knockout K562 cells had similar global gene expression profiles, yet the profiles differed considerably from that of wild-type K562 cells. The gene expression ensemble of mutant K562 cells indicated a general induction of differentiation-promoting genes and attenuation of proliferation-promoting genes.

Conclusions

These results suggest that acetylation/deacetylation of K304 in TRIM28 or TRIM28-K304Q constitutes a switch for regulating its interaction with KRAB-ZNFs and alters the gene regulation of this key epigenetic modifier as demonstrated by the acetylation mimic TRIM28-K304Q.

Background

The evolutionarily conserved, vertebrate-specific tripartite motif protein 28 (TRIM28) also known as KRAB-associated protein 1 (KAP1) and transcription intermediary factor- β (TIF1 β), is an essential developmental regulator—specifically, a universal transcriptional co-repressor for the KRAB-ZNFs, a large family of transcriptional repressors in vertebrates. The mammalian genome encodes >350 KRAB-ZNFs (1–3) involved in growth and differentiation (4,5), the majority of which consists of sequence-specific DNA-binding C2H2-type zinc-finger modules and a KRAB domain that recruits TRIM28.

TRIM28 is composed of an N-terminal unstructured region (amino acid residues 1–62), a RBCC (RING, B1 and B2 boxes, coiled-coil domain) motif, two central unstructured regions (residues 406–623, 673–696),

a PHD finger and bromo domain, and a C-terminal unstructured region (residues 813–835). The RBCC facilitates homodimerization of TRIM28 and specifically interacts with the KRAB domain of ZNFs (6,7). TRIM28 serves as a scaffold for co-repressor proteins including DNA methyltransferases (DNMTs), the Nucleosome Remodeling and Deacetylase (NuRD) complex, the H3K9me3 MTs SETDB1, G9a (EHMT2), and HP1, and this holocomplex contributes to histone modifications and heterochromatin formation to maintain gene silencing (8-12). Through KRAB-ZNFs and other transcription factors (e.g., OCT4, p53, STAT1, STAT3, etc.), TRIM28 can be targeted to chromatin (13). TRIM28 can recruit chromatin writers and readers (DNMTs, SMARCA4, and HP1s) (14-16). The scaffold function of TRIM28 is largely mediated by the intrinsically disordered regions (IDRs). An HP1-binding motif (PXVXL), which is a molecular recognition feature present in IDR (406–623), is responsible for binding HP1 proteins that are essential for histone modifications and facultative heterochromatinization associated with transcriptional repression and progression through cellular differentiation (17). Posttranslational phosphorylation occurs mainly in the IDRs. The IDRs are the most diversified amino acid sequence in TRIM28, whereas the sequences of the RBCC, HP1 box, and PHD and bromo domain are largely conserved evolutionarily. Unlike the two closely related subfamily members TRIM24 and TRIM33, the TRIM28 bromo domain does not bind to lysine-acetylated histones, and consequently the PHD domain cannot recognize the unmethylated histone H3K4 (18,19).

TRIM28 is a maternal-effect protein required for maintenance of genomic imprinting (20,21). The early embryonic lethality resulting from the dysregulation of genomic imprinting in mice lacking maternal TRIM28 attests to its key roles in embryonic development (20,22). Moreover, TRIM28 not only maintains DNA methylation at imprinted regions during early genome-wide reprogramming but also regulates DNA methylation at imprinted gene promoters after reprogramming (23).

Posttranslational phosphorylation and sumoylation of TRIM28 contribute to the regulation of its functions (19,24-29). Recruitment of TRIM28 by KRAB-ZNFs enhances its sumoylation and gene repression activity (30). Phosphorylation and sumoylation of TRIM28 are critical for modulating the increase in production of type I interferons and proinflammatory cytokines during infection with influenza virus (31,32). The acetylation of lysine residues in histones, certain transcription factors, chromatin remodelers, metabolic enzymes, and many regulatory proteins is another widely recognized example of posttranslational modification that significantly alters protein function and cellular regulation. TRIM28 can undergo acetylation in multiprotein complexes that consist TRIM28 and its associated proteins such as NuRD, HP1, SETDB1, KRAB-ZNFs, and DNMTs (33). Although acetylation of histones is widely known to play key roles in regulating gene expression (34-36), the impact of TRIM28 acetylation on its activity remains poorly understood. To address this dearth of knowledge, we targeted three acetylation sites of human TRIM28 (K266, 304 and 340) or mouse Trim28 (K267, 305 and 341) in the coiled-coil domain. Upon mutation of the individual Trim28 lysines to Gln or Arg, only K305Q was compromised with respect to the interaction with KRAB-ZNFs. The gene expression ensemble of the erythroleukemic cell line K562 harboring an endogenous mutation of K304Q differed considerably from that of the parent K562 line. We propose that TRIM28-K304Q could be used as an acetylation mimic for systematic investigation of the

potential effects of acetylation/deacetylation on phenotypic changes at both the cellular and organismal levels in a spatiotemporal manner.

Results

The acetylation mimic TRIM28-K304Q has relatively weak interaction with the KRAB domain

Acetylation of human TRIM28-K266, -K304 and -K340 (equivalent to mouse Trim28-K267, -305 and -K341) in the RBCC region has been identified by MS (33). We produced the acetylation-mimicking mutants of mouse Trim28, namely K267Q, K305Q and K341Q, as well as acetylation-defective mutants K267R and K305R. These constructs were expressed in human embryonic kidney 293T cells to test their interaction with Gal4 DNA-binding domain (Gal4^{DBD})-KRAB and KRAB-ZNF. In a pull-down analysis, only K305Q, but not K267Q or K341Q, had decreased interaction with recombinant Gal4^{DBD}-KRAB (Fig. 1A). Co-IP of recombinant FLAG-tagged Trim28-K267Q, -K267R, -K305Q or -K305R and hemagglutinin (HA)-tagged ZFP57 also demonstrated that K305Q had impaired interaction with recombinant ZFP57 (Fig. 1B). These results may reflect the binding of the KRAB domain by steady-state dimers formed between recombinant and endogenous TRIM28s: K305Q-K305Q, K304-K305Q, and K304-K304 (TRIM28-K304 is endogenous TRIM28). It is likely that both the K305Q homodimer and K304-K305Q heterodimer contributed to the decreased binding to the KRAB domain. In 293T cells, wild-type HA-Trim28 was co-expressed with wild-type or FLAG-K267R, -K267Q, -K305R, or -K305Q. The protein complexes brought down by HA beads were detected by anti-FLAG (Fig. S1A). These results indicated that these mutants can associate with wild-type Trim28. 293T cells were transfected with mouse FLAG-Trim28-K305Q, followed by IP and LC-MS/MS analysis, which identified a specific peptide (residues 32–69) from human TRIM28 (Fig. S1B). The functional luciferase assay demonstrated that K304Q mutant had decreased transcription-suppressor activity (Fig. 1C, upper panel). Western blotting revealed similar protein abundance in each reaction (Fig. 1C, lower panel). These results suggested that the acetylation mimic Trim28-K305Q had decreased interaction with the KRAB domain, both physically and functionally.

Generation of TRIM28-K304Q and TRIM28 knockout K562 cells by CRISPR/Cas9-mediated genomic editing

To explore the physiological consequences of TRIM28 acetylation, we analyzed TRIM28 acetylation in the human leukemia cell line K562 by IP with an antibody against N-terminal TRIM28 (Fig. S2A) followed by LC-MS/MS analysis. Several lysines in the coiled-coil domain located at the same heptad position were acetylated, including K304 (Fig. S2B). To investigate the function of TRIM28 acetylation, human TRIM28-K304Q was knocked-in to K562 cells (Fig. 2A). A SacI restriction site was inserted for genome examination. Several possible clones were further checked by droplet digital PCR (ddPCR)(Fig. S3A). Clones Q1 and Q2 each had three K304Q alleles. After confirmation via Sanger sequencing and western blotting, only Q1 was the correct K304Q knock-in clone, and Q2 lacked TRIM28 expression owing to deletion of certain sequences (Fig. 2B, Fig. S3B). The TRIM28 protein from Q1 was immunoprecipitated with anti-TRIM28-N followed by LC-MS/MS. The TRIM28 sequence coverage was 84%, and the K304Q

mutation was confirmed (Fig. 2C). To further study TRIM28 function, we created TRIM28 knockout (KO) K562 cells using a pair of single guide RNAs (sgRNAs) and Cas9 (Fig. S4A). A clone with homozygous knockout was confirmed by quantitative ddPCR (Fig. S4B). A K562 cell proliferation assay with wild-type TRIM28, K304Q, and TRIM28-KO cells revealed that K304Q cells grew slower than the wild-type cells, and TRIM28-KO cells grew very slowly (Fig. S4C). The sensitivity of these cells to each of the two histone deacetylase (HDAC) inhibitors SAHA (suberoylanilide hydroxamic acid) and imatinib was analyzed by trypan blue staining, which revealed that wild-type cells were more sensitive to imatinib than the other two mutant cells. These results were consistent with the relatively higher proliferation rate of wild-type cells compared with the mutants. TRIM28-KO cells were more sensitive to SAHA than either the wild-type or K304Q cells (Fig. S4D). TRIM28-K304Q cells became attached cells, whereas the wild-type cells remained unattached (Fig. S4E). Trim28-KO cells were transfected with wild-type Trim28, Trim28-K305Q, -K305R, or -R310Q, and the interaction between each Trim28 protein and GAL4^{DBD}-KRAB domain was assessed via IP. The interaction between the GAL4^{DBD}-KRAB domain and Trim28-K305Q was substantially lower than that of wild-type Trim28 (Fig. S4F).

Hemoglobin gene expression in TRIM28-K304Q mutant K562 cells

TRIM28 contributes to the regulation of genetic imprinting through its binding to KRAB-ZNFs (14). TRIM28 is also involved in regulating erythroid cell maturation (37-41). The expression of each of the HBE (ϵ -globin) and HBG (γ -globin) genes was analyzed by quantitative PCR (qPCR; Fig. 3A). Interestingly, compared with wild-type cells, their expression was moderately increased in K304Q cells and highly increased in TRIM28-KO cells. To further demonstrate that TRIM28 represses HBE and HBG expression, we performed ChIP of the LCR (Locus Control Region) of the globin gene (i.e., HS (hypersensitive site)), HBE and HBG promoters. The results clearly showed reduced binding of TRIM28-K304Q to each of the HS3, HBE, and HBG promoters (Fig. 3B).

RNA-sequencing of wild-type, TRIM28-K304Q and TRIM28-KO K562 cells

To assess global gene expression in wild-type, TRIM28-K304Q, and TRIM28 KO cells, we performed RNA sequencing (RNA-seq). The K304Q and KO cells had similar gene expression profiles, but each profile differed from that of wild-type cells (Fig. 4A). In K304Q cells, 442 genes were upregulated and 491 downregulated compared with wild-type cells (Fig. 4B). A Gene Ontology (GO) analysis of K304Q and KO cells revealed the upregulation of DNA-binding proteins such as ZNFs (Fig. 4C and Table S1). Interestingly, the megakaryocytic repressor IKAROS family transcription factors IKZF2 and IKZF3 and the paternally imprinted genes PEG3 and DLK1 were upregulated, and the embryonic and fetal globin repressor SOX6, the maternally imprinted gene MEG3, and melanoma antigen family members MAGEC1, MAGEC2, MAGEB1 and MAGEA1 were downregulated (Table S1). To further verify the up- or downregulation of these genes, we performed RT-qPCR (Fig. 4D), which confirmed the changes in expression of certain genes identified by RNA-seq of TRIM28-K304Q cells. The imprinting gene that encodes the long noncoding RNA H19 was significantly upregulated in TRIM28-K304Q cells, although H19 was not listed in RNA-seq data. Furthermore, the TRIM28-K304Q-induced perturbation of ZNF

expression highlights the important roles of dynamic regulation of acetylation of TRIM28 (as exemplified by the acetylation mimic TRIM28-K304Q) on gene expression.

KEGG pathway analysis revealed significant changes in gene expression pertaining to pathways such as platelet activation, thyroid hormone signaling, and miRNAs in cancer and PI3K/AKT signaling (Fig. 5A). Integrin $\beta 3$ is involved in these pathways, and we verified the upregulation of its mRNA and protein in TRIM28-K304Q cells (Fig. 5B). Fibronectin, which interacts physically with integrin $\beta 3$, was also highly upregulated in K304Q cells (Fig. 5B), which might explain the attached phenotype of K304Q cells (Fig. S4D). Five upregulated and five downregulated genes in K304Q cells were chosen to highlight their roles in the differentiation potential of TRIM28-K304Q K562 cells (Table 1) (42-52). The expression of each of leukemia inhibitory factor receptor (LIFR), activin A receptor (ACVR1C), and inhibin beta E (INHBE) was verified by RT-qPCR (Fig. 5C). Their expression levels in wild-type and TRIM28-K304Q cells were consistent with the RNA-seq results, and LIFR and ACVR1C levels increased whereas INHBE level decreased in TRIM28-K304Q cells. Interestingly, drugs such as the DNMT inhibitor decitabine and HDAC inhibitor SAHA, were used to induce K562 cell differentiation into erythrocytes, resulting in upregulation of LIFR and ACVR1C mRNAs and repression of INHBE mRNA (Fig. 5D). Several ZNFs, e.g., ZNF382, ZNF527, ZNF568, ZNF667, and ZNF829, that were upregulated in TRIM28-K304Q cells were also induced by decitabine in wild-type K562 cells (Fig. S5). Induction of HBE, HBG, and H19 expression by decitabine in wild-type K562 cells is shown in Fig. 5E. Taken together, these results suggested that TRIM28-K304Q-induced or decitabine-induced fetal globin expression in K562 cells may converge on the expression or repression of downstream soluble mediators or transcription factors. The induction of certain ZNFs by TRIM28-K304Q may in fact alter the methylation of promoters/enhancers for ZNF genes and thereby perturb the output of ZNF networks.

Identification of proteins that interact with TRIM28 and TRIM28-304Q

To identify proteins that interact with each of TRIM28 and TRIM28-304Q, we performed IP experiments using anti-TRIM28-N-conjugated protein A beads. The bead-bound TRIM28 or TRIM28-K304Q was washed and eluted with the antibody-generating peptide, and each eluent was analyzed by MS. The majority of TRIM28-interacting proteins were ZNFs; among them, 88 interacted with TRIM28, 54 interacted with both wild-type and TRIM28-K304Q, and 7 interacted with TRIM28-k304Q (Table 2). A number of non-ZNFs that also were specific wild-type TRIM28-interacting proteins were also identified (Table S2), including epigenetic modifiers (EHMT1, EHMT2), protein ubiquitination proteins (GID/CTLH complex), RNA modification factors (YTHD2, ZCCHC4, FBRL), RNA metabolism factors (NEXT complex: MTREX, ZCCHC8, RBM7; mRNA decaying enzyme: EDC4), and a translation regulation factor (GCN1). Both wild-type and TRIM28-K304Q could associate with HP1s (CBX1, CBX3, CBX5), CENPV (centromere protein V), histones (H4, H2B2F, H2B1B; WT-TRIM28-specific: H3.3, H2A1d, H2AV; k304Q-specific: H3.1, H2A1B), RNA-binding and processing proteins (HNRNP M, HNRNP H, HNRNP F, PABP1, PABP4, REXO5, DHX9), and the transcription factor GATA1 (Table S2). These results suggested that wild-type-TRIM28 participates in methylation of each of H3K9 and m6A RNA as well as the nuclear exosome decay pathway, whereas the K304Q mutant retains its ability to interact with HP1 and some RNA-binding

proteins. In addition, we also analyzed the phosphorylation sites of wild-type and TRIM28-K304Q (Table S3). The phosphorylated S473 and S824 were detected in K304Q mutant, indicating the phosphorylation was not affected by K304Q modification.

TRIM28-K304Q mutant interacts relatively weakly with ZNF445 to increase H19 expression

To explore the mechanism by which TRIM28-K304Q regulates H19 expression, we performed ChIP using anti-TRIM28-N and anti-ZNF445. TRIM28 and ZNF445 were enriched at the H19 promoter in wild-type K562 cells but not in K304Q mutant cells (Fig. 6A). TRIM28-associated proteins were analyzed by LC-MS/MS; one such protein was the ICR (imprinting control region)-binding protein, ZNF445 (Fig. 6B, Table 2S, panel A). Co-IP demonstrated that TRIM28-K304Q interacted weakly with ZNF445 (Fig. 6C). Knockdown of ZNF445 in wild-type K562 cells activated H19 expression (Fig. 6D) and decreased the enrichment of TRIM28 at the H19 promoter and the LCR-HS2 (Fig. 6E). These results suggested that the association of TRIM28 with ZNF445 is at least partially responsible for repressing H19 expression and that the weakened interaction between TRIM28-K304Q and ZNF445 can derepress H19 expression.

Discussion

Lysine acetylation is a reversible posttranslational modification of proteins that may regulate their interactions with other macromolecules. Lysine acetylation targets protein complexes such as those containing TRIM28 and others involved in chromatin remodeling, DNA replication, transcription, posttranscriptional mRNA processing, intermediary metabolism and protein turnover (33,36). For example, lysine acetylation within the core histone tail domains has been shown to inhibit nucleosome formation, an effect likely related to its role in facilitating transcription. Experimentally, the effects of acetylation have been studied using recombinant mutants in which lysine residues are substituted with glutamine as a mimic of acetylated lysine, or with arginine as a mimic of unacetylated lysine. The presence of an acetylation-mimic residue within the tail domains of H2B and H4 substantially inhibits self-association and hence nucleosome formation, whereas an acetylation-mimic residue in the H3 tail affects the stability of wrapped DNA within individual nucleosomes (34,35). These studies underscore the validity of the acetyl-lysine mimic, TRIM28-K304Q, for studying TRIM28 activities in vivo. How is the interaction between TRIM28 and KRAB-ZNFs regulated in vivo? One possibility is via the acetylation of TRIM28-K304 as we have demonstrated in our present study. Our observation of reduced binding of the KRAB domain by TRIM28-K304Q, which was not the case for either mutant K266Q or K340Q, suggests that the specific acetylation of K304 may impact the interaction between the KRAB domain and the TRIM28 homodimer (dimerization is mediated by the RBCCs). Molecular modeling failed to pinpoint the interaction at atomic resolution based on published structural studies (6,7,53,54) and our unpublished observations. As such, further structural studies are needed. It is possible that the unstructured C-terminal 23 residues (813–835) or sumoylation of TRIM28 may influence the interaction. Our present results suggest that acetylation/deacetylation of TRIM28-K304 may regulate its interaction with KRAB-ZNFs in a spatiotemporal manner.

TRIM28 regulates the reprogramming of induced pluripotent stem cells and differentiation of pluripotent stem cells. TRIM28 interactions with KRAB-ZNFs alter DNA methylation patterns in addition to H3K9me3 to facilitate gene repression during reprogramming. Interaction between TRIM28 and certain pluripotency-associated ZNFs such as ZNF114, ZNF483 and ZNF589/SZF1 maintains pluripotency of induced pluripotent stem cells. Disruption of any of these interactions causes the pluripotent cells to undergo differentiation (55). Consistent with these results, our present data suggest that TRIM28 interacts more strongly with ZNF589/SZF1 than does TRIM28-K304Q (Table 2). These results are consistent with our finding that TRIM28-K304Q-K562 cells undergo differentiation whereas the parent cells do not.

In addition to the widely recognized co-repressor function of TRIM28 for KRAB-ZNFs, TRIM28 can also interact with SMARCAD1 (SWI/SNF-related, matrix-associated actin-dependent regulator of chromatin, subfamily A, containing DEAD/H box 1) through the CUE (coupling of ubiquitin conjugation to ER degradation) domain of SMARCAD1 (15,56). SMARCAD1 not only binds to and regulates TRIM28 target genes in embryonic stem cells but also appears to compete with KRAB-ZNFs for interaction with TRIM28 (15). Further investigation of both the distinct and overlapping roles of SMARCAD1-TRIM28 and KRAB-ZNFs-TRIM28 or KRAB-ZNFs-TRIM28-K304Q could illuminate their roles in gene specificity and modes of regulation. Interaction between SMARCAD1 and TRIM28-K304Q with the consequent effect(s) on gene expression remains an interesting issue.

During the development of human erythrocytes, there is a switch from expression of the embryonic ϵ -globin gene to the fetal γ -globin gene in utero, and the γ -globin gene is silenced postpartum as β -globin gene expression becomes predominant. BCL11A and SOX6 cooperate to silence γ -globin expression in adult human erythroid progenitors (50). Consistent with these results and the increased expression of γ -globin in TRIM28-K304Q K562 cells, SOX6 expression was found to be reduced in these cells. Using a recombinant construct of BCL11A for tandem affinity chromatography in murine erythroleukemia cells and K562 cells, TRIM28 was found to be among the many BCL11A-associated proteins identified (57). The role of TRIM28 in the context of associating with BCL11A has not been addressed. Interestingly, inducible deletion of *trim28* in adult mouse hematopoietic cells revealed that Trim28 is essential for the cell-autonomous development of immature erythroblasts in bone marrow (40). Trim28 is recruited by the orphan nuclear receptor TR2/TR4 (NR2C1/NR2C2) heterodimer to repress embryonic/fetal globin gene expression. Exactly how the activity of TRIM28 is regulated in vivo to facilitate repression or activation of embryonic and fetal globin genes remains unclear. The observed decreased interaction between TRIM28-K304Q and KRAB-ZNFs suggests that K304 is vital for the TRIM28-mediated modulation of embryonic and fetal globin genes. Alternatively, TRIM28-K304Q may have failed to be recruited by TR2/TR4 in our experimental system.

It has been reported that maternal TRIM28 not only maintains DNA methylation at imprinted regions in the germline during early genome-wide reprogramming but also regulates DNA methylation at imprinted gene promoters after reprogramming (23). The long noncoding RNA H19 has a role in the negative regulation of body weight and cell proliferation in a mouse model (58). H19 also plays a role in the regulation of the imprinted gene network (58). In our present study, H19 RNA was upregulated in TRIM28-

K304Q or TRIM28- KO K562 cells, and the upregulation correlated with the propensity of mutant K562 cells to differentiate. Consistent with a previous report (59), our results showed that, compared with wild-type TRIM28, TRIM28-K304Q had a weaker interaction with ZNF445, leading to upregulation of H19 expression (Fig. 6). In addition, TRIM28-mediated hypomethylation of the differentially methylated region (DMR) of H19/IGF2:IG-DMR (i.e., ICR1) might result in the decreased paternal expression of IGF2 and increased maternal expression of H19 (60). We have confirmed that H19 RNA is dramatically induced by a DNMT inhibitor (Fig. 5E). In contrast to H19, the imprinted gene MEG3 was downregulated in both TRIM28 KO and TRIM28-K304Q K562 cells. It is known that knockdown of TRIM28 results in differential expression of H19 and Gtl2 (Meg3) in sheep fibroblasts (61). However, MEG3 expression is low or undetectable in multiple types of primary tumors and cancer cell lines (62-65). Mechanistic studies have suggested a role for Meg3 in epigenetic regulation by interacting with the chromatin-modifying PRC2 complex, guiding this complex to genomic sites via DNA-RNA triplex formation (66-68). How does TRIM28-K304Q downregulate MEG3 expression? Upregulation of H19 may negatively affect the expression of MEG3 through a noncoding RNA regulatory network.

Conclusions

K562 cells in culture undergo spontaneous differentiation when the cells reach relatively high density. Their differentiation can also be induced by drugs such as HDAC inhibitors (e.g., SAHA and sodium butyrate), DMSO, hemin, rapamycin, imatinib, 5-aza-cytidine/decitabine, and ribavirin (73, and unpublished observations). HDAC inhibitors may promote the opening of chromatin in the region of the fetal globin gene, thereby inducing its expression. The connections between our present results for TRIM28-K304Q-induced and decitabine-triggered differentiation are likely a consequence of the hypomethylation of fetal and embryonic globin enhancers or promoters despite the apparent mechanistic differences, i.e., TRIM28-K304Q has reduced binding affinity for KRAB-ZNFs and DNMTs whereas decitabine inhibits DNMTs. Additionally, the acetylation-mimic TRIM28-K304Q-mediated regulation of ZNFs and differentiation-related genes (Table1) correlates with decitabine-induced genes in K562 cells (Fig. 5). This suggests that TRIM28 acetylation is involved in the differentiation of K562 cells. Further insights into the convergence or divergence of mechanisms of drugs capable of inducing fetal and embryonic globin expression may have therapeutic implications.

Abbreviations

ChIP: Chromatin-immunoprecipitation; CRISPR/Cas: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein nuclease; DMR: Differentially methylated region; DNMT: DNA methyltransferase; ddPCR: droplet digital PCR; GID/CTLH complex: Glucose-induced degradation/C-terminal to LisH complex; HDAC: Histone deacetylase; ICR: Imprinting Control Region; IP: Immunoprecipitation; KRAB-ZFP: Krüppel-associated box zinc-finger protein; KI: knock-in; KO: knock-out; LCR: Locus Control Region; MS: Mass Spectrometry; RT-qPCR: Reverse transcription-quantitative Polymerase Chain Reaction; SAHA: Suberoylanilide hydroxamic acid; TRIM28: Tripartite Motif protein 28.

Methods

Cell lines and plasmids

Human erythroleukemic K562 cells were purchased from American Type Culture Collection (ATCC, CCL-243) and cultured in RPMI 1640 medium supplemented with 15% fetal calf serum (Gibco), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco) at 37°C with 5% CO₂ in a humidified incubator. Human embryonic kidney 293T cells were purchased from ATCC (CRL-3216) and cultured in Dulbecco's modification of Eagle medium with 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Gibco) at 37°C with 5% CO₂ in a humidified incubator. The mouse FLAG-Trim28 expression plasmid was constructed as described (27). The EcoRI-Sall fragment from FLAG-Trim28 was ligated into pCMV-HA-N (Clontech) for HA-Trim28 expression. The TRIM28 mutants were generated with the Q5 Site-Directed Mutagenesis kit (NEB) and primers shown in Table S4A. HA-ZFP57 was cloned via PCR and the sequence confirmed. The ZNF445-DDK-Myc expression plasmid was purchased from OriGene. The GAL4^{DBD}-KRAB expression vector and 5×GAL4^{DBS}-E1bTATA-luciferase reporter were kindly obtained from Dr. Frank J. Rauscher, III.

CRISPR-mediated KO of TRIM28 and generation of mutant TRIM28-K304Q in K562 cells

TRIM28 KO was performed by nucleofection of a Cas9 ribonucleoprotein (RNP). Cas9 sgRNAs, targeting *TRIM28*, were designed using the CRISPR Design Tool at www.benchling.com. Only sgRNAs with high predicted off-target scores (more precise) were selected. The sgRNAs were synthesized by in vitro transcription and purified by denaturing PAGE as described (69). Each purified sgRNA was refolded into a functional structure in buffer containing 20 mM HEPES pH 7.5, 150 mM KCl, 10% glycerol, 1 mM 2-mercaptoethanol, and 1 mM MgCl₂.

The *TRIM28-K304Q* mutant was produced by co-nucleofection of Cas9 RNP and a synthetic DNA repair template for homology-directed repair. A 1490-nt wild-type sequence of TRIM28, including the K304 site, were amplified by PCR using the following primer sets: TRIM28 genomic DNA (gDNA) forward primer 5'-CTCTACATCTTCCCAATAAATGGCCCAGTG-3' and reverse primer 5'-TGTGAACAAAGCAGAACCTCTGCCTCAGT-3'. The PCR reaction contained 200 ng genomic DNA and Taq polymerase. The PCR DNA fragment was ligated into pGEM-T Easy (Promega) to construct a pGEM-T Easy/hTRIM28 wild-type plasmid.

Site-directed mutagenesis was then performed to introduce the K304Q mutation in the plasmid using the primer set of the Q5 Site-Directed Mutagenesis kit (New England Biolabs) and primer sets (hTRIM28-K304Q site-directed forward 5'-GCTCAATAAGCGGGGCCGTGTG and reverse 5'-TCCTGCATGATCTGCAGGATGGCC). The resulting plasmid, pGEM-T Easy/hTRIM28-K304Q, carried the K304Q (AAG>CAG) mutation as well as a nearby silent mutation encoding a SacI restriction sequence for screening. The DNA repair template was amplified from the plasmid by PCR using the primer sets K304Q homology-directed repair (HDR) template forward 5'-CTACCTAGCCTGACCTGCTGTG and reverse 5'-CTCACCCCAGACAGCATCATCA. Recombinant Cas9 was purified as described (DOI: 10.1002/cpmb.43).

Cas9 RNP was prepared by incubating purified recombinant Cas9 and sgRNA at 1:1.2 molar ratio at 37°C for 10 min. The DNA repair template was then added to the RNP mixture. Nucleofection of human CML K562 cells was performed in a Lonza 4D Nucleofector system using SE Cell Line 4D-Nucleofector™ kit and FF-120 pulse (Lonza). After nucleofection, the cells were incubated at 37°C for 48 h, and single cells were sorted by a BD FACSJazz automated cell sorter at the Flow Cytometry Core Facility of the Institute of Biomedical Sciences, Academia Sinica. The same primer set (TRIM28 gDNA primers) that is complementary to the flanking regions of the HDR region was used to amplify the target region of individual single clones. The co-integrated SacI restriction site was used to screen for the insertion of the K304Q mutation.

Antibodies, preparation of extracts, co-IP, and western blotting

Cells were harvested and washed one time with PBS and lysed with whole-cell extract buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM DTT, protease inhibitor, and phosphatase inhibitor). Cell lysates were shaken at 4°C for 30 min and centrifuged at 12,000×*g*, 4°C for 5 min. The extracts were pre-cleared with protein G agarose (Sigma Aldrich) for 1 h at 4°C and immunoprecipitated with anti-FLAG M2 agarose (Sigma Aldrich) or anti-HA agarose (Sigma Aldrich) for 2 h at 4°C. Unbound proteins were removed by washing three times with whole-cell extract buffer. SDS-PAGE sample buffer (diluted 1:4) was added to each agarose bead sample, with subsequent heating at 100°C for 5 min, SDS-PAGE, semi-dry transfer to a polyvinylidene difluoride membrane (Millipore), and western blotting with appropriate primary antibodies and horseradish peroxidase–conjugated secondary antibodies. The immunocomplexes were visualized by enhanced chemiluminescence (Western Lightning, PerkinElmer Life Sciences) with subsequent exposure to x-ray film (FUJIFILM Corp.). The primary antibodies used were specific for the following proteins: ZNF445 and HA (Bethyl Laboratories), integrins and β-actin (Cell Signaling Technology), Gal4 (Santa Cruz Biotechnology), FLAG (mouse; Sigma Aldrich), and FLAG (rabbit; (70)). Rabbit anti-human TRIM28 was generated by immunizing rabbits with a peptide encompassing residues 14–43 (anti-TRIM28-N). The antibody was purified with a peptide-agarose affinity column. Monoclonal anti-human TRIM28 (clone 20A1) was produced as described (27) and obtained from Biolegend. The secondary antibodies were goat anti-mouse (KPL, 474-1806) and goat anti-rabbit (KPL, 474-1516).

Identification of associated proteins from immunoprecipitated TRIM28 and TRIM28-K304Q

Peptide identification by MS was performed by the Mass Spectrometry Common Facility at the Institute of Biological Chemistry, Academia Sinica, using a LTQ-Orbitrap Velos system (Thermo Fisher). Data interpretation and correlations between the spectra and amino acid sequences within a human EST database and customized FLAG-Trim28 sequence were analyzed using Mascot (Matrix Science software package).

RNA-sequencing

Total cellular RNA was extracted using TRIzol Reagent (Invitrogen). Total RNA of each sample was quantified with a 2100 Bioanalyzer (Agilent Technologies), NanoDrop (Thermo Fisher Scientific), and 1% agarose gel electrophoresis. Total RNA (1 µg) with a RNA integrity value >6.5 was used for library preparation. Libraries were prepared for next-generation sequencing according to the manufacturer's protocol (Agilent Technologies). Poly(A) mRNA was isolated using the Poly(A) mRNA Magnetic Isolation Module or rRNA removal kit (New England Biolabs). The mRNA was fragmented and primed using First Strand Synthesis Reaction Buffer (New England Biolabs) and random primers. First-strand cDNA was synthesized using ProtoScript II Reverse Transcriptase (New England Biolabs), and second-strand cDNA was synthesized using the Second Strand Synthesis Enzyme Mix (New England Biolabs). Bead-purified double-stranded cDNA was then treated with End Prep Enzyme Mix to repair both ends, and dA-tailing was carried out in the same reaction, followed by T-A ligation to add adaptors to both ends. Size selection of adaptor-ligated DNA was then performed using beads, and fragments of ~420 bp (insert size ~300 bp) were recovered. Each sample was then amplified by PCR for 13 cycles using primers P5 and P7, each of which carried sequences that could anneal with the flowcell to perform bridge PCR; P7 carried a six-base index to allow multiplexing. The PCR products were cleaned up using beads, validated using a Qsep100 (Bioptic), and quantified with a Qubit3.0 fluorometer (Invitrogen). Then, libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument (Illumina). Sequencing was carried out using a 2 × 150-bp paired-end configuration, and image analysis and base calling were conducted with HiSeq Control Software (HCS)+ OLB +GAPipeline-1.6 (Illumina) on the HiSeq instrument.

Lentivirus-mediated gene knockdown

Lentiviral vector-mediated short hairpin RNA technology was used for *ZNF445* knockdown. Two human *ZNF445* short hairpin RNA target sequences, namely GCGCTATAAATGTAATCTATG (TRCN0000415362) and ATCAAACCTTTACTCGTCATAT (TRCN0000435973), and scrambled control pLKO.1-shLuc (TRCN0000072243) were obtained from the National RNAi Core Facility at Academia Sinica. Viruses were produced using calcium phosphate-mediated transfection. Early subcultures of 293T cells were co-transfected with 14 µg pPGK-GFP, pLKO.1-shLuc, or pLKO.1-sh*ZNF445* and 14 µg pCMVΔR8.91 and 2 µg pMD.G. After 8 h, the medium was replaced with K562 cell maintenance medium to collect virus. Then, K562 cells in a 6-well plate were infected with viral supernatants in the presence of 8 µg/ml polybrene for 48 h and further selected with 3 µg/ml puromycin for 1 week. Knockdown efficiency was determined based on RT-qPCR and western blotting.

Reverse transcription-quantitative PCR

K562 cells in a 6-cm dish were harvested, and total RNA was prepared with TRIzol reagent (1 ml per dish). After quantification by measuring absorbance at 260 and 280, 2 µg RNA was treated with DNase I (Invitrogen) and then reverse transcribed into cDNA using Superscript IV (Invitrogen). The quantitative PCR (qPCR) was performed with the Corbett Research RG-6000 Real Time PCR Thermocycler (Qiagen). The total volume was 20 µl including QuantiNova SYBR Green master mix (Qiagen), 20-fold diluted cDNA, and 0.3 µM each of the forward and reverse primers (Table S4B). The amplification conditions were 60

cycles of 95°C for 10 s and 60°C for 15 s. The results were analyzed by the $2^{-\Delta\Delta C_t}$ relative quantitation method. All experiments were independently repeated three times.

Chromatin-immunoprecipitation (ChIP)

K562 cells were cultured to a density of 2×10^6 /ml and harvested for crosslinking with 1% formaldehyde in medium at room temperature for 10 min. Glycine was added (final concentration, 125 mM) to quench unreacted formaldehyde for 5 min. Cells were pelleted by centrifugation, and the pellet was washed with 5 ml cold PBS. Cells (2×10^7) were lysed in 400 μ l lysis buffer (5 mM HEPES pH 8.0, 85 mM KCl, 0.5% (v/v) NP-40, and protease inhibitor cocktail) on ice for 15 min, and the cell suspension was mixed gently every 5 min. Nuclei were collected by centrifugation at $9000 \times g$ for 5 min at 4°C, resuspended in 200 μ l nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, and protease inhibitor cocktail), and kept on ice for 10 min. The nuclear lysates were sonicated using the middle setting of an ice-water Bioruptor (Diagenode) for 10–15 min total (30 s on, 30 s off). The resulting sheared chromatin (2 μ l) was resolved by electrophoresis through a 2% agarose gel to check that the length of DNA fragments was 200–600 bp. Each sheared chromatin sample was centrifugated at $12,000 \times g$ for 5 min at 4°C, and the supernatant was diluted 10-fold with buffer containing 20 mM Tris-HCl pH 8.1, 167 mM NaCl, 0.01% SDS, 1% Triton X-100, 1 mM EDTA, and protease inhibitors. Each chromatin complex sample was divided into two equivalent volumes for IP with either normal IgG or anti-TRIM28-N. After incubating with antibody at 4°C overnight, protein A/G magnetic beads (20 μ l) were added for an additional 2 h. The beads were washed one time each with 0.5 ml of each of following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% (v/v) IGEPAL CA630, 1% deoxycholic acid–sodium salt, 1 mM EDTA, 10 mM Tris pH 8.1), and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Each wash step was done for 5 min on a rotating platform at 4°C. Chromatin complexes were eluted with 100 μ l elution buffer (1% SDS, 0.1 M NaHCO₃, containing 1 μ l proteinase K) for 2 h at 62°C with mixing, with subsequent incubation at 95°C for 10 min to de-crosslink the chromatin complexes. Samples were cooled to room temperature, and the supernatant was transferred to a new tube. After extraction by phenol/chloroform, the DNA was precipitated by ethanol and analyzed by qPCR with specific primers (Table S4C).

Statistical analysis

All data are presented as the mean $\pm$ SD of at least three independent experiments. Statistical significance (* $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$) was determined by the one-tailed Student's t-test.

Declarations

- Ethics approval and consent to participate

Not applicable.

- Consent for publication

Not applicable.

- Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

- Competing interests

The authors declare that they have no competing interests.

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- Authors' contributions

YJC and SL designed and performed the CRISPR/Cas9 genome editing experiments and produced Fig.1B. ZK performed RNA-seq and produced Fig. 3 and Fig. 5. BJS and WHT purified anti-TRIM28-N and performed IP for LC-MS/MS analysis. WCC produced Fig. 6D and 6E. HPL produced Fig. 1C. SWL expressed and purified recombinant proteins. SYL performed LC-MS/MS. SJC performed quantitative ddPCR. CWL produced Fig. 1A. YJH performed qPCR presented in Fig. 4D. HHW provided study materials. CJC conceived and coordinated the study and wrote the paper. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Impaired interaction between Trim28-K305Q and KRAB domain-containing proteins. (A) Pull-down analysis of K267Q, K305Q, and K341Q of TRIM28 with Gal4DBD-KRAB. Each Trim28 protein contained an N-terminal FLAG tag, and each was expressed in 293T cells and immunoprecipitated by anti-FLAG agarose. Each bead-bound FLAG-Trim28 protein was then incubated with extract of 293T cells that overexpressed Gal4DBD-KRAB. The pulled-down protein complexes were analyzed by western blotting with antibodies against FLAG and Gal4. WT, wild type. (B) Co-IP of HA-ZFP57 with each of WT Trim28, K267Q, K267R, K305Q, and K305R. (C) Luciferase reporter assay by cotransfecting 293T cells with 5×Gal4DBS-E1bTATA-driven luciferase and Gal4DBD-KRAB and WT or mutant TRIM28 K305Q (Q) or K305R (R) expression plasmids. *P < 0.05. Lower panel: western blotting with antibodies against FLAG and Gal4.

Figure 2

Generation and characterization of TRIM28-K304Q knock-in in K562 cells. (A) Sequences of target, sgRNA, and homology-directed repair (HDR) donor DNA for TRIM28-K304Q knock-in. (B) mRNA and protein expression of knock-in clones Q1 and Q2. RNAs and proteins were isolated from wild-type (WT) and TRIM28-K304Q knock-in clones for qPCR analysis and western blotting with anti-TRIM28 (monoclonal, 20A1), respectively. (C) MS analysis of TRIM28-K304Q knock-in cells. A 304Q-containing peptide (residues 297–308) was identified. (D) Amino-acid sequence confirmed by MS.

A **B** **Figure 3**

Differential expression of HBE and HBG upon expression of TRIM28 or its mutants. (A) qPCR analysis of globin gene expression in wild-type (WT), K304Q and TRIM28-KO cells. Total RNA from K562 cells was isolated and used for RT-qPCR to assess the expression of γ -globin (HBG) and ϵ -globin (HBE). (B) WT, but not TRIM28-K304Q, enrichment in a specific regulatory region. ChIP was performed with anti-TRIM28-N and normal IgG in WT and TRIM28-K304Q. The precipitated genomic DNA was analyzed by qPCR with the indicated primers from gene regulatory regions. The results are represented as fold-enrichment using the signal of anti-TRIM28-N normalized by the signal of normal IgG. Results represent the mean $\pm$ SD of data from two independent experiments, each with triplicate reactions. ***P < 0.001.

Figure 4

RNA-seq analysis. (A) RNA-seq correlations. The Pearson correlation coefficient indicates the degree of linearity between two variables. The greater the absolute value (ranging between 0 and 1), the stronger the linear relationship. Wild-type (WT): K562; MUT-KI: TRIM28-K304Q knock-in; MUT-KO: TRIM28 KO. (B) Differential expression volcano plot. Red and blue dots represent genes that were significantly upregulated or downregulated, respectively. x axis: log2 fold-change of gene expression. y axis: statistical

significance of the differential expression in terms of log10(P-value). (C) GO enrichment histogram. The number of differentially expressed genes in each GO term is shown along with the specification of the relevant biological process, cellular component, or molecular function. Shown are the top 30 most prominent GO categories. Upper: P-value histogram; lower: number of differentially expressed genes in each GO category. (D) RT-qPCR assays verified the identity of certain ZNFs, imprinting genes, and transcription factors in WT and TRIM28-K304Q KI cells. *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant.

TRIM28-mediated pathways. (A) KEGG enrichment histogram. x axis: gene number. y axis: pathway term. (B) Integrin- β 3 expression in wild-type (WT) and K304Q KI K562 cells. (C) qPCR analysis of LIFR, ACVR1C, and INHBE in WT and TRIM28-K304Q cells. (D) LIFR, ACVR1C, and INHBE mRNA expression levels in decitabine-treated or SAHA-treated K562 cells. (E) qPCR analysis of HBE, HBG and H19 RNAs in WT K562 cells treated with decitabine. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Interaction between TRIM28 and ZNF445 inhibits H19 gene expression. (A) ChIP by anti-TRIM28 and anti-ZNF445 and analyzed with H19 promoter in wild-type (WT) and TRIM28-K304Q K562 cells. (B) Schematic presentation of ZNF445. It contains SCAN domain, KRAB domain and 14 Zinc fingers. (C) Immunoprecipitation analysis. TRIM28-K304Q had decreased interaction with ZNF445. The asterisk indicates a non-specific signal. The red arrow indicates the specific ZNF445 band. (D) Knockdown of ZNF445 in K562 cells increases H19 expression. Left panel: western blotting confirmed ZNF445 knockdown. The red arrow indicates the specific ZNF445 band. Right panel: qPCR analysis. (E) Knockdown of ZNF445 in K562 cells decreases TRIM28 enrichment at the H19 promoter and locus control region-HS2. *P < 0.05, **P < 0.01, ***P < 0.001.

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