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碩士論文

Institute of Biochemical Sciences
College of Life Science
National Taiwan University

Master Thesis

在 K562 中藉由 TRIM28 調節的基因調控

TRIM28-mediated gene regulation in erythroleukemia cell line K562

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Abstract

Tripartite motif-containing 28 (TRIM28), also known as KRAB (Krüppel-associated box repression) domain associated protein 1 (KAP1) or transcriptional intermediary factor 1 beta (TIF1β), is a ubiquitously expressed protein that plays a role in many important physiological phenomena such as cellular differentiation and proliferation. It associates with KRAB-Zinc finger proteins (ZNFs) via N-terminal RBCC domains (RING domain, two B-box zinc fingers and a coiled coil domain), recruiting corepressor complexes by C-terminal PHD-Bromo domains and forms heterochromatin through interacting with HP1. The corepressor activity of TRIM28 was regulated by post-translational modifications. We have demonstrated that acetylation-mimic TRIM28-K304Q mutant weakens the interaction with KRAB-ZNFs. The TRIM28-K304Q knock-in K562 mutant and *TRIM28* homozygous knock-out K562 cells have been created by CRISPR-Cas9 gene editing. In this study, we performed real-time PCR and RNA-seq to delineate gene expression in TRIM28 K304Q K562 cells. We found that lncRNA H19, embryo epsilon globin and fetal gamma globin genes were up-regulated, while adult beta globin, and a globin gene repressor SOX6 were down-regulation in the TRIM28 K304Q mutant. The expression levels of megakaryocyte marker integrin beta3 and some of differentiation-related genes were increased in TRIM28 K304Q cells, indicating constant K304 acetylation might promote cell differentiation. Interestingly, the mRNA expression of MAGEC2, a binding partner of TRIM28 for E3 ligase activity regulation, was down-regulated in K304Q and homozygous knock-out cells through unclear mechanism. Rabbit anti-TRIM28-N

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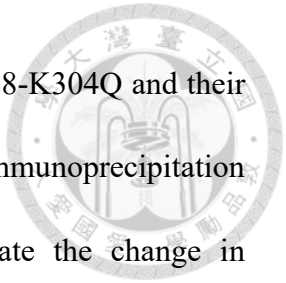

(N-terminal) antibody was used for isolation of TRIM28 and TRIM28-K304Q and their associated proteins precipitate TRIM28 from K562 cells by immunoprecipitation followed by mass spectrometry LC/MS/MS analysis to investigate the change in TRIM28 association protein. GID/CTLH E3 ligase complexes could only be detected in wild-type K562 while compared with the TRIM28-K304Q mutant. The protein-protein interactions of them have been further confirmed by immunoprecipitation analysis. Taken together, our results suggest that the TRIM28-K304 acetylation may modulate TRIM28-mediated gene expression via regulation of its interaction with KRAB-ZNFs.

Key words: TRIM28, K562, acetylation

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摘要

TRIM28 又稱作 KAP1 或是 TIF1β，是一個在細胞中大量表現并參與在細胞分化以及生長等多種重要生理反應的一個蛋白質。它通過其 N 端的 RBCC domains (RING domain, 兩個 B-box zinc fingers and a coiled coil domain)來和帶有 KRAB domain 的鋅指蛋白結合，並且通過 C 端的 PHD-Bromo domains 把輔抑制物複合體帶過來，並且通過和 HP1 結合來誘導異染色質產生。TRIM28 的這種輔抑制能力是經由轉譯後修飾來調控的，因而我們認為如果有 TRIM28 賴氨酸 304 換成穀氨醯胺來模擬乙醯化的細胞，我們應該可以看到 TRIM28 和鋅指蛋白的這種結合會受到影響。在 K562 細胞中，用 CRISPR-Cas9 技術製造了一個敲除了 TRIM28 的細胞株，並且也製造了敲入了一個在賴氨酸 304 的位置換成穀氨醯胺的 TRIM28 來模擬賴氨酸的乙醯化的細胞株。我們通過即時聚合酶鏈式反應和 RNA 定序來觀察穀氨醯胺 304 突變株的基因的表達發生了怎樣的變化。我們在穀氨醯胺 304 突變和 TRIM28 敲除突變中觀察到長非編碼 RNA H19，胚胎期表現的ε球蛋白和胎兒時期表現的γ球蛋白的表現量上升；成年期表現的β球蛋白，球蛋白抑制物 SOX6 的表現量下降。我們也觀測到巨核細胞分化的標記物——整合素β3 和其他一些跟細胞分化有關的基因表現量上升了，這暗示賴氨酸 304 的乙醯化可能會促進細胞的分化。有趣的是，TRIM28 的重要結合蛋白質 MAGEC2 的信使 RNA 的量在穀氨醯胺 304 突變株和 TRIM28 敲除細胞株中也下降了，具體機制目前不明。我們自己純化出了一個 anti-TRIM28-N 抗體用來做免疫沉澱的實驗，並把抗體抓到的蛋白質送去做質譜分析，看看正常 TRIM28 和突變的 TRIM28 各自抓到的蛋白質會有哪些不同。我們找到了一個叫做 GID/CTLH 複合物的蛋白質，這個蛋白質只能在正常的 TRIM28 的質譜資料中找到。我們進一步利用免疫沉澱法證實了這兩個蛋白質真的

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會相互結合而不是無選擇性抓到的雜質，這是這兩個蛋白質第一次被報導會有相互作用。綜合來看，TRIM28 在賴氨酸 304 的乙醯化可能通過影響到那些會跟 TRIM28 相互作用的蛋白質來影響到那些被 TRIM28 調節的基因的表現。

關鍵詞：TRIM28, K562, 乙醯化

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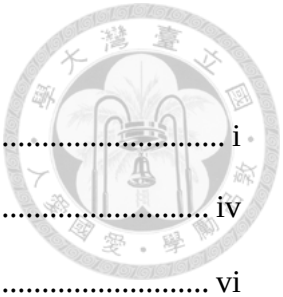

Contents

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1.Introduction

1.1 Tripartite motif-containing protein 28

Tripartite motif-containing protein 28 (TRIM28), is also called KRAB-associated protein 1 (KAP1) or transcriptional intermediary factor 1-beta (TIF1β), belongs to tripartite motif (TRIM) family. TRIM28 plays an important role in cell differentiation and development [1-4]. In the N-terminal of TRIM28, there are four conserved domains, a RING finger domain is a known ubiquitin E3 ligase domain [5], two different type B box domains, and a coiled coil domain, these domains could interact with zinc finger protein, and they are collectively called RBCC domain [6]. Structure analysis showed that TRIM28 forms anti-parallel dimer and interacts with KRAB domain of zinc finger proteins through coiled-coil domain. [7, 8]. In the central of TRIM28 is a PxVxL pentapeptide region which was responsible for interaction with Heterochromatin protein 1 (HP1) [9]. In the C-terminal of TRIM28, there are two functional domains, a Bromo domain and a plant homeodomain (PHD) domain, and these two domains cooperate as a transcription co-repressor through recruit NuRD HDAC (histone deacetylation) complex or histone H3 lysine 9-specific methyltransferase SETDB1 and finally result in gene silencing [10-12].

TRIM28 may be subjected to multiple protein posttranslational modifications, including phosphorylation and SUMOylation [13, 14]. The acetylated peptides analysis in the acute myeloid leukemia cell line MV4-11 treated with histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) has identified serval conserved acetylation residues on TRIM28 RBCC domain and Bromo domain include lysine 304

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acetylation [15].

TRIM28 is a crucial regulator in development and differentiation. Knockout of *Trim28* gene results in embryonic lethality, indicating that it plays essential roles for embryonic development [16]. It is required for the maintenance and pluripotency of embryonic stem cells [17, 18] and regulating gene networks controlling hematopoietic stem cell development including T-cell, B-cell and erythropoiesis [1-3, 19, 20]. Recent reports showed that TRIM28 haploinsufficiency in both mouse and human leads to lean and obese phenotypes arising from the identical genotypes through dysregulation of an imprinting gene network [21, 22].

1.2 Krüppel-associated box (KRAB) zinc finger protein (ZNF)

Krüppel-associated box (KRAB) domain is a transcription repression domain consist of 75 amino acid residues [23], which is present in 400 human zinc finger proteins [24]. Cys2-His2 (C2H2) zinc finger proteins are the largest regulatory protein family in mammals which have a sequence-specific DNA binding motif to bind to promoter regions [25]. KRAB-ZNFs bind to DNA through an array of C2H2-type zinc fingers, and recruit TRIM28 through the interaction between KRAB domain and TRIM28 coiled-coil domain, leading to gene repression [6, 26]. KRAB domain only appear in zinc finger proteins containing multiple C2H2 zinc fingers at C-terminal [27].

1.3 MAGEC2

MAGE (melanoma-associated antigen) family consists of about 60 genes including

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type I: MAGE-A, -B, and -C and type II: MAGE-D, -E, -F, -G, -H, -L2 and Necdin families, serving as cancer biomarkers and immunotherapeutic targets due to their specific expression pattern in various cancers [28]. The MAGE (melanoma antigen) family proteins assemble with RING E3 ligase to enhance ubiquitination activity [29]. The RING domain of TRIM family proteins serves as an E3 ubiquitin ligase [30], and it is known that MAGE-A2, -A3, -A6, and -C2 interact with TRIM28 [31], WH-A and WH-B domain of MAGEC2 are found to interact with coiled-coil domain of TRIM28 [32]. TRIM28-MAGE complex promotes p53, AMPK, and FBP1 (fructose-1,6-bisphosphatase) ubiquitination and degradation [32-35]. It is also reported that TRIM28 controlled MAGEC2 protein stability and no effect on mRNA expression levels [36]. A previous report shows that MAGEC2 is regulated by DNA methylation and post translation modification of histone [37]. These regulatory axes demonstrate that TRIM28 and MAGEC2 play functions in cancer cell proliferation and metabolism.

1.4 GID/CTLH (glucose-induced degradation-deficient/multi-subunit C-terminal to LisH) complex

GID/CTLH complex is a large ubiquitin E3 ligase specifically degrading the surplus of gluconeogenic enzymes, such as Fructose-1,6-bisphosphatase 1 (FBP1) and Phosphoenolpyruvate carboxykinase 1 (PCK1) in yeast [38, 39], and this complex degrades other substrates, such as High Mobility Group Box Transcription Factor 1 (HBP1) [40] and AMPK [41] in mammals. GID complex was first found in yeast, and is composed of RanBP9 which was reported to interact with numerous proteins and was

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involved in numerous biological pathways, Mkln1, TWA1, WD40-containing protein WDR26, ARMC8, YPEL5 which was reported as a pro-apoptotic role, and the RING domain-containing proteins RMND5A and MAEA [40, 42-47].

1.5 Globin gene expression and Human erythroleukemia K562 cells

Hemoglobin is a symmetric tetramer consist of alpha globin dimer and beta globin dimer. During erythroid differentiation, globin gene transcription initiated at erythroblast stage. Beta globin gene cluster undergoes sequential expression of the embryo epsilon globin genes, then fetal gamma globin genes, and then the adult beta globin genes [48]. Transcription factor like SOX6 can bind with promoter of epsilon/gamma globin to inhibit its expression [49, 50]. Some papers report that TRIM28 is important for erythroblast differentiation in mice [1] and takes part in beta and epsilon globin regulation [51].

Human erythroleukemia K562 cells were derived from the pleural effusion of a patient with chronic myelogenous leukemia (CML) in terminal blast crisis [52]. K562 is a model to study cellular differentiation, and K562 can undergo further differentiation into megakaryocytic or erythroid lineages depending on the stimuli [53]. For examples, DMSO, histone deacetylase (HDAC) inhibitors, 5-aza-2'-deoxycytidine (decitabine) and nucleoside analog such as ribavirin (1-β-ribofuranosyl-1,2,4-triazole-3-carboxamide) induce K562 into erythroid cells. It has been known that K562 usually expressed epsilon and gamma globin [54]. Some protein complexes are reported to regulate differentiation of K562, such as activin A mediates growth inhibition and hemoglobin

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synthesis in K562 [55], while inhibin inhibits K562 spontaneous differentiation to erythroid lineage [56]. The differentiation of K562 cells is also under the control of transcription factors such as GATA1, GATA2, EGR1, TAL1 and EKLF1 for differential lineages [53, 57].

Dr. Yao-Jin Chang had created TRIM28 homozygous knock-out K562, TRIM28 heterozygous knock-out K562 and TRIM28-K304Q mutant K562 cells using Crispr-Cas9. Since K304 is located at coiled coil domain of TRIM28, we speculate K304Q mutation might disrupt the interaction between TRIM28 and some known associate proteins such as zinc finger proteins and MAGEC2, and further affect downstream biological pathways to regulate the differentiation of K562.

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2.MATERIALS AND METHODS

2.1 Cell lines and cell culture

Human erythroleukemia cell line K562 were cultured in RPMI 1640 (Hyclone), supplemented with 10% FBS (Gibco), 100 U/mol penicillin/streptomycin (Gibco), 1mM sodium pyruvate (Gibco), in T75 flask. HEK293T cells were cultured in DMEM (Hyclone), supplemented with 10% FBS and 100 U/mol penicillin/streptomycin in 10cm dish. Both of them were cultured in 5% CO_2 incubator at 37℃. Before subculture, all solution was prewarmed at 37℃ in water bath. Cell number was counted by LUNA™ Automated Cell Counter.

2.2 Cell extract preparation, western blotting, immunoprecipitation and nuclear extract preparation

K562 was centrifuged at 1500 rpm for 5 min from its medium in 15 ml centrifuge tube, 293T was centrifuged at 6000 rpm for 5 min in 1.5 ml centrifuge tube. Both fresh pellet and the pellet stored in -80 ℃ for following western blotting or immunoprecipitation, but only fresh pellet was used in nuclear extraction. All pellet was washed twice by 1 ml 1x PBS before frozen or lysis.

Cell extract preparation

The cell pellet was treated by lysis buffer [20 mM Hepes (pH7.9), 200 mM KCl, 1% NP40, 1 mM EDTA, 10% glycerol and 100x protease inhibitor cocktail] on ice for 20 min, and vortex 4 times during the 20 min, the volume of lysis buffer was dependent on

doi:10.6342/NTU202004036

the volume of pellet. Then centrifuge the lysate at 12000 rpm for 10 min and discard the pellet. Add one third volume of 4x SDS sample buffer [200 mM Tris (pH 6.8), 8% SDS, 0.4% bromophenol blue,40% glycerol, 400 mM β-mercaptoethanol], boiling at 100℃ for 5 min.

Immunoprecipitation

For immunoprecipitation, 200 μl of cell extracts from one 6-cm dish or $5x10^6$ cell culture were added by 16 μl of 50% protein A beads, rotated at 4°C for 1 h to pre-clear. Centrifuge the complex in 5000 rpm for 1 min, and 10 μl of supernatant was collected as direct loading. Then 25 μl of 30% M2 beads or25 μl of 30% HA beads or 50 μl of anti-TRIM28-N antibody with 20 μl of 50% Protein A was added to the remaining supernatant. This complex was gently rotated at 4℃ for 2 h, then washed by 0.5 ml of 1X PBS for three times. The precipitated complexes were added with 7 μl of 4x sample buffer, boiling at 100℃ for 5 min.

Nuclear extract preparation

The cell pellets were suspended in five-fold cell pellet volume cytosol extraction buffer (10 mM Hepes, 10 mM KOAc, 1.5 mM $MgCl_2$, 2.5 mM DTT, 0.075% NP40, 10% Glycerol, 1mM TSA, 1mM PMSF, 100x protease inhibitor cocktail, 20 μM MG132, 200x NEM, 20x phosphatase inhibitor), gently mixed without vortex, on ice for 5min, then centrifuge at 9000 rpm for 1min. The supernatant was cytosol fraction, then the pellets were added with nuclear extraction buffer [20 mM Tris (pH7.4), 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA 10% Glycerol, 1 mM DTT, 1 mM TSA, 1 mM PMSF,

doi:10.6342/NTU202004036

100x protease inhibitor cocktail, 20 μM MG132, 200x NEM, 20x phosphatase inhibitor], on ice for 20 min with vortex. The volume of nuclear extraction buffer was one half volume of cytosol extraction buffer. After centrifuge at 12000 rpm for 10 min, the supernatant was collected as nuclear fraction.

SDS-PAGE

SDS-PAGE was prepared with recipe showed below.

		lower gel		upper gel
gel concentration	7.50%	10%	12.50%	4.50%
lower gel buffer (ml)	1	1	1	
upper gel buffer (ml)				0.5
30% acryamide (ml)	1	1.3	1.7	0.3
ddH_2O (ml)	2	1.7	1.3	1.2
10%APS (ul)	40	40	40	15
TEMED (ul)	4	4	4	5

Lower gel buffer: 1.5 M Tris/HCl (pH 8.8), 0.4% SDS

Upper gel buffer: 0.5 M Tris/HCl (pH 6.8), 0.4% SDS

Western blot assay

Electrophoresis condition: 100 V, 15 min; 120 V, until the dye is going to pass through the bottom of the gel. One piece of polyscreen® PVDF transfer membrane (blossombio) and six pieces 3MM papers (Whatman) were cut in correct size depend on your gel, and were soaked in transfer buffer (48 mM Tris-Cl, 39 mM glycine, 0.037% SDS, 20% methanol), for 5 min before membrane transfer. Proteins were transferred to PVDF membrane in 2 mA/cm^2 for 1h by C.B.S. Scientific EBU-4000. After transfer,

doi:10.6342/NTU202004036

membrane was blocked with 5% milk [in 1x PBST (0.1% Tween-20 in PBS)] for 30 min at room temperature. Antibodies were diluted in blocking milk. Primary antibodies were incubated with membranes at 37℃ for 1 h, or 25℃ for 2 h, or 4℃ overnight depending on antibodies. The secondary antibody was incubated with the membrane at 37°C for 1 h. Membranes were washed with 1x PBST for 5 min and repeated for 3 times after each antibody incubation. Membranes were exposed to X-ray film (Fujifilm) with Western Lightening Plus Enhanced Chemiluminescence reagent (Perkin Elmer).

2.3 DNA extraction and semi-quantitative PCR

Plasmid DNA was extracted by Geneaid™ Midi Kit and Geneaid™ Mini Kit. The genomic DNA from cell culture was extracted by Quick-DNA™ Miniprep Plus Kit, and purified by FB PCR/Gel Extraction Kit. KAPA HiFi HotStart ReadyMix PCR Kit was used for K304R selection. PCR condition used for K304R selection: initial denaturation: 95°C 3min, denaturation: 98°C 20 s, annealing: 62°C 15 s, extension 72°C 15 s for 35 cycles (T-3000 -Thermal Master). The PCR fragment was separated on 1% agarose gel.

2.4 RNA isolation, reverse transcription and real-time PCR

RNA was extracted by TRIzol™ and concentration was measured by Nano Vue Plus (GE). 2 μg RNA was treated with deoxyribonuclease I (Invitrogen) and used to prepare cDNA with FIREScript Reverse Transcriptase (Solis BioDyne) at 37°C for 30 min, and then at 85°C for 5 min in 20 μl. 5 μl cDNA product was diluted into 100 μl for following RT-PCR. Real-time PCR was performed in RG-6000 (Corbett) or CFX Real Time PCR system (BIO-RAD). Real-time PCR condition: 95°C hold for 5 min, 95°C

doi:10.6342/NTU202004036

for 10 s, 60°C for 30 s for 40 cycles (for HD 2X SYBR GREEN qPCR MIX), or 95°C hold for 2 min, 95°C for 10 s, 60°C for 15 s for 45 cycles (for SYBR® GREEN PCR KIT). The relative level of target gene expression was calculated according to the comparative Ct method using the $2^{-\Delta\Delta Ct}$ formula, the expression of GAPDH was regarded as an endogenous control. Statistically significant values were calculated by multiple t-test. All real-time PCR primers are showed in Table.1. p-value analysis was calculated by multiple t-test via GraphPad Prism.

2.5 Transfection

293T cells were seeded to the 6 cm dish with 3 ml medium at the day before transfection. Cells were transfected by 37.5ul of 1M $CaCl_2$ and 150 ul of 2x HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM Dextrose, 50 mM HEPES pH 7) with 2 μg plasmid while the cell was about one third confluency. The cells also can be transfected by TurboFect™ Transfection Reagent (Thermo Fisher Scientific) in 70-90% confluency. Cells were harvested after 24 or 48 h for cell extract isolation.

K562 cells were seeded to the 6-well dish with 2 ml medium (without p/s, $2x10^5$ cells/ml) in each well. After 24 h, the cells were transfected by Lipofectamine® LTX & PLUS™ Reagent (Invitrogen) with 3 μg plasmid and was harvested after 48 h.

2.6 Antibody

Anti-TRIM28-N antibody was purified via modified Thermo Scientific™ SulfoLink™ Coupling Resin.

doi:10.6342/NTU202004036

Coupling resin

3 ml sulfolink™ coupling resin was added to the column, equilibrated column with 25 ml coupling B (CB, 50 mM Tris, 5mM EDTA-Na; pH 8.5), then 200 ul peptide [dissolved in 5 ml CB and 161 ul of 25 mM (final concentration) TCEP] was added, mixed for 15 min. After incubation for 30 min, the column was washed with 4.5 mL CB, and 6 mL NAC (0.0079 g/ml, dissolved in CB) was added to the column, mixed for 15 min and rested for 30 min., The column was drain, washed with 15 ml NAC, and incubated in 4 ml storage buffer (10 mM EDTA, 0.05% NaN3, 50% glycerol) at 4℃ overnight.

Affinity purification

Transferred 50ml serum to prepared column, collect the flow through, storage at 4°C. Washed column by 20 ml wash buffer (0.1M Na_2HPO_4, 5mM EDTA-Na Ph 6.0), added 300 ul of 1M Tris (pH 8.0) in a syringe, connect the syringe to the bottom of the column, washed column by 3ml 0.1M glycine (pH 2.4), mixed and pull out the solution by syringe, collect the solution (#1), repeat above, to collect #2~#6, all eluted solution was storage at 4°C. Washed column by 20ml PBS and storage the column in storage buffer at 4°C.

The effect of the purified antibody was confirmed with both purified TRIM28 and K562 whole cell extract by western blotting, and the antibody was stored in 40% glycerol. This antibody was used in TRIM28 immunoprecipitation assay and western

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blot assay.

Other antibodies

Rabbit anti-Flag antibody was provided by Dr. Sheng-Chung Lee, Other antibodies were showed in Table 2.

2.7 CRISPR-Cas9

Site-directed mutagenesis has been performed to introduce the K304R mutation on the plasmid [pGEM®-T Easy Vector Systems (Promega) ligated with wild type TRIM28] using the primer set Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and primer sets (hTRIM28 K304R site-directed forward 5' –GCT CAA TAA GCG GGG CCG TGT G, and hTRIM28 K304R site-directed reverse 5' – TCC TGC ATG ATC TGC AGG ATG GCC). The resulting plasmid pGEM-T Easy/hTRIM28 K304R carries the K304R (AAG>AGG).

Cas9 protein was reacted with sgRNA at 1:1.2 molar ratio at 37°C for 10 min. Electroporation was performed in Lonza SE Cell Line 4D-Nucleofector^{TM} Kit under FF-120 program on 4D-Nucleofector^{TM} system. After culture for 48 hours, cells were isolated by BD FACSJazz automated cell sorter. SacI was used to select K304R mutant.

2.8 RNA-seq (offered by manufacture)

mRNA sequencing by Illumina HiSeq

Total RNA of each sample was extracted using TRIzol Reagent (Invitrogen)/RNeasy Mini Kit (Qiagen)/other kits. Total RNA of each sample was

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quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agarose gel. 1 μg total RNA with RIN value above 6.5 was used for following library preparation. Next generation sequencing library preparations were constructed according to the manufacturer's protocol. The poly(A) mRNA isolation was performed using Poly(A) mRNA Magnetic Isolation Module or rRNA removal Kit. The mRNA fragmentation and priming was performed using First Strand Synthesis Reaction Buffer and Random Primers. First strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA by beads was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a 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 (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 13 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using beads, validated using an Qsep100 (Bioptic, Taiwan, China), and quantified by Qubit3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2x 150 bp paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control

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Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. The sequences were processed and analyzed by GENEWIZ.

Data analysis

Quality Control:

In order to remove technical sequences, including adapters, polymerase chain reaction (PCR) primers, or fragments thereof, and quality of bases lower than 20, pass filter data of fastq format were processed by Cutadapt (V1.9.1) to be high quality clean data.

Mapping:

Firstly, reference genome sequences and gene model annotation files of relative species were downloaded from genome website, such as UCSC, NCBI, ENSEMBL. Secondly, Hisat2 (v2.0.1) was used to index reference genome sequence. Finally, clean data were aligned to reference genome via software Hisat2 (v2.0.1).

Expression analysis

In the beginning transcripts in fasta format are converted from known gff annotation file and indexed properly. Then, with the file as a reference gene file, HTSeq (v0.6.1) estimated gene and isoform expression levels from the pair-end clean data.

Differential expression analysis

Differential expression analysis used the DESeq2 Bioconductor package, a model

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based on the negative binomial distribution. the estimates of dispersion and logarithmic fold changes incorporate data-driven prior distributions, Padj of genes were setted <0.05 to detect differential expressed ones.

GO and KEGG enrichment analysis

GOSeq(v1.34.1) was used identifying Gene Ontology (GO) terms that annotate a list of enriched genes with a significant padj less than 0.05. And topGO was used to plot DAG.

KEGG (Kyoto Encyclopedia of Genes and Genomes) is a collection of databases dealing with genomes, biological pathways, diseases, drugs, and chemical substances (http://en.wikipedia.org/wiki/KEGG). We used scripts in house to enrich significant differential expression gene in KEGG pathways.

Novel transcripts prediction

After assembling a transcriptome from one or more samples, Cuffcompare, a tool of Cufflinks v2.2.1, compared assembly to known transcripts. Then, novel transcripts can be predicted via results of Cuffcompare.

Alternative splicing analysis

Asprofile v1.0 is a suite of programs for extracting, quantifying and comparing alternative splicing (AS) events from RNA-seq data. It took a GTF transcript file created by Cufflinks as its input.

SNV analysis

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Samtools v0.1.19 with command mpileup and Bcftools v0.1.19 were used to do SNV calling.

Differential exon usage

By differential exon usage (DEU), we mean changes in the relative usage of exons caused by the experimental condition. The relative usage of an exon is defined as number of transcripts from the gene that contain this exon number of all transcripts from the gene.

The basic concept can be summarized as follows. For each exon (or part of an exon) and each sample, we count how many reads map to this exon and how many reads map to any of the other exons of the same gene. We consider the ratio of these two counts, and how it changes across conditions, to infer changes in the relative exon usage. In the case of an inner exon, a change in relative exon usage is typically due to a change in the rate with which this exon is spliced into transcripts (alternative splicing). Note, however, that DEU is a more general concept than alternative splicing, since it also includes changes in the usage of alternative transcript start sites and polyadenylation sites, which can cause differential usage of exons at the 5' and 3' boundary of transcripts. To analysis DEU, we used Bioconductor package DEXSeq (V 1.21.1).

Principal component analysis

This is another way to visualize sample-to-sample distances. In this ordination method, the data points (here, the samples) are projected onto the 2D plane such that they spread out in the two directions that explain most of the differences (Figure below).

doi:10.6342/NTU202004036

The x-axis is the direction that separates the data points the most. The values of the samples in this direction are written PC1. The y-axis is a direction (it must be orthogonal to the first direction) that separates the data the second most. The values of the samples in this direction are written PC2. The percent of the total variance that is contained in the direction is printed in the axis label. Note that these percentages do not add to 100%, because there are more dimensions that contain the remaining variance (although each of these remaining dimensions will explain less than the two that we see). This analysis has been dealed with R language.

Protein-protein interaction

Protein-protein interaction plays key role in predicting the protein function of target protein and drug ability of molecules. The majority of genes and proteins realize resulting phenotype functions as a set of interactions. Protein-protein interactions (PPIs) handle a wide range of biological processes, including cell-to-cell interactions and metabolic and developmental control. Protein-protein interaction is becoming one of the major objectives of system biology. We simply analysis with String Database, in which containing known PPIs, or we can predict novel by blast against known sequences.

2.9 Mass-spectrum

Nuclear extraction was performed in pellet of wild type K562 and K304Q mutant, and Anti-TRIM28-N antibody was used to perform immunoprecipitation in nuclear fractions of wild type K562 and K304Q mutant, followed by an SDS-PAGE (described in method 2.2). The membrane was stained with Sypro Ruby, and TRIM28

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was cut from the membrane, each sample contained about 3 μg TRIM28. Sample was sent to Academia Sinica Common Mass Spectrometry Facilities at IBC for following treatment and mass-spectrum analysis.

Sample was cut into 1 cubic millimeter and distained with 200 μl 50% acetonitrile (ACN) and 25 mM ammonium bicarbonate (ABC) for 15 min, then soaked in 200 μl 25% ACN and 25 mM ABC. Dry the gel in speed-Vac for 15 min and soaked in 100 μl 100 mM dithioerythreitol (DET) and 25 mM ABC prevent light at 37 °C for 1 hour. Centrifuge and remove DET, then add 100 μl 100 mM iodoacetamide (IAM) and 25 mM ABC at 25°C for 1 hour. Removed IAM, and washed by 200 μl 50% ACN and 25 mM ABC for 15 min for 4 times. Remove wash buffer, add 100 μl 100% ACN for 5 min then dried in speed-Vac for 5 min. Incubate the gel with Lys-C in 25 mM ABC at 37 °C for 3 hours, then add trypsin, incubated at 37°C, overnight (protein: enzyme = 50:1). Collect the supernatant and extracted residual gel with 50 μl 5% Trifluoroacetic acid and 50% ACN, sonicated on ice for 200 s (repeat extraction for 2 times), and combine with supernatant, dried in speed-Vac for 1.5 hours, dissolved in 10 μl 0.1% formic acid. Wash 10 μL C18 Zip-Tip with 10 μl 50% ACN for 2 times, 10 μl 0.1% formic acid and 50% ACN for 1 time and 10 μl 10% formic acid for 3 times. After the wash, absorbed the sample with Zip-Tip, washed Zip-Tip with10 μl 0.1% formic acid for 15 times, elute sample with 10 μl 10% formic acid and 50% ACN for 3 times, collect the sample with methanol washed microcentrifuge PP tube and dried in speed-Vac, keep the sample in -20°C.

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Mass-spectrum analysis was performed by Academia Sinica Common Mass Spectrometry Facilities on LTQ-Orbitrap Velos system (Thermo Fisher). Data were analyzed by Mascot (Matrix Science), and the software was offered by Academia Sinica Common Mass Spectrometry Facilities.

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3.Result

3.1 Anti-TRIM28-N antibody purification

To explore TRIM28-associated proteins in K562 cells, a good anti-TRIM28 for immunoprecipitation is needed. The two anti-TRIM28 antibodies, a polyclonal 71-3, and a monoclonal 20A1, were not useful in immunoprecipitation. Thus, we decided to generate an antibody to perform immunoprecipitation. A short peptide in N-terminus of TRIM28 was synthesized and injected into rabbits, and we purified an antibody called anti-TRIM28-N (see method 2.6). The effect of anti-TRIM28-N has been confirmed by an immunoprecipitation experiment (Fig.1). We can hardly detect any TRIM28 signal in post-IP soup, indicating that our anti-TRIM28-N antibody has a good efficiency in an immunoprecipitation experiment.

3.2 K304Q mutation disrupts the interaction between TRIM28 and its associated protein

The anti-TRIM28-N was used to perform immunoprecipitation assay in wild type K562 and K304Q mutant, followed by LC-MS/MS analysis. We have identified many zinc finger proteins only detected in wild type K562,whereas few zinc finger proteins were only detected in K304Q mutant (Table.3a). A complicated ubiquitin E3 ligase called GID/CTLH complex was only detected in wild type TRIM28 (Table.3b). Two proteins in MAGE family, MAGEA9 and MAGEC2, were only detected in the precipitated proteins by wild type TRIM28 (Table.3c). The interaction between wild type TRIM28 and GID/CTLH complex or MAGEA9 or MAGEC2 was verified by immunoprecipitation in 293T (Fig.2). The results showed that the Flag-Myc-tagged

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GID/CTLH complex component including YPEL5, RanBP9, WDR26, MAEA and GID8, and its E2 UBE2H and MAGEA9/MAGEC2 could precipitate endogenous TRIM28, which implied their protein-protein interactions.

To further investigate the interacting domain between GID/CTLH complex and TRIM28, we constructed some TRIM28 deletion mutants including deleting RING domain, BB box, coiled-coil domain, RBCC domain, or C-terminal 20 amino acids (C20) (abridged general view sees Fig.3a, sequence design see table.4). We then transfected these HA-tagged deletion mutants and Flag-tagged RanBP9 into 293T cells, and performed a co-IP assay to monitor this interaction (Fig.3b). The result shows that the coiled-coil domain of TRIM28 is important for the interaction between TRIM28 and RanBP9. To investigate the function of this interaction, we've measured some substrates of GID/CTLH complex, and find that the protein expression of PCK1 has been down-regulated in K562 (Fig.3c).

Because MAGEC2 is a well-known TRIM28 partner, we would like to know how TRIM28 regulates the MAGEC2 expression. The protein and mRNA expression levels of MAGEC2 were measured in wild type K562, K304Q mutant, TRIM28 homozygous knock-out and TRIM28 heterozygous knock-out K562 (Fig.4a and 4b). The protein expression of MAGEC2 is significantly down-regulated in K304Q mutant and TRIM28 homozygous knock-out K562. The mRNA expression level of TRIM28 in these cell lines were consistent with their protein expression. As shown in Fig.4c, the TRIM28 expression levels are equal in wild type K562 and K304Q mutant (Fig.4c). To confirm this TRIM28 K304Q-mediated MAGEC2 down-regulation effect is not due to

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CRISPR-Cas9 off-target, we transfected plasmids encoding TRIM28 wild type, or TRIM28 K304Q or TRIM28 K304R mutation into TRIM28 homozygous knock-out K562with or without treatment of proteasome inhibitor MG132 to prevent degradation of MAGEC2 (Fig.5a). The MAGEC2 protein and mRNA expression was examined by western blotting and real-time PCR, respectively (Fig.5a and 5b). The wild type TRIM28 but not TRIM28 K304Q or TRIM28 K304R can restore the MAGEC2 protein and mRNA expression. The MAGEC2 proteins were not increased under MG132 treatment, indicating the down-regulation of MAGEC2 is not regulated via protein degradation (Fig.5a). As shown in Fig.5b, the mRNA expression levels of two globin genes were significantly up-regulated and that of *MAGEC2* was significantly down-regulated in cells transfected with K304Q, and reverse results were observed when transfected with wild-type TRIM28. This result further testifies that the down-regulation of MAGEC2 in K304Q mutant is not an off-target effect. In general, TRIM28 represses gene expression through epigenetic regulators recruitment by its C-terminal PHD domain and Bromo domain. Thus, wild type K562, K304Q mutant, TRIM28 homozygous knock-out and TRIM28 heterozygous knock-out K562 cells were treated with decitabine (DNA methylation inhibitor), SAHA (HDAC inhibitor) and TSA (HDAC inhibitor) to investigate the epigenetic regulation of MAGEC2 (Fig.6). The results show that none of the inhibitors could rescue the expression of MAGEC2.

3.3 RNA-seq analysis

Since TRIM28 is a well-known co-repressor, we also perform an RNA-seq (sequencing) in wild type K562, K304Q mutant and TRIM28 homozygous knock-out

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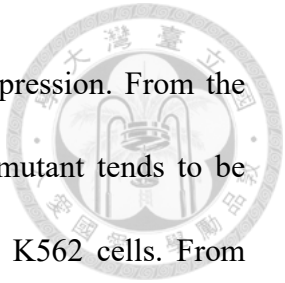

K562 to investigate the effect of K304Q mutant in global RNA expression. From the matrix heat map (Fig.7) the mRNA expression pattern of K304Q mutant tends to be more similar with TRIM28 homozygous knock-out than wild type K562 cells. From group-DE (differential expression) up-down (Fig.8) and GO (gene ontology) bar plot (Fig.9a; Fig.9b), we found more were up-regulated genes in TRIM28 homozygous knock-out K562 than those in K304Q mutant. This indicates that K304Q mutation may have its own specific regulation and serves as a switch of gene expression.

From RNA-seq data, the mRNA expression levels of integrin beta 3 and its receptor fibronectin are significantly up-regulated about 11.79-fold and 5.57-fold, respectively, in K304Q mutant (Fig.10a). Real-time PCR and western blot were performed to confirm the mRNA and protein expression of integrin beta 3 (Fig10.b). On the other hand, we demonstrated the mRNA expression levels of epsilon globin and gamma globin were significantly up-regulated meanwhile the mRNA expression levels of beta globin and *SOX6* were significantly down-regulated in K304Q mutant (Fig.11). Beside above, some differentiation-related genes were up- or down-regulated in TRIM28 K304Q k562 (Table.5a). *LIF* (leukemia inhibitory factor) has been reported to be induced while treating K562 with sodium butyrate and PMA [58]. The expression of *LIF* and *LIFR*（encodes the receptor of LIF） are significantly up-regulated in K304Q mutant in real-time PCR analysis (Fig.12a). Activin A could induce K562 differentiation into erythroid lineage [59], we found the receptor of activin A was upregulated in K304Q mutant, and this observation was also confirmed by real-time PCR assay (Fig.12b). Inhibin can inhibit K562 spontaneous differentiation to erythroid lineage [56].

doi:10.6342/NTU202004036

In our RNA-seq data, one of inhibin, *INHBE* has been down-regulated, and it also has been confirmed by real-time PCR assay (Fig.12b). Imprinting gene *DLK1* is up-regulated and *MEG3* is down-regulated in RNA-seq analysis data of K304Q mutant, and these two gene are regulated by TRIM28 [60, 61]. Real-time PCR assay was performed to confirm the mRNA expression level (Fig.12c), showing both *DLK1* and *MEG3* are significantly regulated via TRIM28 K304Q.

3.4 TRIM28-ZNF445-H19 regulatory axis in K562 cells

Another imprinting gene *H19*, a long noncoding RNA, is regulated by TRIM28 [60, 62], and *ZNF445* is involved in the regulation of the imprinting gene *H19* [63]. We first performed a real-time PCR assay to testify *H19* expression in wild type K562, K304Q mutant (Fig.13a). The result showed that *H19* was modestly increased in K304Q K562 and dramatically increased in TRIM28-KO K562. Since we have identified ZNF445 in our mass spectrum data in both wild type K562 and K304Q mutant (Table.3a), we decided to test the interaction of ZNF445 and TRIM28 through an immunoprecipitation experiment (Fig.13b). The result shows that the interaction between ZNF445 and TRIM28 K304Q is significantly decreased. Moreover, we knocked down *ZNF445* in K562, and western blot was displayed the knockdown efficiency (Fig.13c left panel) The real-time PCR assay was performed to testify the RNA expression of *TRIM28*, *ZNF445* and *H19* (Fig.13c right panel). The *H19* expression tendency in ZNF445 knock-down K562 is accordance to K304Q mutant, but the variation extent is more intense than K304Q mutant.

3.5. TRIM28-mediated gene expression during K562 differentiation

doi:10.6342/NTU202004036

To further investigate the mechanism of TRIM28 mediated regulation in K562 differentiation, we treated wild type K562 with SAHA, TSA and decitabine, and performed a real-time PCR to measure mRNA expression, which was up- or down-regulated in TRIM28 K304Q K562 (Fig.14). The results show that mRNA expression tendencies in drug treated K562 are similar with mRNA expression tendencies in K304Q. This implies that the differentiation of K562 cells is modulated by TRIM28-mediated gene expression. We also identified that the expression of some zinc finger proteins have been regulated (Table.6), four zinc finger proteins have been down-regulated, and eighteen zinc finger proteins have been up-regulated. Among these up-regulated zinc finger proteins, ZNF737 and ZNF98 have been detected in K304Q association protein mass spectrum data (Table.3a).

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4.Discussion

K304 is located on coiled-coil domain of TRIM28, this domain is important for TRIM28 to interact with MAGEC2 and zinc finger proteins. Coiled-coil domain of TRIM28 starts from E246 to L376. From blast aliment (see appendix), we find that the sequence of coiled-coil domain is conserved not only in animals have a close genetic relationship to human such as mouse but also in animals are not closely relate to human such as king cobra. This might suggest that the modification of K304 plays an important role in normal physiological state. Our current study is focused on the functional effect of K304 acetylation in the differentiation of human erythroleukemia K562 cells, and it also could be investigated in other systems, such as mouse 3T3-L1 preadipocyte.

We've purified anti-TRIM28-N antibody to perform immunoprecipitation followed by LC/MS/MS to analyze TRIM28 protein modifications.The acetylation is detected on K304 (data not shown). Since this acetylation might be a transient modification, we need a specific antibody to investigate when this acetylation occurs and its functional effects.

Compared to wild-type TRIM28, TRIM28 K304Q mutant decreased the interaction with ZNF445, MAGEC2 and GID/CTLH complex. The associated-proteins analysis also showed the preference in wild-type TRIM28 interacting with zinc finger proteins (Table 3a). The co-repressor function of TRIM28 might be disrupted due to the decreased interaction between TRIM28 and zinc finger proteins. In RNA-seq data, the mRNA expression of many zinc finger proteins is regulated (Table.5b). These might suggest that TRIM28 K304Q mutation weakens the interaction with zinc finger proteins

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and might auto-regulate zinc finger protein gene expression. SETDB1, TRIM28 and ZNF274 co-localize at 3' exons of ATRX-bound ZNF genes and form a complex with ATRX in K562 [63], and ATRX-TRIM28 complex was reported to promote heterochromatin formation [64]. Since TRIM28 binds to both 3′-coding exons of ZNF genes and promoter regions [65], how the K304 acetylation regulates zinc finger protein expression need to be testified.

The interaction between GID/CTLH complex and TRIM28 is a novel observation. They have been reported to be involved in cell cycle control [40] and gluconeogenesis [33, 64], and these two ubiquitin E3 ligases even have two common substrates, FBP1 [33] and AMPK [40, 66]. More details need to be figured out, such as which GID/CTLH component directly interacts with TRIM28 coiled-coil domain, and what are their substrates in K562. Our preliminary results show the purified recombinant YPEL5 but not RanBP9 could be pull-down by GST-TRIM28 (data not shown). More components of GID/CTLH complex will be expressed and examine their direct interaction with TRIM28. We also want to find out the spatiotemporal regulation manner in these two E3 ligases interactions. Since YPEL5 locates at centrosome [67], and TRIM28 has been reported take part in centrosome regulation [68], we speculate these two proteins might interact during mitosis.

MAGE family protein is regarded as a cancer biomarker. TRIM28 K304Q dramatically decreases MAGEC2 expression through an unclear mechanism, perhaps this regulation could be served as a drug target. MAGEC2 is regulated by DNA methylation, post translation modifications of histone and noncoding RNA [36]., In our

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study, the DNA methylation and HDAC inhibitors can't restore MAGEC2 expression in TRIM28-K304Q cells. Thus, we will examine whether TRIM28 K304Q mutation regulates MAGEC2 expression through noncoding RNA. The TRIM28-mediated noncoding RNA expression and TRIM28-associated RNA are to be identified for further understanding of this regulation.

The up-regulation of gamma globin and epsilon globin in TRIM28 K304Qis accordance to normally observed drug induced erythrocyte differentiation of K562. Combined with phenotype observation, TRIM28 K304Q cells are similar to erythrocytes. This regulation might through TRIM28 K304Q-mediated down-regulation of transcription repressor SOX6. But, in normal condition, cell doesn't express globin and integrin beta 3 at the same time, the reason remains to be further investigated.

It seems that when TRIM28 K304 is acetylated, the interaction between TRIM28 and some of its association protein such as GID complex and some zinc finger proteins is decreased. This might further affect the downstream substrate degradation of GID complex, and t the co-repressor activity of TRIM28. When TRIM28 co-repressor activity is decreased, some downstream genes are up-regulated such as lncRNA H19, integrin beta3, and some downstream genes are down-regulated such as SOX6 and MAGEC2. Interestingly, K304 acetylation not only affects the interaction between TRIM28 and zinc finger proteins, it also regulates the expression of zinc finger proteins. These zinc finger proteins might recruit TRIM28 to specific gene targets and finally result in the data we observed (Fig.15).

doi:10.6342/NTU202004036

5.Figures

1.

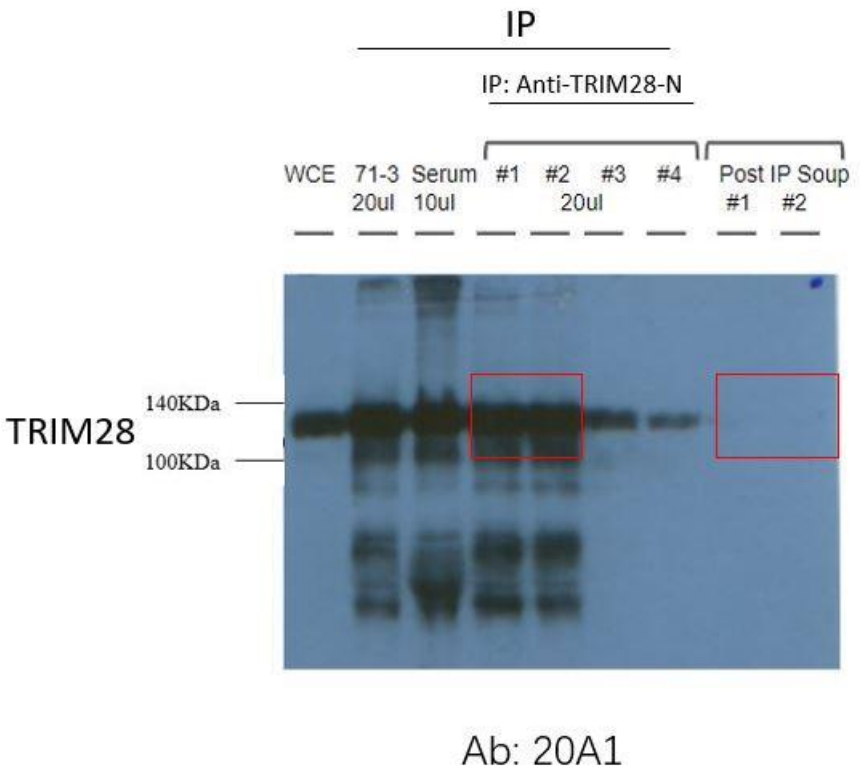

Figure.1

Anti-TRIM28-N fractions (#1~#4) were purified from rabbit serum (see method) and were used in immunoprecipitation of TRIM28. WCE means whole cell extract direct loading, 71-3 is a TRIM28 polyclonal antibody, 20A1 is a TRIM28 monoclonal antibody.

Post IP soup (#1~#2) were used to testify the IP efficiency of anti-TRIM28-N antibody.

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2.

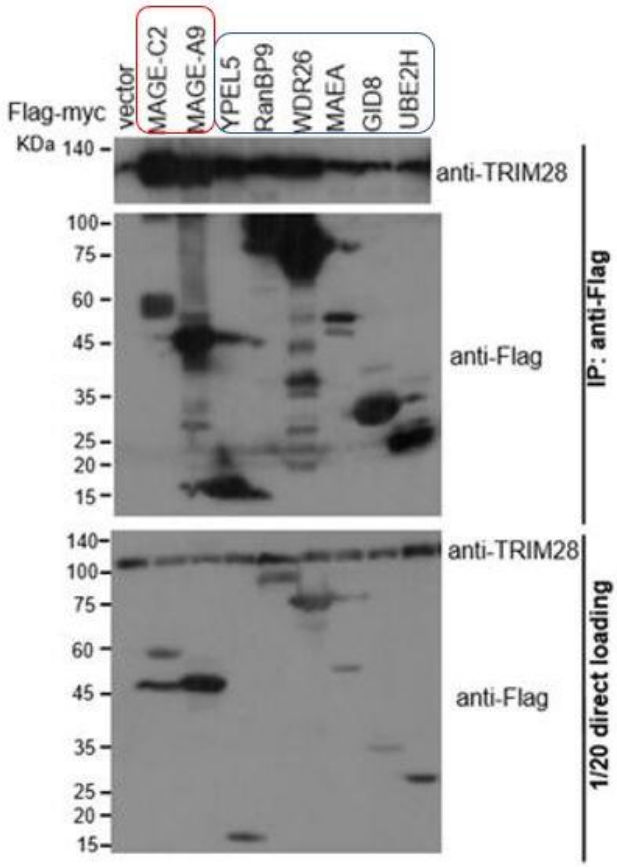

Figure.2

Flag-myc-tagged GID complex components as indicated and UBE2H (circumscribed in blue), and MAGEC2, MAGEA9 (circumscribed in red) were expressed in 293T cells and precipitated with endogenous TRIM28. Immunoprecipitation was performed by M2 beads (anti-Flag) and western blotting was detected by αTRIM28 (20A1).

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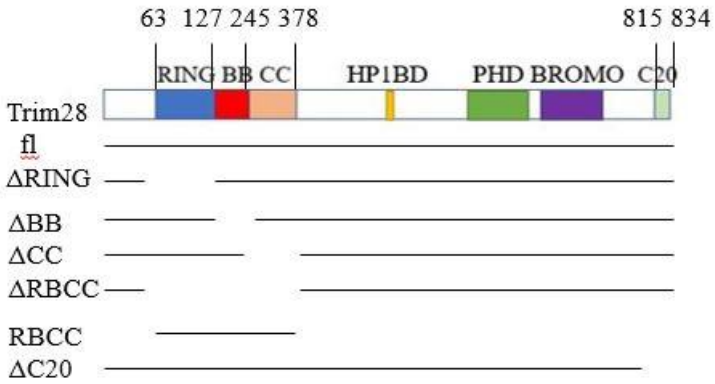
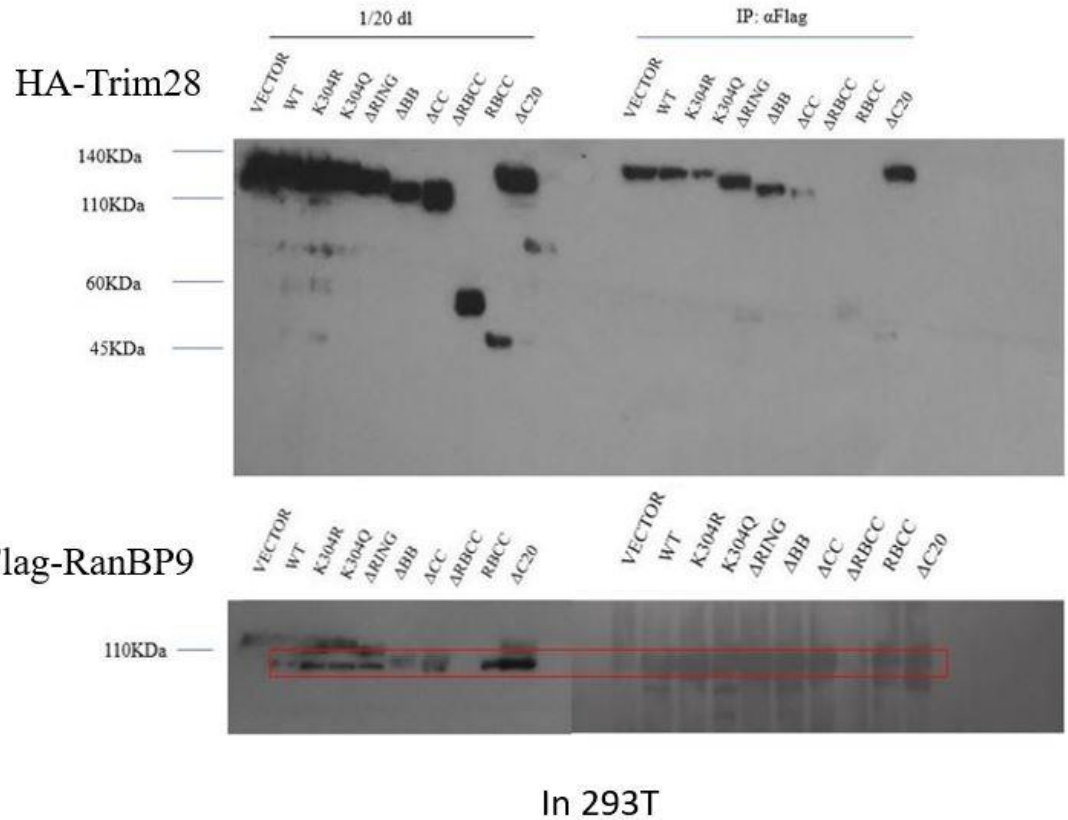
3.

a



b



In 293T

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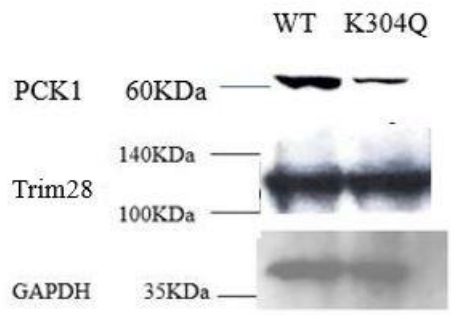
c



In K562

Figure.3

a. Constructs of mouse Trim28 deletion mutants

b. Co-IP of RanBP9 and Trim28 deletion mutants. Two micro grams of HA-TRIM28 deletion construct and one micro gram of Flag-Myc-RanBP9 construct were transfected into 293T cells. Immunoprecipitation was performed by M2 beads (anti-Flag).

c. PCK1, a substrate of GID/CTLH complex was measured by western blot in wild type K562 and K304Q mutant.

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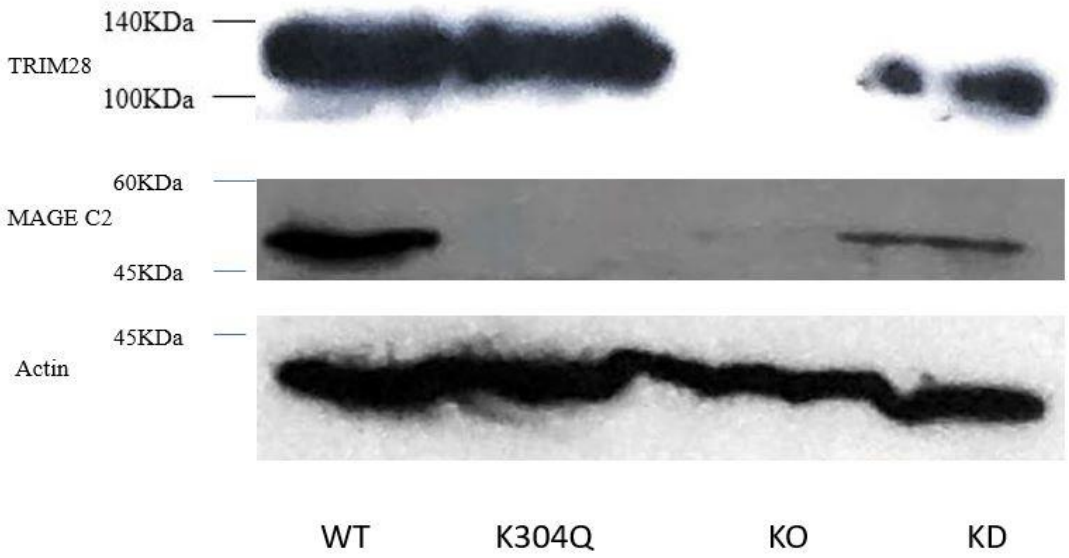
4.

a.



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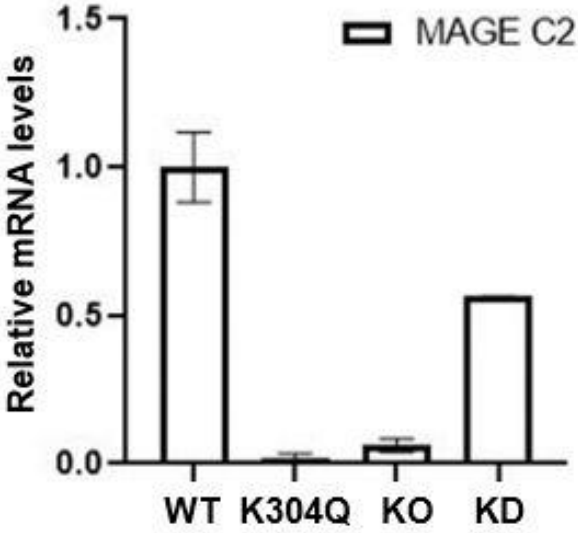

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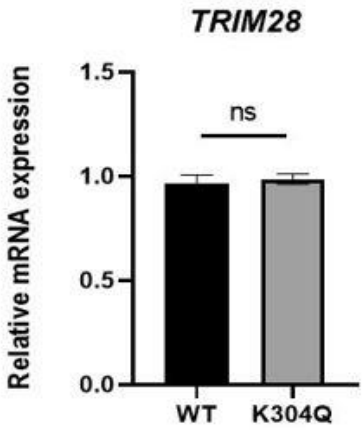

Figure.4

a. Western blot was performed in wild type K562, K304Q mutant, TRIM28 homozygous knock-out K562 and TRIM28 heterozygous knock-out K562, beta actin is used as a control.

b. Real-time PCR was performed in wild type K562, K304Q mutant, TRIM28 homozygous knock-out K562 and TRIM28 heterozygous knock-out K562 to test the mRNA expression of MAGEC2.

c. Real-time PCR was performed in wild type K562, K304Q mutant to testify the *TRIM28* mRNA expression level. p-value, ns $P > 0.05$

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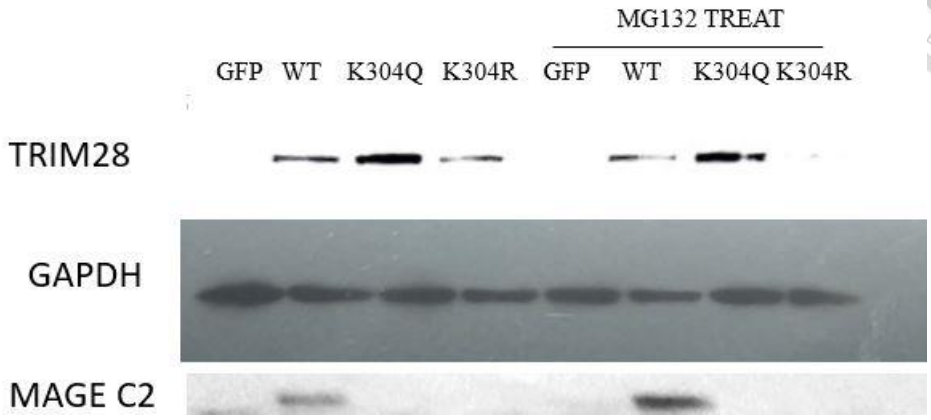
5.

a.



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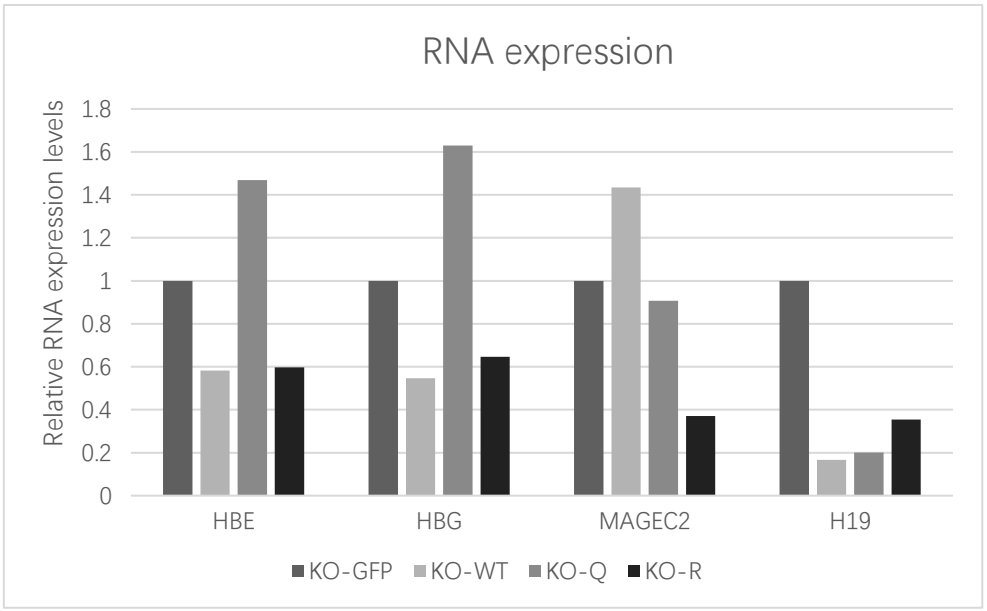

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Figure.5

a. Flag-tagged wild type TRIM28, K304Q mutant and K304R mutant were constructed and transfected into *TRIM28* homozygous knock-out K562. A group of K562 cells have been treated with 20 μM MG132 for 6 hours to prevent proteasome activity. The cells were transfected with GFP expression vector as a negative control and a transfection efficiency indicator.

b. Real-time PCR was performed to determine the mRNA expression level of *HBE*, *HBG*, *MAGEC2* and *H19* in TRIM28 homozygous knock-out K562, transfected with GFP, TRIM28 wild-type, TRIM28 K304Q or TRIM28 K304R expression plasmid.

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6.

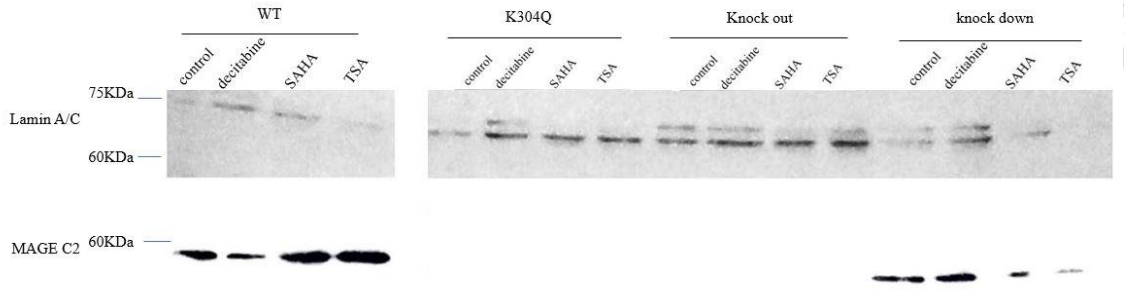

Figure.6

Wild type K562, K304Q mutant, TRIM28 homozygous knock-out K562, and TRIM28 heterozygous knock-out K562 were treated with decitabine (5 μM), SAHA (2 μM), and TSA (200 nM) for 4 days. Nuclear extracts were isolated for western blot assay with anti-MAGEC2. Lamin A/C was used as a nuclear internal control.

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7.

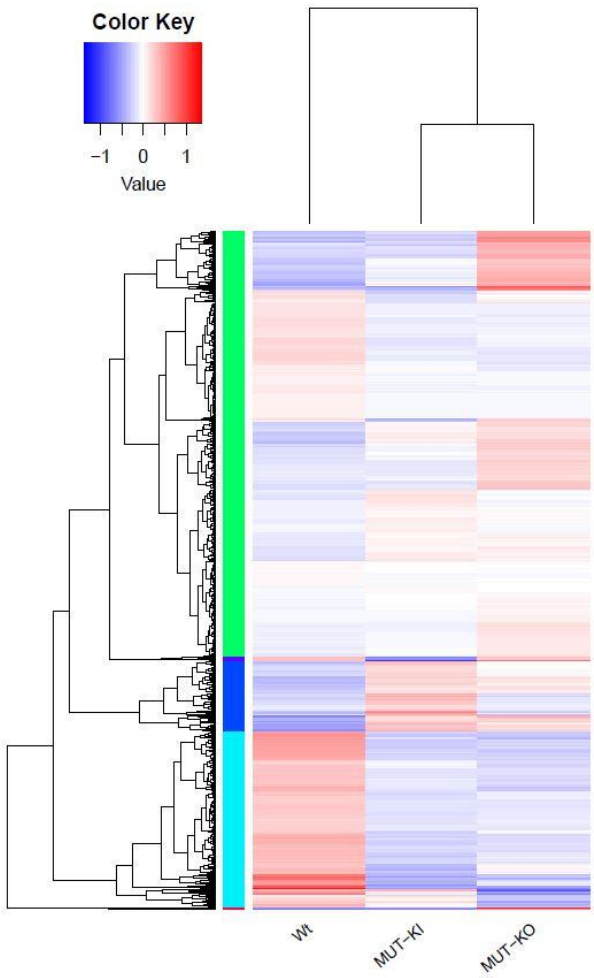

Figure.7

Heatmap of top 1147 differential expression genes upon wild type K562, K304Q mutant and *TRIM28* homozygous knock-out K562. Red: up regulated. Blue: down regulated. The intensity of the color reflects the degree of gene regulation.

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8.

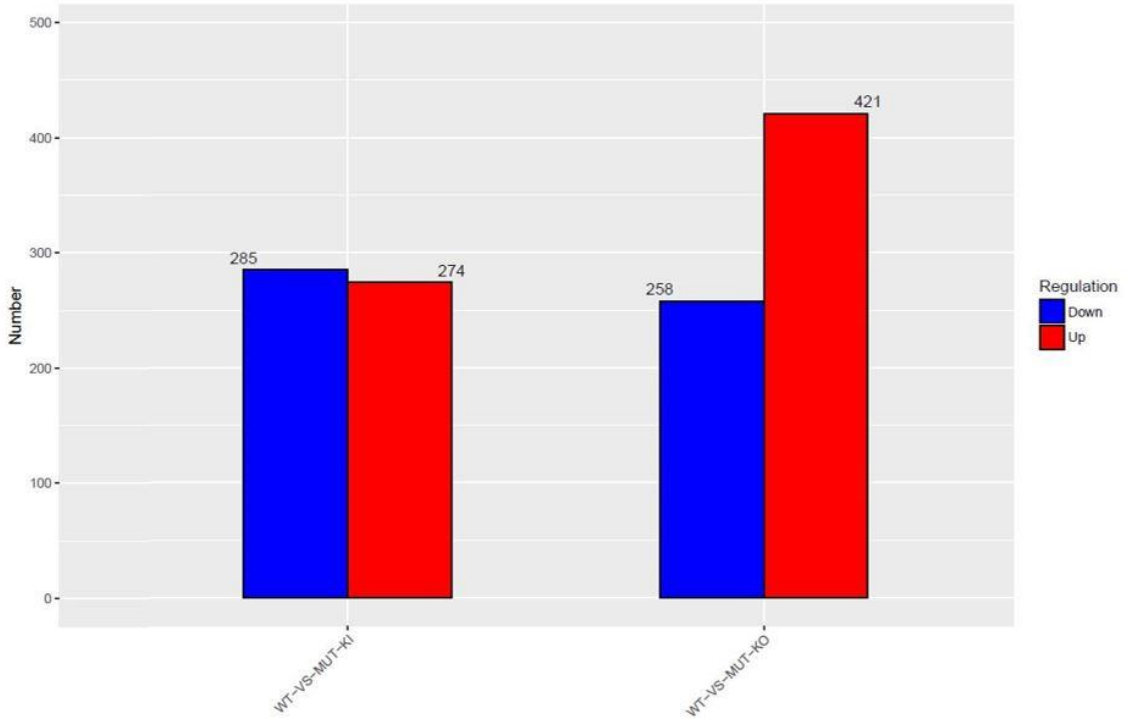

Figure.8

Bar plots of differential expression genes of wild type K562 vs K304Q mutant and wild type K562 vs *TRIM28* homozygous knock-out K562. Red: up regulated. Blue: down regulated. The intensity of the color reflects the degree of gene regulation. Y axis: the number of differential expression genes.

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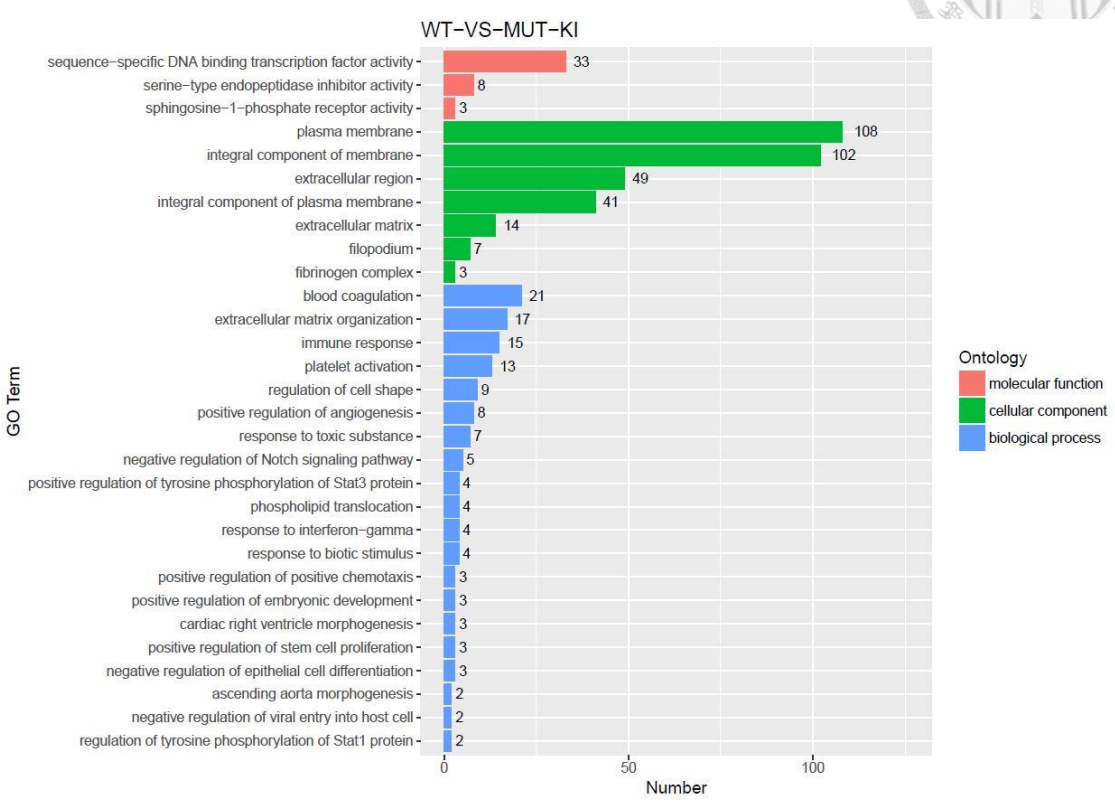
9.

a.



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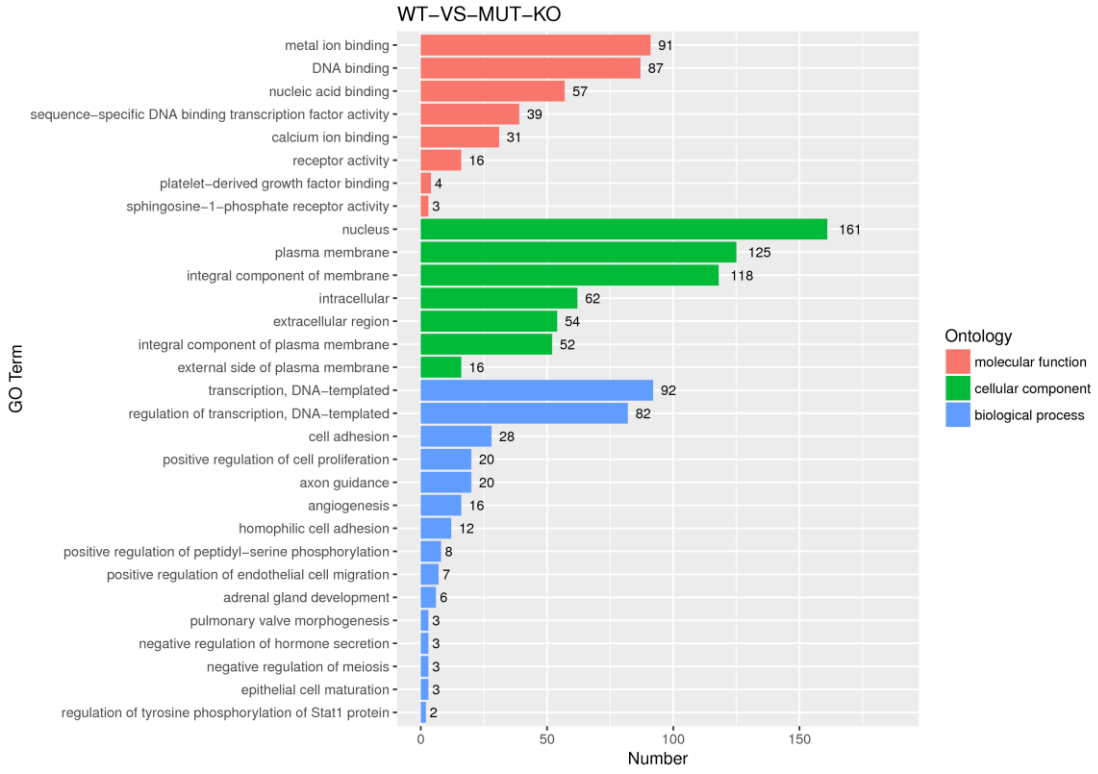

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Figure.9

a. Bar plot of gene ontology analysis upon wild type K562 and K304Q mutant.

b. Bar plot of gene ontology analysis upon wild type K562 and *TRIM28* homozygous knock-out K562

Red: molecular function. Green: cellular component. Blue: biological process.

Y axis: pathway which differential expression gene involve in.

X axis: the number of differential expression genes.

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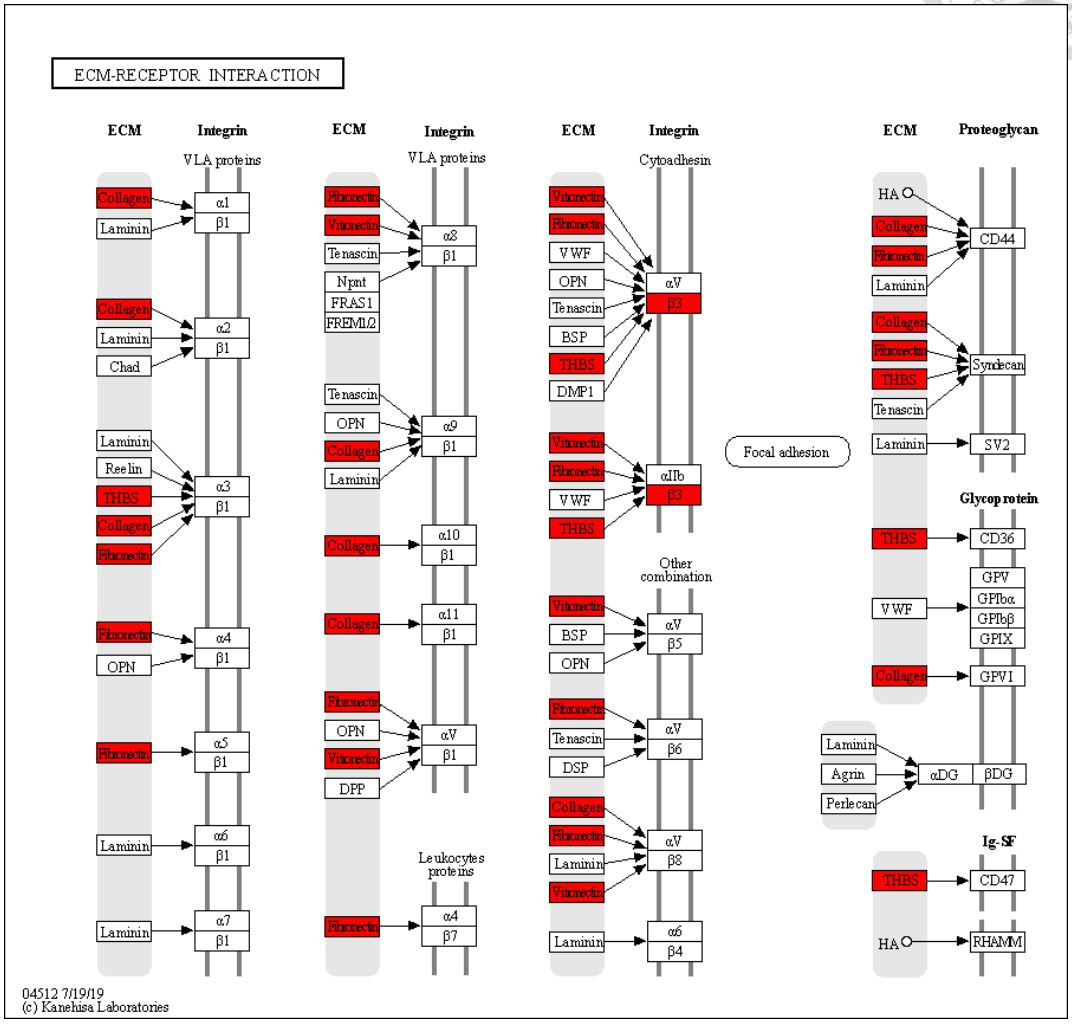
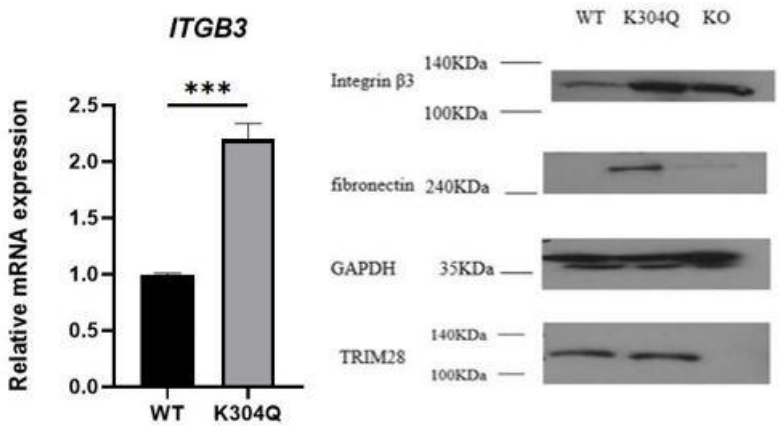
10.

a.



b.



doi:10.6342/NTU202004036

Figure.10

a. KEGG pathway of extracellular matrix-receptor interaction upon K304Q mutant and wild type K562. Red: up regulated.

b. Real-time PCR was performed to testify the mRNA expression of integrin beta 3 in wild type K562 and K304Q mutant. The protein expression of integrin beta 3 and fibronectin was examined by western blotting in wild type K562, K304Q mutant and *TRIM28* homozygous knock-out K562. P-value, *** $p < 0.001$.

doi:10.6342/NTU202004036

11.

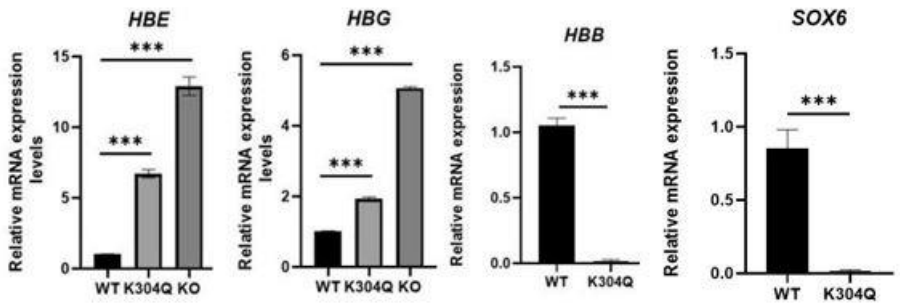


Figure.11

Real-time PCR was performed to measure the mRNA expression of epsilon globin, gamma globin, beta globin and *SOX6* in K562. P-value, *** p< 0.001

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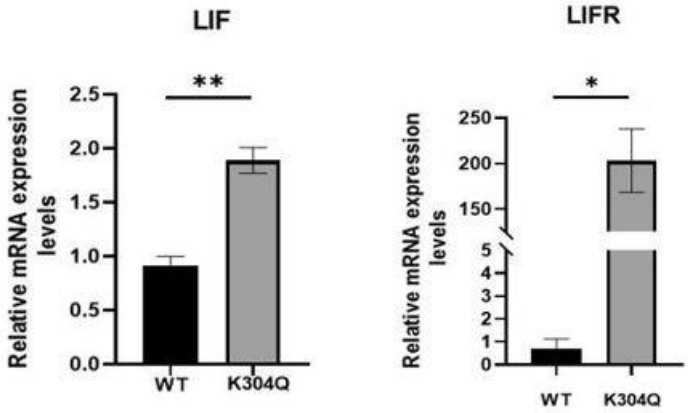
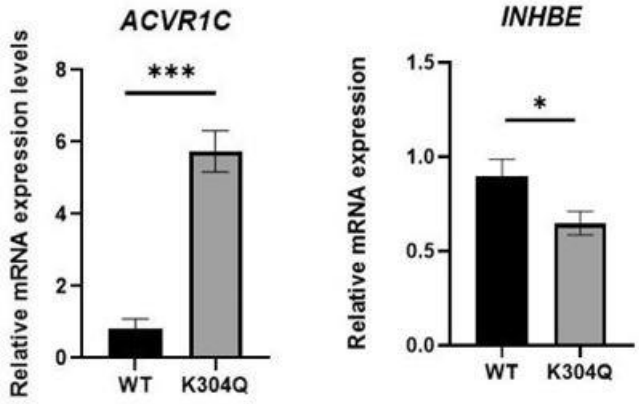
12.

a.



b.



c.

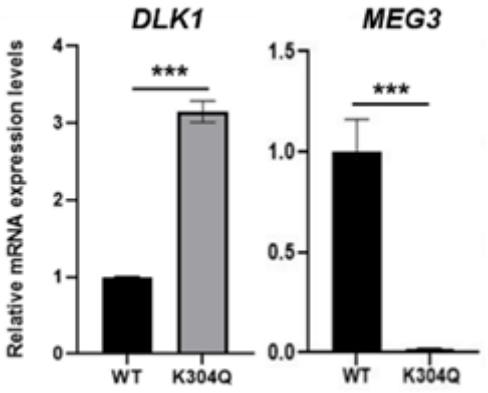


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Figure.12

a. Real-time PCR was performed to measure the mRNA expression of *LIF* and *LIFR* in K304Q mutant and wild type K562. p-value, *p< 0.05, p-value, **p<0.01.

b. Real-time PCR was performed to measure the mRNA expression of *ACVR1C* and *INHBE* in K304Q mutant and wild type K562. p-value, *p< 0.05, p-value, **p<0.01, P-value, *** p< 0.001.

c. Real-time PCR was performed to measure the mRNA expression of *DLK1* and *MEG3* in K304Q mutant and wild type K562, p-value, *** p< 0.001.

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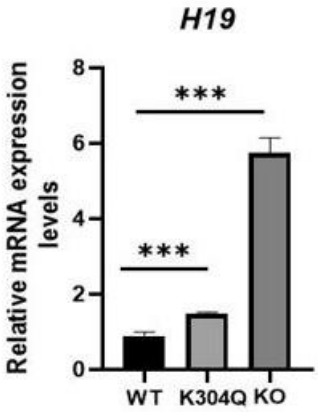
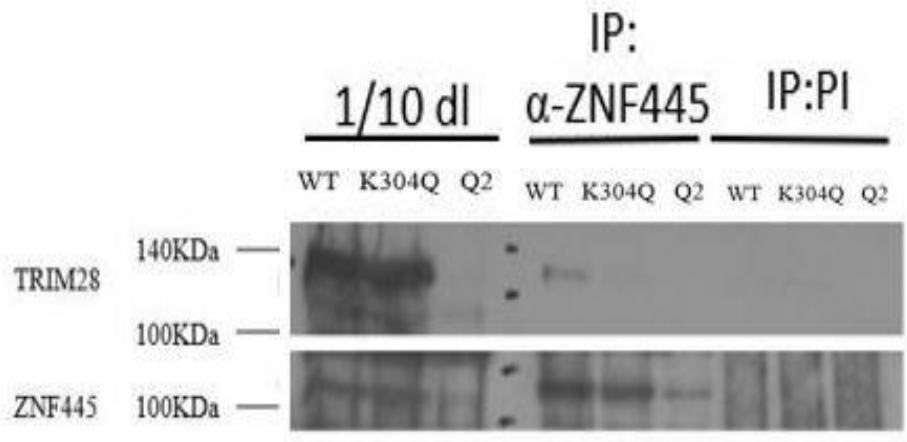
13.

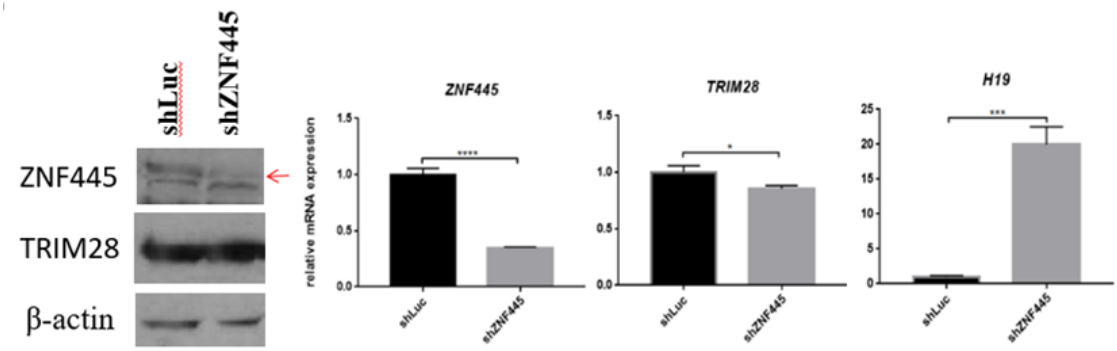
a.



b.



c.



doi:10.6342/NTU202004036

Figure.13

a. Real-time PCR was performed to measure the mRNA expression of *H19* in K304Q mutant and wild type K562. P value, *** p< 0.001.

b. Immunoprecipitation of ZNF445 in wild type K562, K304Q mutant and Q2 (a knock-out cell line) mutant. PI is pre-immune serum.

c. Real-time PCR was performed to measure the mRNA expression of *TRIM28, ZNF445* and *H19* in ZNF445 Knock-down and wild type K562. The knock-down efficiency of ZNF445 expression was monitored by western blotting. The red arrow bar indicates ZNF445 protein position, and TRIM28 and beta-actin is internal controls. p-value, *p< 0.05, P-value, ***p< 0.001, p-value, P ≤ 0.0001.

doi:10.6342/NTU202004036

14.

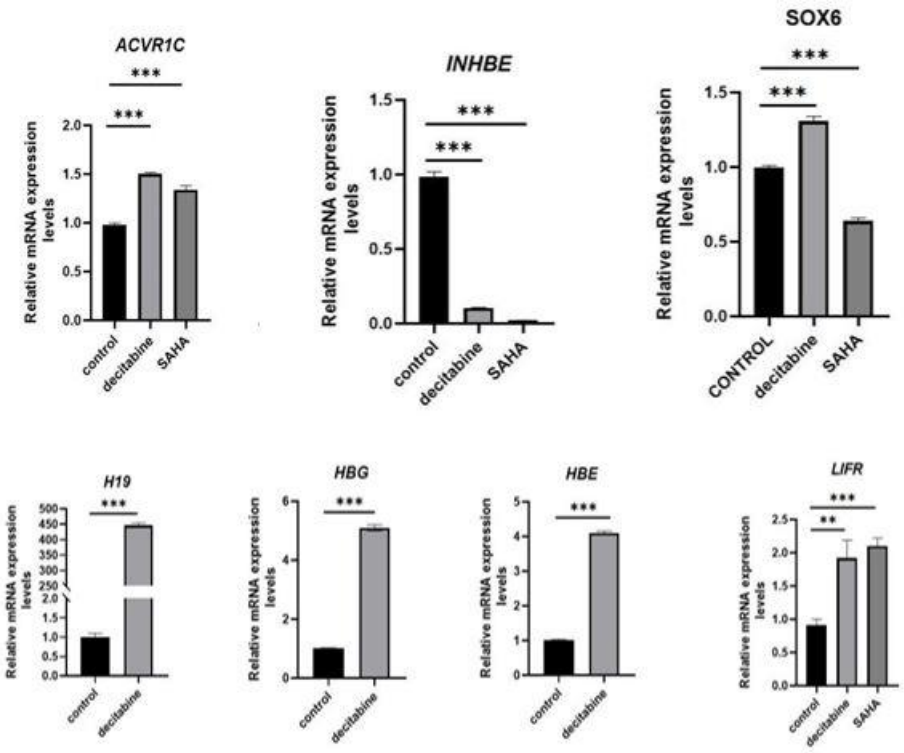


Figure.14

Real-time PCR has been performed to measure the mRNA expression of *ACVR1C, INHBE, SOX6, HBG, HBE, LIFR* and *H19* in decitabine or SAHA induced wild type K562. p-value, **p< 0.01, P-value, ***p< 0.001, p-value, P <0.0001.

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15.

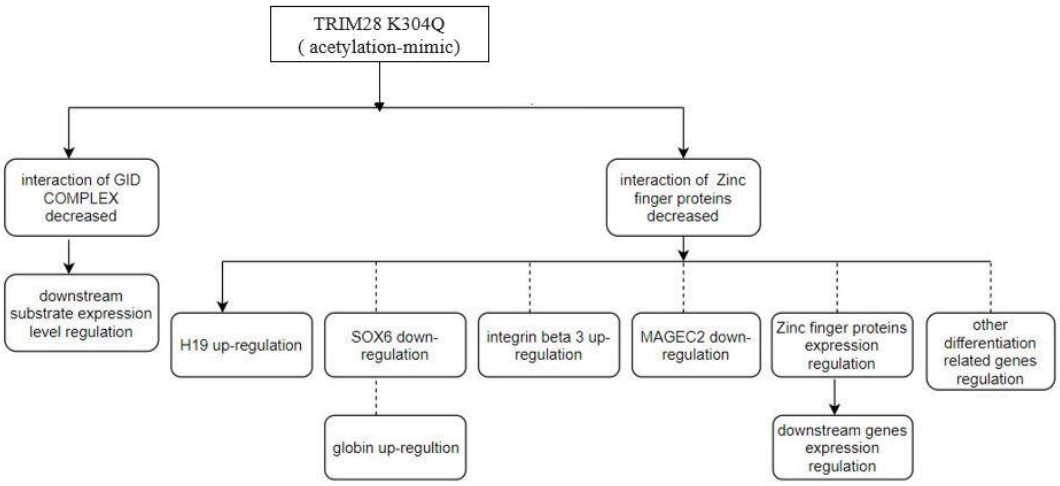

Figure.15

TRIM28 K304Q-mediated regulation in K562 cells. Dotted line denotes unclear mechanism or unconfirmed hypothesis.

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6.Tables

1. Primes used for qPCR

Gene	Forward (5'-3')	Reverse (5'-3')
ZNF516	GCACACTCAGTGGTGTTTGAG	GGACATCGTGAGGGTACTGC
ZNF667	TGTGACAAGTTCTTCAGGCG	GGATGAATGCCGATTGCAGAC
ZNF667-AS1	GGGAGTGTCCGCCATAAAGT	CTACACAAACGCGCGATCAA
ZNF382	CCTGCTCAGAAGGCGCTTTACA	CTCTGTGTCCATAGCTCTTCTCC
ZNF568	AAGAGTCTGCCCTTTCCGAGGA	GCAGGTTTCATTTGCTCCCACTC
ZNF829	ATGGGAATGCCTGGACGCTGAT	CCAGGGCTCTTTTCCTTGTTCC
ZNF527	GAGTGGGAATGGCTGAAGCCAT	CCAGTAAGGAGATCATGTTGGGC
ZNF91	AGGAGTGGCAATGTCTGGACAC	CAGGGCTCTTTTCCTTGCTCCA
ZNF445	AGCTCCAGGAGACCATGACT	GAATGGTCCCACCAGGGAAG
ZFP57	AAGCCAGAGGTCCATCCAG	GGGCAACAGAAGACATTGAG
H19	TGCTGCACTTTACAACCACTG	ATGGTGTCTTTGATGTTGGGC
IGF2	GGGCAAGTTCTTCCAATATGA	TCACTTCCGATTGCTGGC
PEG3	CGGAACAGAAGAGAGTCCTCAC	TGCTTCTTGGGTTCCTGGTGTG
MEG3	TTTTGTGCCCAAGGCTCCTGGA	AGGGACTCAAGGAGCCAGGTTA
DLK1	GCACTGTGGGTATCGTCTTCC	CTCCCCGCTGTTGTACTGAA
ITGB3	AGAGCCAGAGTGTCCCAAG	GGCCTCTTTATACAGTGGGTTGT
ITGA4	TTCCAGAGCCAAATCCAAGAGTAA	AAGCCAGCCTTCCACATAACAT
ITGA5	GCCTGTGGAGTACAAGTCCTT	AATTCGGGTGAAGTTATCTGTGG
MAGEA2	TGCAGTGGTTCTAGGATCTGC	GGTTCCTGGAGCTCCTGATT
MAGEA3	GATTCTCGCCCTGAGCAACG	CCCACTGGCAGATCTTCTCC
HBG(1/2)	TGGATGATCTCAAGGGCAC	TCAGTGGTATCTGGAGGACA
HBB	CTGAGGAGAAGTCTGCCGTTA	AGCATCAGGAGTGGACAGAT
HBE1	GCAAGAAGGTGCTGACTTCC	ACCATCACGTTACCCAGGAG
SOX6	CGGTCTACCTACTGGGATAA	GCTTTTGTTTGGCAGATTGA
IKZF2	ACACTCTGGAGAGAAGCCGTTC	CCAGTGAACTGCGCTGCTTGTA
LIF	AGATCAGGAGCCAACTGGCACA	GCCACATAGCTTGTCCAGGTTG
LIFR	CACCTTCCAAAATAGCGAGTATGG	ATGGTTCCGACCGAGACGAGTT
FOSL2	AAGAGGAGGAGAAGCGTCGCAT	GCTCAGCAATCTCCTTCTGCAG
AVR1C	TGCTAGTGGTCTGGCACACCTT	CTTCACAGCCAACCCTAAGTCC
SETBP1	GGAGCTATGAAGGCTTTGGAACG	ATGCACTGCTGTCTGGTCCACT
INHBE	CCCAGAATAACTCATCCTCCACC	GGACAGGTGAAAAGTGAGCAGG
TRIM28	AAGGACCATACTGTGCGCTCTAC	ACGTTGCAATAGACAGTACGTTCAC
GAPDH	CAACAGCGACACCCACTCCT	CACCCTGTTGCTGTAGCCAAA
ACTIN	GCACCAGGGCGTGATGG	GCCTCGGTCAGCAGCA

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2. Antibodies used in this paper

antigen	company	Cat No.	host
TRIM28	BioLegend	619302	mouse
RanBP9	abcam	ab64275	rabbit
HBP1	abcam	ab83402	rabbit
MAGEA9	abcam	ab191568	rabbit
FBP1	abcam	ab109732	rabbit
HA tag	BETHYL	A190-108A	rabbit
YPEL5	invitrogen	PA5-34351	rabbit
integrin	Cell signaing techonology	4749T	rabbit
GFP	Fine Test	FNab03433	rabbit
rabbit IgG	abcam	ab6721	goat
EHMT2/G9A	abcam	ab185050	rabbit
PCK1	abcam	ab70358	rabbit
MAEA	abcam	ab65239	rabbit
MAGEC2	abcam	ab209667	rabbit
SOX6	Abclonal	A7115	rabbit
integrin β1	Merck	MAB1951Z	mouse
HA tag	genetex	GTX115044	rabbit
Flag tag	sigma	A2220	mouse
acetyl lysine	novusbio	NB100-78428	mouse
lamin A/C	Cell signaing techonology	4C11	mouse
mouse IgG	KPL	474-1806	goat
Gal4	Santa cruz biotechnology	sc-577	rabbit
rabbit IgG	KPL	474-1516	goat
actin	novusbio	AC-15	mouse
GAPDH	proteinbiotech	60004-1-ig	mouse

doi:10.6342/NTU202004036

3. association protein

a. zinc finger proteins identified in mass spectrum

ZFPs associated with wild-type TRIM28 only	ZNF7	ZNF12	ZNF17	ZNF20	ZNF22	ZNF25	ZNF33A	ZNF33B
	ZNF41	ZNF44	ZNF45	ZNF57	ZNF84	ZNF101	ZNF107	ZNF121
	ZNF136	ZNF181	ZNF184	ZNF197	ZNF208	ZNF211	ZNF227	ZNF229
	ZNF235	ZNF257	ZNF264	ZNF311	ZNF317	ZNF320	ZNF324B	ZNF354B
	ZNF383	ZNF419	ZNF440	ZNF468	ZNF480	ZNF490	ZNF493	ZNF512
	ZNF529	ZNF543	ZNF550	ZNF552	ZNF554	ZNF555	ZNF558	ZNF563
	ZNF564	ZNF567	ZNF569	ZNF583	ZNF587B	ZNF589	ZNF607	ZNF611
	ZNF615	ZNF620	ZNF624	ZNF644	ZNF655	ZNF678	ZNF684	ZNF697
	ZNF699	ZNF701	ZNF707	ZNF718	ZNF721	ZNF726	ZNF749	ZNF766
	ZNF776	ZNF778	ZNF780A	ZNF785	ZNF789	ZNF791	ZNF799	ZNF805
	ZNF813	ZNF841	ZNF845	ZFP1	ZFP82	ZBT11	ZKSC1	ZKSC8
ZFPs associated with wild-type and K304Q TRIM28	ZNF8	ZNF34	ZNF74	ZNF77	ZNF90	ZNF91	ZNF92	ZNF93
	ZNF100	ZNF124	ZNF160	ZNF195	ZNF250	ZNF253	ZNF267	ZNF273
	ZNF274	ZNF316	ZNF324A	ZNF354A	ZNF417	ZNF430	ZNF432	ZNF441
	ZNF445	ZNF460	ZNF485	ZNF486	ZNF561	ZNF562	ZNF566	ZNF587
	ZNF595	ZNF614	ZNF627	ZNF649	ZNF669	ZNF670	ZNF680	ZNF688
	ZNF689	ZNF708	ZNF724	ZNF728	ZNF736	ZNF738	ZNF764	ZNF792
	ZNF808	ZNF816	ZNF823	POGK	ZFP92	RBAK		
ZFPs associated with K304Q TRIM28 only	ZNF98	ZNF140	ZNF182	ZNF626	ZNF681	ZNF737	ZNF878	

doi:10.6342/NTU202004036

b. GID/CTLH complex identified in mass spectrum

Gene Name	Score	Unique peptides	emPAI	MW
GID E3 ubiquitin ligase complex				
GID8 (TWA1)	1438	27	96.82	26732
MAEA (GID9)	1041	29	14.24	45258
YPEL5	312	9	13.87	13833
WDR26 (GID7)	1993	37	11.08	72079
RANBP9 (GID1)	2284	30	6.21	77798
RMD5A (GID2)	548	18	5.28	43964
RMD5B	141	7	0.96	44386
RBP10	711	21	3.3	67214
ARMC8 (GID5)	629	21	2.28	75460
MKLN1(Muskelin1)	315	18	1.48	84713
GID4	66	3	0.46	33493

c. MAGE family proteins identified in mass spectrum

Gene Name	Score	Unique peptides	emPAI	MW
MAGE proteins				
MAGEA9	521	13	3.82	35066
MAGEC2	675	13	3.24	41137

doi:10.6342/NTU202004036

4. TRIM28 deletion mutation sequence design

Trim28-delta C20-F
5'-TGA AGC TGG GGC TCT TGT GG-3'
Trim28-delta C20-R
5'-TGG TGG TGG TTC TAC CAG CAC-3
mTrim28(delta RING)-F
5'-TAC TCC AAA GAC ATC GTG GAG-3
mTrim28(delta RING)-R
5'-AAG CTC CTG CGC CTC GCC AC-3'
mTrim28(delta BB)-F
5'-TTT TTG GAA GAT GCA GTG AG-3'
mTrim28(delta BB)-R
5'-GCA CTG GTT AGC ATC CTG GG-3'
mTrim28(RBCC)-F
5'-TGA AGC TGG GGC TCT TGT GG-3'
mTrim28(RBCC)-R
5'-TTT GAG GGC CCG ATG CAG CTG-3
mTrim28(delata CC)-F
5'-ATG ATT GTG GAT CCT GTG GAG-3
mTrim28 (delta CC)-R
5'-CTG GTA CTG ATG GTC CTT GTG-3'

doi:10.6342/NTU202004036

5. Cellular differentiation-related gene expression in RNA-seq analysis

a.

Gene symbol	Description	LogFC[a]	P val	Regulation
LIF	Leukemia inhibitory factor	9.427395	6.4E-09	Up
ZNF516	Znc finger protein 516	4.369112	3.6E-05	Up
LIFR	Leukemia inhibitory factor receptor	4.078841	5.24E-08	Up
FOSL2	FOS-like antigen 2	3.655176	2.1E-06	Up
ACVR1C	Activin A receptor	2.817457	0.000258	Up
INHBE	Inhibin beta E	-5.94428	6.6E-13	down
SETBP1	SET binding protein 1	-3.37967	0.000622	down
NUPR1	Nuclear protein transcriptional regulator 1	-3.25139	0.000416	down
SOX6	SRY (sex determining region Y)-box 6	-3.10725	2.87E-05	down
ESRRB	Etrogen-related receptor beta	-2.64624	0.00018	down

b.

logFC	logCPM	pval	FDR	Regulation	Chr	Start	End	Strand	GeneSymbol
-3.79698	1.976963	3.97E-06	0.001172	Down	19	35248981	35264134	-	ZNF599
-7.32672	-0.67391	0.000291	0.034134	Down	3	21459915	22414812	-	ZNF385D
-7.32672	-0.67391	0.000291	0.034134	Down	2	1.85E+08	1.86E+08	+	ZNF804A
-6.44354	3.229586	1.60E-11	4.52E-08	Down	X	84498997	84528368	+	ZNF711
5.381284	3.121825	6.60E-10	8.50E-07	Ups	19	20188803	20237885	+	ZNF90
8.769645	0.674928	2.57E-07	0.000118	Ups	19	23015003	23042224	+	ZNF723
5.913337	1.044747	2.84E-07	0.000125	Ups	19	22817126	22850472	+	ZNF492
5.140074	1.199657	6.45E-07	0.000247	Ups	19	20718631	20748615	-	ZNF737
5.635164	0.788516	1.42E-06	0.000476	Ups	19	22573821	22715287	-	ZNF98
4.247812	0.914955	1.34E-05	0.003156	Ups	5	1.78E+08	1.79E+08	+	ZNF354C
3.711928	1.357113	2.04E-05	0.0044	Ups	19	23158270	23185978	-	ZNF728
3.739116	1.136684	2.95E-05	0.005661	Ups	19	20107867	20150315	-	ZNF682
4.369112	0.504134	3.60E-05	0.006593	Ups	18	74069644	74207146	-	ZNF516
7.747529	-0.24428	4.53E-05	0.007953	Ups	19	56950694	56989434	-	ZNF667
3.202858	2.253861	4.68E-05	0.008174	Ups	19	58038693	58068910	+	ZNF549
2.943878	3.452231	5.76E-05	0.00972	Ups	19	20278037	20311299	+	ZNF486
7.584836	-0.38456	9.64E-05	0.014493	Ups	19	22867969	22869534	+	ZNF849P
4.707224	-0.03565	0.000127	0.018248	Ups	10	48355024	48373866	+	ZNF488
7.401436	-0.54022	0.000218	0.0272	Ups	4	331603	378653	+	ZNF141
4.515418	-0.19857	0.000306	0.034983	Ups	19	37095719	37119499	+	ZNF382
2.710521	2.273042	0.000345	0.038355	Ups	19	21106028	21133503	+	ZNF85
3.034924	0.977851	0.000398	0.042793	Ups	19	37407231	37489602	+	ZNF568

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8. Appendix

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H3AC48/139-269          DNAVENQRKALGLLVKQLGERSTSLQKSSKEVRTTIRSISEMQKRIQVEVRMAILMIMKE      60
A0A5G3KEA7/210-340      EDAVKNQRKVLSALVKRLGDKHTALQKSTKDVRTSIRQVSDLQKRLQVDVKMAILHIMKE      60
V8NNJ1/222-352          DDAVKNQRKMLASLVKRLGDKNANLQKSTKEVRTSIRQVADVQKRVQVDVKMSILQIMKE      60
Q62318/247-377          EDAVRNQRKLLASLVKRLGDKHATLQKNTKEVRSSIRQVSDVQKRVQVDVKMAILQIMKE      60
A0A484GIG4/250-380      EDAVRNQRKLLASLVKRLGDKHATLQKNTKEVRSSIRQVSDVQKRVQVDVKMAILQIMKE      60
Q13263/246-376          EDAVRNQRKLLASLVKRLGDKHATLQKSTKEVRSSIRQVSDVQKRVQVDVKMAILQIMKE      60
                        ::**.**** *.  ***:**:: : ***.:*:**::**.::::***:**:*:*:** ****

H3AC48/139-269          LNKRGKSLMNDSQKFTEAHQEKLERQHWSMTKLQRHYEHVIRFGSWALSSDNNTALLLCK      120
A0A5G3KEA7/210-340      LNKRGKLLVNDMQKVTEGHQDKLEKQHWAMNKLQKHQEHILRFATWALESDNNIALLLSK      120
V8NNJ1/222-352          LNKRAKVLVSDAQKVTEGQQEKLERQHWAMTKLQRHQEHILRFANWALESDNNTALLLSK      120
Q62318/247-377          LNKRGRVLVNDAQKVTEGQQERLERQHWTMTKIQKHQEHILRFASWALESDNNTALLLSK      120
A0A484GIG4/250-380      LNKRGRVLVNDAQKVTEGQQERLERQHWTMTKIQKHQEHILRFASWALESDNNTALLLSK      120
Q13263/246-376          LNKRGRVLVNDAQKVTEGQQERLERQHWTMTKIQKHQEHILRFASWALESDNNTALLLSK      120
                        ****.: *:.* **.**.:*::**:***:*.*:*:* **::**..***.**** ****.*

H3AC48/139-269          KMISYQLQRAL      131
A0A5G3KEA7/210-340      KLIHFQLHRAL      131
V8NNJ1/222-352          KLIYFQLHRAL      131
Q62318/247-377          KLIYFQLHRAL      131
A0A484GIG4/250-380      KLIYFQLHRAL      131
Q13263/246-376          KLIYFQLHRAL      131
                        *:* :**:***
```

An alignment displays the following symbols denoting the degree of conservation observed in each column:

An * (asterisk) indicates positions which have a single, fully conserved residue.

A : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix.

A . (period) indicates conservation between groups of weakly similar properties - scoring =< 0.5 in the Gonnet PAM 250 matrix.

Human K304 and its homologs are highlighted in red.

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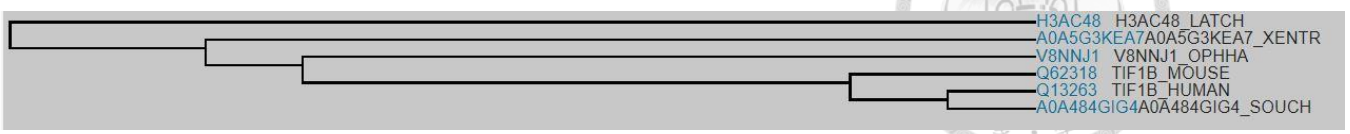
Cladogram:



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