2014 年臺灣國際科學展覽會
優勝作品專輯

作品編號　**080006**

參展科別　生物化學

作品名稱　**HDAC 抑制劑及 Ribavirin 誘發 K562 細胞分化的研究**

得獎獎項　大會獎：四等獎

就讀學校　臺北市立第一女子高級中學

指導教師　許一懿、呂勝春

作者姓名　羅瑞恩

關鍵字　**K562 細胞、SAHA、Ribavirin**

作者簡介



我是羅瑞恩，目前就讀於北一女中數理資優班。我喜歡涉獵各領域的知識，從校內的國畫優等、北市生物能力和英語單字競賽得到三等獎以及生物奧林匹亞初選通過等，我接受了許多跨領域的挑戰。專題研究對於我更是嶄新的經歷，從蒐集資料、閱讀論文、操作實驗、整理資料、到撰寫報告等，都是我十分難能可貴的經驗。從青培計畫一步步走上來，在臺大醫院分醫所計時器催人的嗶剝聲中度過無數充實的日子，終於站在國際科展的舞台上，一路上的鼓勵與挫折都鞭策著我勇往直前。這都要感謝十分關切我的呂教授、老師們和實驗室學長姐，以及永遠支持我的朋友和爸爸媽媽。

摘要

K562 細胞是一種人類慢性骨髓性白血病細胞株，普遍用於研究幹細胞分化的先驅細胞，可以藉不同誘導劑分化成紅血球或巨核細胞。此研究聚焦於兩種臨床藥物：抗病毒藥物 Ribavirin 和組蛋白去乙醯化抑制劑 SAHA，探討其誘導 K562 細胞紅血球分化機制。其中針對一個在許多分化機制扮演重要角色的染色質修飾蛋白 TIFβ來研究，它可以召集其他轉錄因子在基因上形成抑制複合體。我們藉由定點突變探討 TIFβ 上的 bromo 或 RBCC domain 的乙醯化狀態對於 TIFβ與抑制複合體的轉錄因子交互作用的影響。研究結果發現乙醯化 RBCC domain 大幅影響了 TIFβ與轉錄因子的親和力，並且降低 TIFβ三聚體的形成，可能導致抑制複合體無法形成進一步開啟了分化的通道。

Abstract

K562 is a human erythroleukemia cell line and has been used as a common progenitor for studying stem cell differentiation. It can be induced into erythrocytes or megakaryocytes by many inducing agent. This study investigated the mechanism of the K562 cell erythroid differentiation induced by two clinical medicines, including ribavirin, treatment for virus infection, and SAHA, an HDAC inhibitor. My research focused on one epigenetic modifier, TIF1β, which involved in many other pluripotent cell differentiation. TIF1β can recruit other epigenetic modifiers to form a repressive complex on genes. By performing site-direct mutagenesis, we analyze the effects of acetylated bromo or RBCC domains of TIF1β on the interaction between TIF1β and the protein of the repressive complex. Our results showed that acetylated RBCC domain significantly affected the affinity between TIF1β and the modifiers, and the formation of TIF1β homotrimer, suggesting that it may hinder the formation of the repressive complex and lead to the differentiation pathway.

I. Introduction

A. Literature Review

1. K562 cells

Human erytholeukemia K562 cells were deprived from the pleural effusion of a patient with chronic myelogenous leukemia (CML) in terminal blast crisis (1), having the Philadelphia chromosome t(9;22 chromosomal translocation) (2). They have been used as a model of common progenitor for studying stem cell differentiation. When treated with ribavirin, hemin, HDAC inhibitors (i.e. SAHA) or other reagents (i.e. mycophenolic acid), K562 cells can be differentiated into erythrocytes. When treated with 12-O-tetradecanoylphobol-13-acetate (TPA, PMA), K562 cells differentiate into megakaryocytes (3, 4, 5). During cell differentiation, the pattern of gene expression changes (3).

2. TIF1β

Transcription intermediary factor 1-beta (TIF1β) is an epigenetic modifier with 834 amino acids, also called TRIM28 (tripartite motif-containing protein 28), KAP1 (KRAB-associated protein 1), or KRIP1 (KRAB-A-interacting protein 1). TIF1β is a critical regulator of normal development and differentiation. TIF1β is also involved in maintaining pluripotency, and has been associated with promoting and inhibiting differentiation of different adult cell types (6).

TIF1β contains four domains (Fig.1), including RBCC, HP1 box, PHD,

and bromo domains (6). It is known that TIF1β represses genes by recruiting SETDB1 (SET domain, bifurcated 1, an H3K9me3-specific histone methyltransferase), HP1 (heterochromatin protein 1), and the NuRD (nucleosome remodeling and histone deacetylation) complex (6). Posttranslational modifications such as phosphorylations of TIF1β (i.e., Ser473 and Ser 824) are known to have profound effects on its activity. Phosphorylations sites of a protein are generally located in the unstructured region (as exemplified by TIF1β/Ser473 and Ser824) while acetylations are in the domain region. The known acetylation sites of TIF1β are located in the RBCC and bromo domains, respectively. It would be important to address whether these acetylations have impacts on the functions of TIF1β.

TIF1β is also known to play important repressive function for the control of Moloney murine leukemia virus (M-MLV) replication in embryonic carcinoma (EC) and embryonic stem (ES) cells. Proviral DNAs are silenced at the level of transcription by TIF1β that binds to the primer-binding site (PBS) of M-MLV and repress transcription from the viral promoter (11). Furthermore, the results of inducible depletion of TIF1β in ES cells showed a modest increase in LINE1 (long interspersed nuclear elements 1) transcripts after TIF1β-removal, but a marked upregulation of endogenous retroviruses (ERVs), in particular IAP element (12). Thus, TIF1β-mediated repression could contribute to the control of ERVs. Ribavirin, a guanosine analog, could potentially inhibit proviral DNA transcription of retrovirus. This action could be linked to the repressive function of TIF1β.

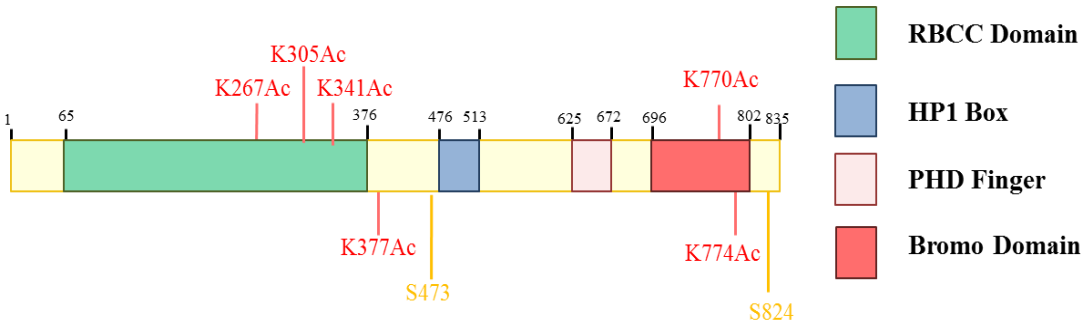


Fig.1. Schematic diagram of TIF1β

3. RBCC domain

RBCC (Ring (really interesting new gene) finger, two B-box zinc fingers, and a coiled coil) is necessary for TIF1β to binds as a homotrimer via the interaction of TIF1β with the KRAB repression module of KRAB-ZNFs (7).

4. HP1 box

TIF1β-HP1 complex can be formed via the interaction between HP1 box on TIF1β and the chromoshadow domain of HP1. TIF1β-HP1 complex have high affinity to H3K9me3 because of the ability of HP1 to bind to H3K9me3 (6).

5. PB domain

PB domain (PHD (Plant Homeo Domain) and bromodomain), functioning as transcriptional repression, can interact with NuRD complex and SETDB1 (6). Bromo domain is found in transcriptional activators and recognizes acetylated lysine residues such as those on the N-terminal tails of histones. This recognition is often a prerequisite for protein-histone association and chromatin remodeling.

6. Ribavirin

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been widely used for the treatment of DNA and RNA virus infections such as severe RSV, acute myeloid leukemia (AML) and hepatitis C. It is a member of the nucleoside anti-metabolite drugs that interfere with viral replication, yet the mechanism is still unknown. Its activities against viruses were first described nearly 40 years ago. In combination with interferon, it has become part of the standard for the treatment of hepatitis C infection (8). The hemoglobin produced in the ribavirin-treated K562 cells consisted of approximately 60% fetal hemoglobin and its acetylated equivalents. The adult-type α-globin was found, while no β-globin chains were demonstrated. Thus, accumulation of fetal hemoglobin and production of α-globin chain in ribavirin-treated K562 cells are different from the pattern of hemoglobins induced by hemin (9).

7. SAHA

SAHA (or called Vorinostat) is an HDACi and is marketed under the name Zolinza for the treatment of cutaneous T cell lymphoma (CTCL) when the disease persists, gets worse, or comes back during or after treatment with other medicines. Since SAHA is a HDAC inhibitor, the SAHA-induced K562 cell differentiation is believed mainly through the inhibition of HDACs (i.e., HDAC1 and 2) and histone acetylation. However, its effects on epigenetic modifiers other than histones remain poorly understood.

B. Motivation

In recent years, HDAC inhibitors have emerged as potential treatments for cancer. However, the mechanism of how they work remains unclear. SAHA, one of

the HDAC inhibitors now in clinical use, is known to induce K562 cells, a cancer cell line which is an excellent model of progenitor for differentiation, into erythrocytes. By studying the functions of acetylated lysine on the domain of TIF1β, a well-known regulatory protein, the mechanism of SAHA-induced K562 could be partially explained. On the other hand, ribavirin, also a clinical treatment, is known to induce K562 cells into erythrocytes as well. The differences and similarities between these two systems of differentiations are also unknown.

II. Materials and Methods

A. Research Flow

Compare the effects on K562 cell growth and differentiation between SAHA, Ribavirin, PMA treated and control
✧ Treat K562 cells with Ribavirin/SAHA/PMA → Morphologic analysis → Plot growth curve → RT-PCR→Compare the induction of α-, β-and γ-globin
Study the roles of acetylated TIF1β in K562 differentiation
Site-directed mutagenesis : Create TIF1β–RBCC/ bromodomain acetylation mimetics ✧ Plasmids of wild type TIF1β→PCR→DnpI→Clean up→DH5α Transformation→mini plasmid prep.→sequencing→Transfect into 293T cells→midi plasmid prep.
Analyze the binding between acetylated peptides and TIF1β–bromodomain acetylation mimetics ✧ Transfect 293T cells with TIF1β–bromodomain acetylation mimetics→ nuclear extract→pull down with acetylated peptides→SDS-PAGE
Analyze the binding between HP1 and TIF1β–RBCC/ bromodomain acetylation mimetics ✧ Transfect 293T cells with TIF1β–RBCC/ bromodomain acetylation mimetics and HP1→ nuclear extract→immunoprecipitate by M2 beads→SDS-PAGE
Analyze the binding between SETDB1 and TIF1β–RBCC/ bromodomain acetylation mimetics ✧ Transfect 293T cells with TIF1β–RBCC/ bromodomain acetylation mimetics and SETDB1→ nuclear extract→immunoprecipitate by M2 beads→SDS-PAGE
Analysis of the fractionated nuclear extracts of TIF1b-WT/ 3Q ✧ Nuclear extracts of TIF1b-WT/ 3Q were fractionated by FPLC and probed with anti-Flag, anti-TIF1β, anti-S473 antibodies.
Electroporation
Mutant K562 differentiation

B. Cells and virus

1. K562 cells (Human erythromyeloblastoid leukemia cell line)
2. *Escherichia coli* (strain:DH5α)
3. 293T cells

C. Reagents

1. Iscove's modified Dulbecco's medium (IMDM)(from GIBCO)
2. glutamine (200 mM stock) (from GIBCO)
3. penicillin/streptomycin (10^4 units) (from GIBCO)
4. Ribavirin (from Sigma-Aldrich)
5. SAHA (from Sigma-Aldrich)
6. PMA (Biomed)
7. Monoclonal antibodies to TIF1β (clone 20A1), acetylated-Lysine (clone 15G10) , Rabbit anti-epitope-tag FLAG, and histone H4

D. Methods

1. Cell Cultures

K562 cells were cultured in IMDM supplemented with 10% FCS, 100 units penicillin/streptomycin/ml and 2 mM glutamine. The cells were kept at approximately 5×10^6/ml in a T-75 flask at 37℃, 5% CO_2 in a humidified incubator.

2. Induction of K562 cells differentiation

a. Morphologic examination of Ribavirin, SAHA, PMA-treated and control K562 cells

Ribavirin, SAHA, PMA-treated and control K562 cells were cultured for 4 days and harvested. The colors and the appearances of the cells were examined.

b. Compare the growth curve of Ribavirin, SAHA, PMA-treated and control K562 cells

Ribavirin-treated and control K562 cells were harvested on day1, 3, 5 and 7. SAHA, PMA-treated and control K562 cells were harvested on day1, 3 and 5. The cells were calculated by use of hemacytometer.

c. Compare the expression levels of mRNA of α-, β-and γ-globin between Ribavirin, SAHA, PMA-induced and control K562 cells

Ribavirin-treated and control K562 cells were harvested on day1, 2 and 3. SAHA, PMA-treated K562 cells were harvested on day 3. Total RNA was extracted by TRIZOL reagent, and converted to cDNA using the High-Capacity cDNA Reverse Transcription Kits. Target sequence was probed and amplified by PCR with α-, β-and γ-globin former and reverse primers. After agarose gel electrophoresis, samples were stained by EtBr and exposed to UV light.

3. Site-directed mutagenesis

Site-directed mutagenesis was made to analyze the effects of acetylated TIF1β-bromo and RBCC domain. Two acetylation sites in bromodomain, K770

and 774, and three in RBCC domain, K267, 305 and 341, have been found. Site-directed mutants were created on those sites to mimic acetylated status. The lysines of acetylation sites of bromo or RBCC domains were changed into glutamines or arginines to mimic acetylated or original lysines respectively.

A pair of complementary mutagenic primers was used to amplify the entire plasmid by PCR using pfu polymerase. Methylated DNA produced by *E. coli* was digested by DpnI, a restriction enzyme which is specific for methylated DNA. Therefore the mutated plasmid generated by PCR would be left intact. Once the mutated plasmids were cleaned up, they were transformed into DH5α competent cells. Mutated plasmids produced by DH5α were sequenced. The mutant plasmids were transfected into 293T cells for producing mutant recombinant TIF1β.

4. The interaction between acetylated peptides and TIF1β-bromodomain acetylation mimetics

Since bromodomain is known to interact with acetylated lysine, Pull-down assay is performed to test whether wild type or TIF1β-bromodomain acetylation mimetics can bind to acetylated peptides, using p300/K1020, p300/K1024 and H3K9Ac peptides. The acetylated peptides were immobilized to the beads via sulfolink and used to pull-down the recombinant TIF1β. The captured recombinant proteins were then analyzed by Western blot.

5. The interaction between HP1γ/SETDB1 and TIF1β-RBCC/ bromodomain acetylation mimetics

To assess the effects of acetylation of RBCC/ bromodomain of

TIF1β on its interaction with HP1/ SETDB1, Flag-tagged wild type or TIF1β acetylation mimetics were co-transfected with HA-HP1-γ/SETDB1 into 293T cells. Nuclear extracts were immunoprecipitated by M2 beads. The immunoprecipitated complex was probed with rabbit anti-Flag and -HA antibodies.

6. Preparation of nuclear extracts

HEK293T cells were transfected with 4 μg of pCMV-Flag-TIF1β -WT or pCMV-Flag-TIF1β -3Q/10-cm plate with Turbofect at ~50% confluent culture. After 16 hours of transfection, the cultures were changed to fresh DMEM/10% FCS medium and the cultures were kept for additional 24 hours. Cells pooled from three plates of each plasmid transfectant were washed with 10 ml of cold PBS, centrifuged at 1,500 xg for 5 min. The cell pellets were resuspended in 1 ml PBS in microcentrifuge vial and centrifuged for 15 seconds at 8,000 rpm. The pellets were resuspended in 400 μl of ice cold buffer A (10 mM Hepes, pH7.9, 10 mMKCl, 0.1 mMEDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mMPMSF) and pipetting gently with yellow tip. The suspended cells were kept on ice for 15 min before adding 25 μl of 10% NP-40. The cells were vortexed rigorously for 10 seconds and centrifuged for 30 seconds in a microcentrifuge at 8000 rpm. The pellets were resuspended in 200 μl of ice-cold buffer C (20 mM Hepes, pH7.9, 0.42M NaCl, 1 mM EDTA, 1 mM DTT and 1 mMPMSF), vortexed vigorously for 30 seconds and kept on ice for 15 min. The nuclear extracts were collected by centrifuged at 12,000 rpm for 30 min.

7. Fractionation of TIF1β by Superdex200 FPLC

All operations were performed at 4^{o}C. Superdex200 column (1.0 x 30 cm)

was equilibrated with buffer C. 0.2 ml (~1 mg) nuclear extracts (in buffer C) prepared from HEK293T cells transfected with pCMV-Flag-TIF1β -WT or pCMV-Flag-TIF1β -3Q were loaded onto the column. The column was eluted with buffer C at 0.4 ml/min and 0.5 ml fraction was collected.

The Superdex 200 column was calibrated with thyroglobulin (670 kDa), IgG (160 kDa), ovalbumin (44 kDa) and myoglobin (17 kDa) (Appx.H).

8. Immunoblotting analysis of the fractionated nuclear extracts

The elution profiles of recombinants Flag-TIF1β–WT and Flag-TIF1β-3Q were assayed by Western blot using anti-Flag, anti-TIF1β (clone 20A1) and anti- phospho-TIF1β/Ser473 antibodies. 0.4 ml of each fraction was precipitated with 5% (v/v) TCA for 30 min on ice, centrifuged in a microcentrifuge for 10 min at 12000 rpm. The pellets were washed with ice cold 95% ethanol. The pellets were dried briefly in a 50^{o}C dry bath. The pellets were dissolved in 40 μl of SDS sample buffed, heated at 95^{o}C for 5 min. For immunoblotting, 5μl sample was separated in 7.5% SDS and blotted onto the membrane and probed with antibodies.

III. Results

A. Ribavirin- and SAHA-induced K562 cells differentiate into erythrocytes

Morphological observation of induced K562 cells indicated that ribavirin- or SAHA-treated cells appeared reddish while the control cells were yellowish (Fig.2). The ribavirin- and SAHA-treated K562 cells showed similar and significant growth retardation compared to control (Fig.3, 4). All K562 cells express α-globin, while both ribavirin- and SAHA-treated cells express relatively higher level of α-globin (Fig.5, 7). No β-globin was delectable in any of the cells (Appx.B, C). Both ribavirin- and SAHA-treated K562 cells showed distinct expression of γ-globin (Fig.6, 8).

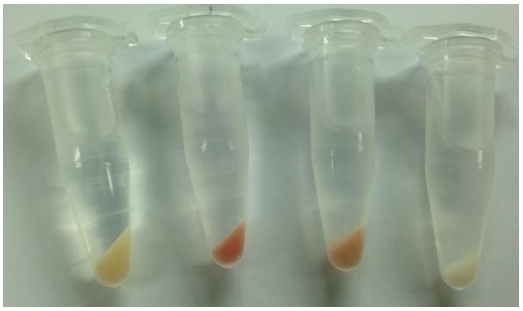


Fig.2. Left to right: control, Ribavirin, SAHA, and PMA-treated K562 cells for five days. PMA-treated K562 cell is known to differentiate into megakaryocytes.

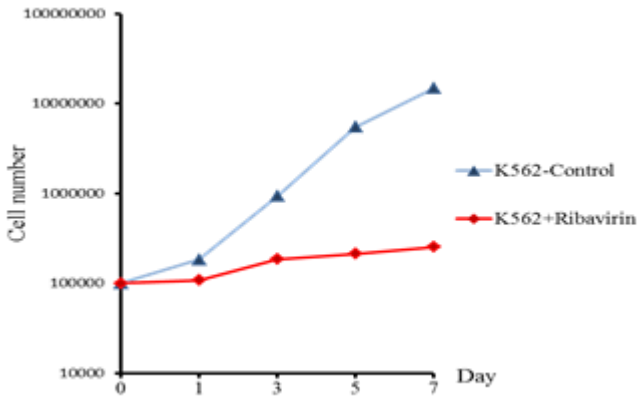


Fig.3. Growth curve of Ribavirin-treated and control K562 cells

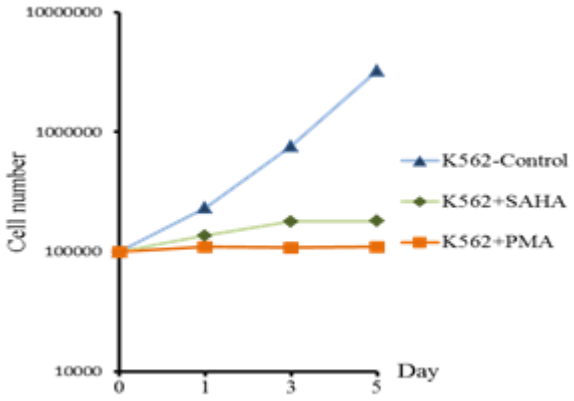


Fig.4. Growth curve of SAHA, PMA -treated and control K562 cells

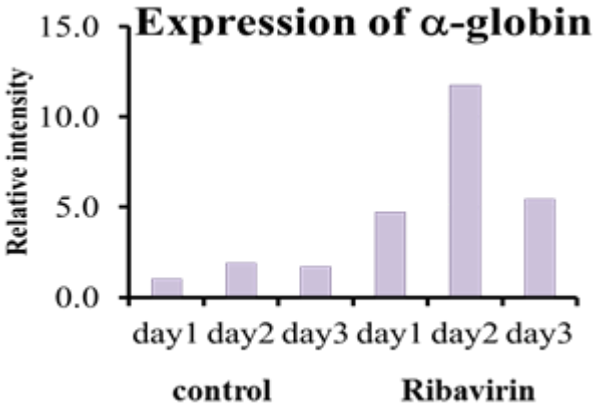


Fig.5. The relative expression levels of α-globin in control and ribavivin-treated K562 cells.

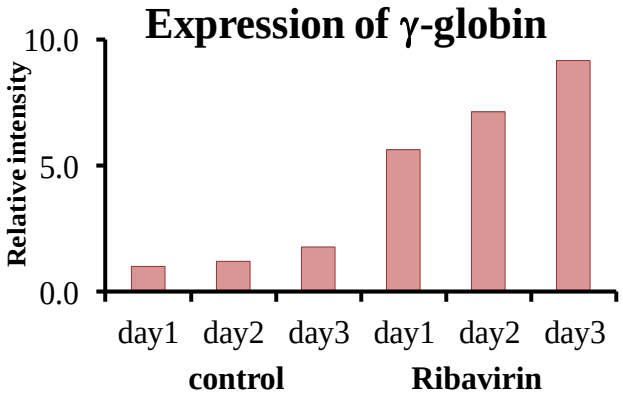


Fig.6. The relative expression levels of γ–globin in control and ribavivin-treated K562 cells.

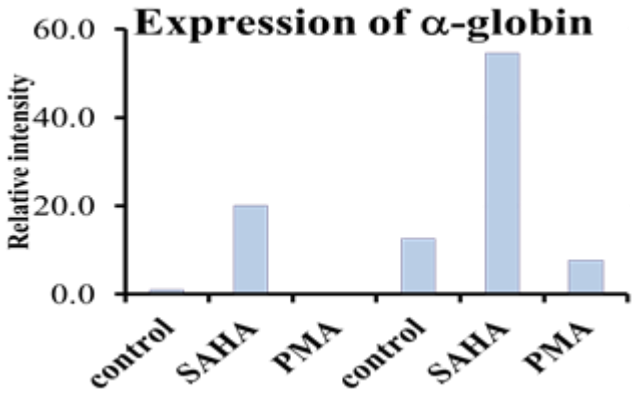


Fig.7. The relative expression levels of α–globin in control, SAHA- and PMA-treated K562 cells.

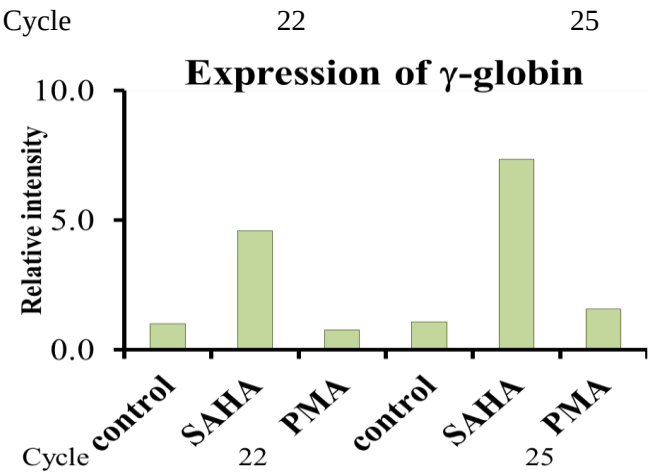


Fig.8. The relative expression levels of γ–globin in control, SAHA- and PMA-treated K562 cells.

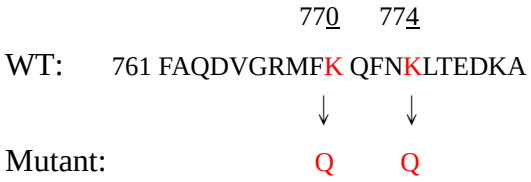
B. Site-directed mutagenesis of TIF1β (Mus musculus) for generating acetylation mimetic mutants

The sequences of these mutants were shown below.

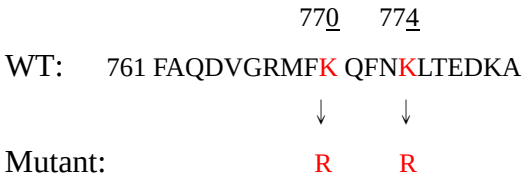
1. TIF1β–RBCC domain acetylation mimetics sequence:

	267	305	341
WT:	261 VKRLGDKHAT	301 LQIMKELNKR	341KHQEHILRFA
	↓	↓	↓
Mutant:	Q	Q	Q

2. TIF1β–bromodomain acetylation mimetics sequence:



3. TIF1β–bromodomain original mimetics sequence:



C. Interaction between WT-TIF1β, TIF1β–bromodomain acetylation mimetics and acetylated peptides

The interaction between WT-TIF1β, TIF1β–bromodomain acetylation mimetics and acetylated p300/K1020, p300/K1024 and H3K9Ac peptides were negative (Appx. E).

D. Interaction between TIF1β, acetylation mimetics of TIF1β and SETDB1 or HP1-γ

The immunoprecipate assay indicated that TIF1β–RBCC domain acetylation mimetics, abbreviated as 3Q, significantly impaired its ability to interact with either SETDB1 or HP1-γ. TIF1β–bromodomain acetylation mimetics, abbreviated as 2Q, had no effect on its interaction with SETDB1, but enhanced its interaction with HP1-γ (Fig.9, 10).

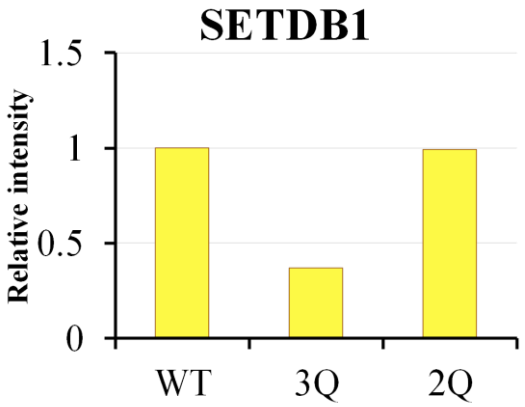


Fig.9. Relative binding affinity with SETDB1 between TIF1β-WT, 3Q and 2Q.

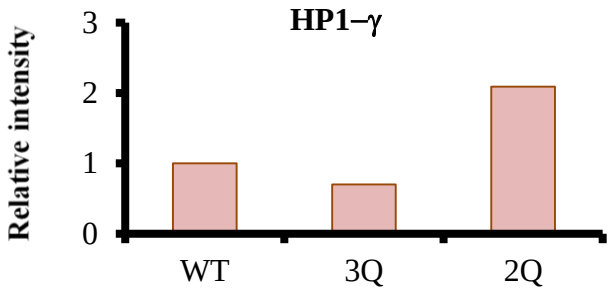


Fig.10. Relative binding affinity with HP1-γ between TIF1β-WT, 3Q and 2Q.

E. Elution profiles of recombinant TIF1β–WT and TIF1β-3Q

The elution profiles of nuclear extracts from Superdex200 column were shown below (Fig. 11, 12). When probed with anti-Flag antibody, the recombinant TIF1β–WT appeared mainly in fractions 19-21 with peak at fraction 19 and 21. However the distribution of TIF1β–WT could be found from fractions17-23 (Fig. 13, Appx. I). The distribution of TIF1β-3Q was found from fractions 16-23 with peak at fractions 21-22. Peak fraction 19 appeared to be trimeric form while fractions 21 appeared to be monomeric form (Fig. 14, Appx. J). The wide distribution from high molecule weight to monomeric form fractions of both TIF1β–WT and TIF1β-3Q suggest that they can associate with many other proteins.

When probed with anti-phospho-TIF1β/Ser473 monoclonal antibody, the signals could be detected in fractions 20 and 21 with major signal in fraction 21.

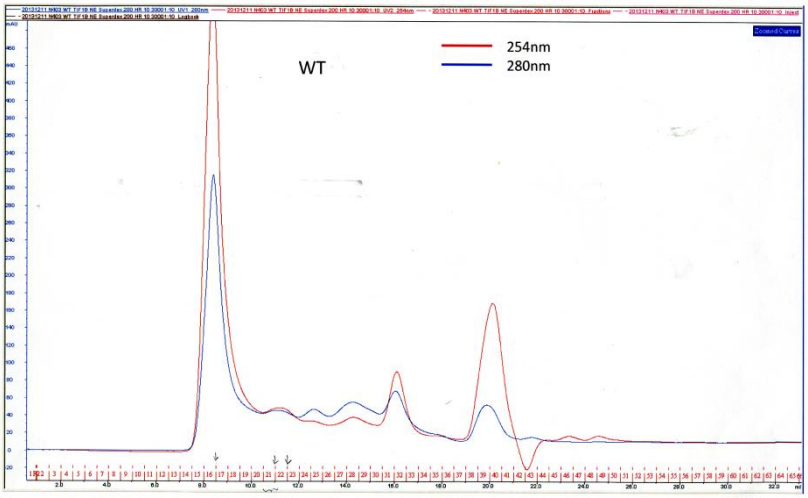


Fig.11. The elution profiles of nuclear extracts of TIF1β-WT

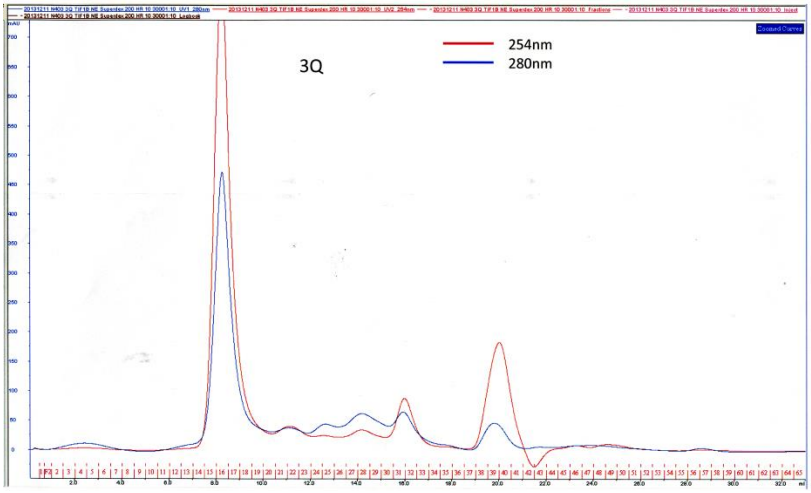


Fig.12. The elution profiles of nuclear extracts of TIF1β-3Q

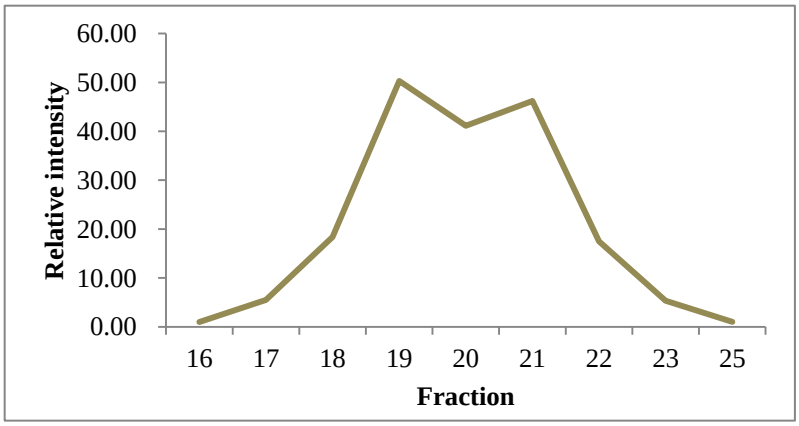


Fig.13. The relative intensity of TIF1β in the nuclear extracts of TIF1β-WT

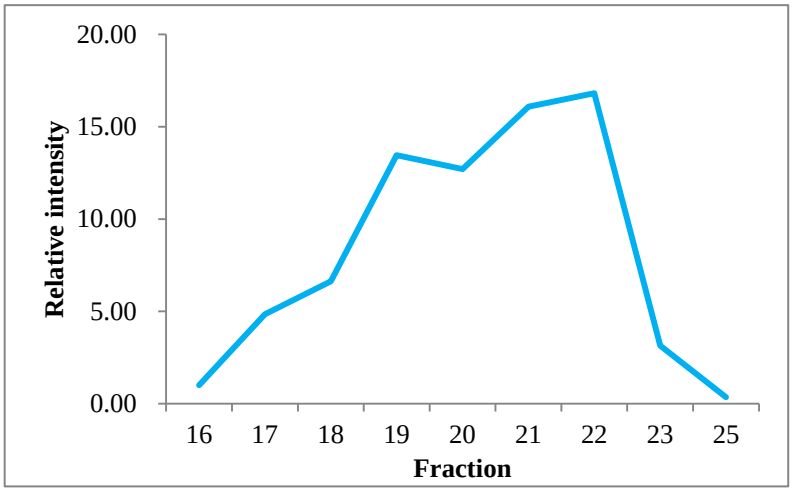


Fig.14. The relative intensity of TIF1β in the nuclear extracts of TIF1β-3Q

F. The expression level of S473 phosphorylation of recombinant TIF1β–WT and TIF1β-3Q

The elution profiles of nuclear extracts from Superdex200 column were shown below (Fig. 15, 16, Appx. K). When probed with anti-S473 antibody, the recombinant TIF1β–WT and TIF1β-3Q appeared mainly in fractions 20-21 with peak at fraction 21.

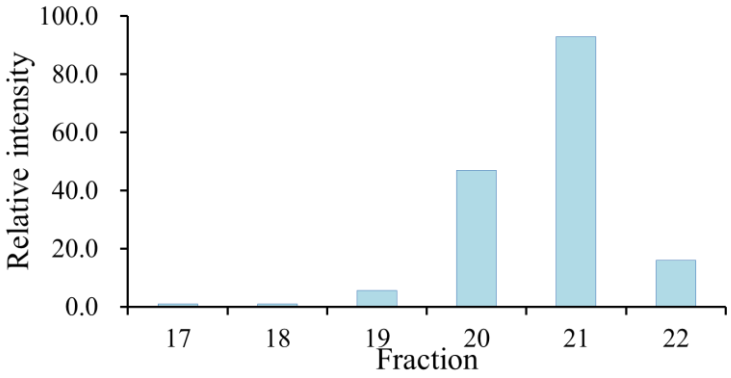


Fig.15. The relative level of phosphorylation at S473 in the nuclear extracts of TIF1β-WT

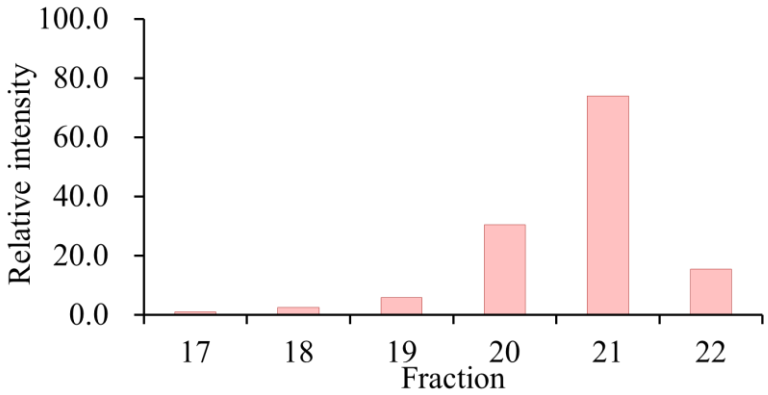


Fig.16. The relative level of phosphorylation at S473 in the nuclear extracts of TIF1β-3Q

IV. Discussion

A. The differentiation pathway of SAHA-treated K562

The results showed that acetylated RBCC domain of TIF1β could negatively affect its interaction with SETDB1 and HP1-γ. Since RBCC domain is necessary for TIF1β to form active homotrimer, it is likely that these acetylated TIF1β failed to mature to fully active form.

RBCC domain of TIF1β is its co-repressor functions by binding to KRAB domain of KRAB domain-containing zinc-finger proteins (ZFPs). RBCC domain is also known to mediate trimer formation and thus activation of TIF1β. Therefore, posttranslational modifications of TIF1β RBCC domain may have profound effects in its activity. The functions of acetylations in the RBCC domain are completely unknown up until now. We have found that acetylation mimetic TIF1β-3Q appeared to favor monomeric form in the cells. Thus, acetylation/deacetylation in the RBCC domain may constitute of a switch from monomeric to trimeric (or oligomeric) form; i.e., from the form unable to bind to chromatin to able to bind to chromatin. This switch may also reflect the availability of energy-rich acetyl-CoA. In the highly proliferated cells with abundant acetyl-CoA, it is likely easier to switch from trimeric to monomeric form and vise versa. Additional layer of regulation may be at phosphorylation of TIF1β/Ser473. Intriguingly, the crosstalk between these acetylation and phosphorylation events could be a very precise regulatory mechanism that controls the TIF1β-mediated gene expression in the proliferation and differentiation of embryonic stem cells.

A complex between TIF1β and KRAB-domain-containing DNA-binding zinc

finger protein, ZFP57 has recently been shown to interact with DNA methyltransferases providing a likely mechanism for its function in the maintenance of DNA methylation (13, 14). These results reminisce the loss of TIF1β activity via acetylation of its RBCC domain, depletion of TIF1β, or treatment with 5-azacytidine (to inhibit DNA methylation). Together, our present results are highly significant in that we have uncovered the physiological switch for down-regulation of TIF1β activity through acetylation and modulation of gene expression by epigenetic mechanisms. These conclusions are depicted as a model (Fig.17).

This phosphorylated TIF1β/Ser473 apparently correlated with the peak fraction of both TIF1β–WT and TIF1β-3Q. The peak fraction of phosphorylated TIF1β/Ser473 appeared to be monomeric form of recombinant TIF1β–WT and TIF1β-3Q. Taken together, these results suggest that only monomeric form of TIF1β can be phosphorylated at Ser473. The Ser473 phosphorylated monomeric form of TIF1β is unable to bind to HP1 and associated with chromatin (15). Consistent with this notion, the interaction between TIF1β-3Q and SETDB1 is compromised as compared to TIF1β–WT. When K562 cells were induced differentiation to megakaryocytes by TPA, the level of phosphorylated TIF1β/Ser473 is reduced suggesting that binding of trimeric (or oligomeric) form of TIF1β to chromatin is mainly associated with proliferating cells. These results further suggest that proper silencing of differentiation-related genes by co-repressor TIF1β is important events for cell differentiation.

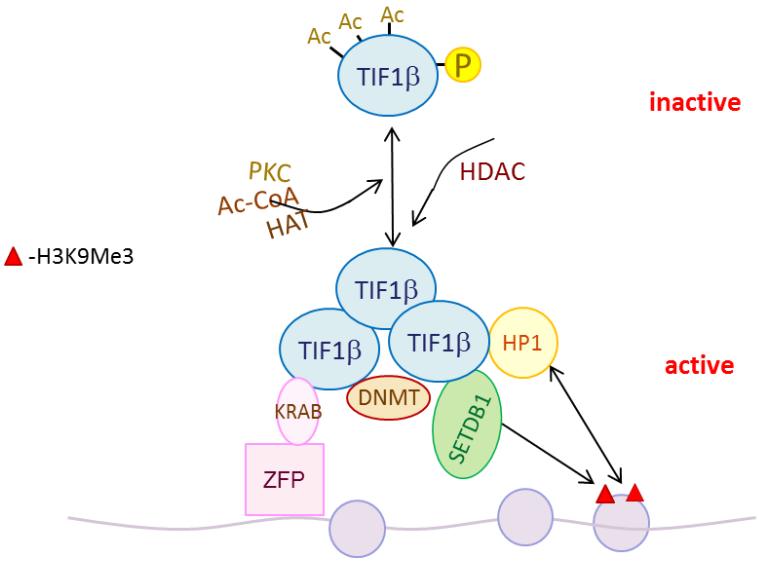


Fig.17. Model depicting how the activity of TIF1β is regulated by acetylation of RBCC domain and phosphorylation at S473

B. The differentiation pathway of ribavirin-treated K562

There are two potential mechanisms listed below of how ribavirin induce K562 cells differentiate into erythrocytes.

1. First, the differentiation may be linked to the ability of ribavirin to interfere with virus replication. Since ribavirin resembles one of the nucleotides, guanosine, it may be inserted into RNA genome of virus during replication and induce hypermutation. Ribavirin is then converted to ribavirin 5'-monophosphate and inhibits cellular inosine monophosphate dehydrogenase (IMPDH), a key enzyme for purine nucleotides synthesis. This may explain why ribavrirn can inhibit DNA viral replication. Since both ribavirin and mycophenolic acid could induce K562 cell differentiation, the energy metabolism, including ATP and acetyl-CoA levels, is severely affected by IMPDH inhibition. The reduced level of acetyl-CoA could affect the activities of other metabolic enzymes and epigenetic modifiers.

2. Second, the differentiation may result from the epigenetic regulation of

endogenous retrovirus (ERV). TIF1β has been identified as the retroviral silencing factor in pluripotent cells (10, 11), and its deletion was shown to lead to a marked upregulation of a range of ERVs in mouse embryonic stem (ES) cells and in early embryos (12). Although the etiological link between potential retrovirus and derivation of K562 cells is unknown, it cannot be ruled out that some retroviral silencing mechanisms may be involved. The converged impact of ribavirin and TIF1β on epigenetic silencing may be tested by assaying the transcription of ERV genes (e.g. the expression of LINE1) by combination of depletion of TIF1β and treatment with ribavirin. Finally, the acetyltransferase(s) responsible for the acetylation of TIF1β should be identified.

V. Conclusions

A. SAHA-induced K562 cell differentiation may be linked to the acetylation of TIF1β RBCC domain, affecting the formation of homotrimer, leading to failure for forming repressive complex and activating erythroid genes expression. The acetylation and phosphorylation events could be a very precise regulatory mechanism that controls the TIF1β-mediated gene expression in the proliferation and differentiation of embryonic stem cells.

B. Ribavirin-induced K562 cell differentiation may result from the ability of ribavirin to interfere with virus replication or from the epigenetic regulation of endogenous retrovirus (ERV).

VI. References

1. Lozzio CB, Lozzio BB. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45: 321-334.

2. Bütler TM, Ziemiecki A, Friis RR. (1990) Megakaryocytic Differentiation of K562 Cells Is Associated with Changes in the Cytoskeletal Organization and the Pattern of Chromatographically Distinct Forms of Phosphotyrosyl-specific Protein Phosphatases. Cancer Res 50: 6323-6329.

3. Huo XF, Yu J, Peng H, Du ZW, Liu XL, Ma YN, Zhang X, Zhang Y, Zhao H.L, Zhang JW. (2006) Differential expression changes in K562 cells during the hemin-induced erythroid differentiation and the phorbol myristate acetate (PMA)-induced megakaryocytic differentiation. Molecular and Cellular Biochemistry 292: 155–167.

4. Tsiftsoglou A S, Pappas I S, Vizirianakis I S. (2003) Mechanisms involved in the induced differentiation of leukemia cells. Pharmacology & Therapeutics 100: 257–290.

5. Osti F, Corradini FG, Hanau S, Matteuzzi M and Gambari R. (1997) Human leukemia K562 cells: induction to erythroid differentiation by guanine, guanosine and guanine nucleotides. Haematologia 82:395-401.

6. Iyengar S and Farnham JP. (2011) KAP1 Protein: An Enigmatic Master Regulator of the Genome. JBC VOL 286, NO. 30, pp.26267-26276.

7. Peng H, Feldman I, Rauscher F J 3rd. (2002) Hetero-oligomerization among the TIF family of RBCC/TRIM domain-containing nuclear cofactors: a

potential mechanism for regulating the switch between coactivation and corepression.J. Mol. Biol. 320, 629–644

8. Taunton J., Collins J.L., Schreiber S.L. (1996) Synthesis of Natural and Modified Trapoxins, Useful Reagents for Exploring Histone Deacetylase Function. J. Am. Chem. Soc 118: 10412-10422.

9. Yu J, Smith RD. (1986) Sequential alterations in globin gene chromatin structure during erythroleukemia cell differentiation. J Biol Chem 260:3035-3040.

10. Ellis J, Hotta A, Rastegar M. (2007) Retrovirus silencing by an epigenetic TRIM. Cell 131:13-14.

11. Wolf D, Goff SP. (2007) TRIM 28 mediates primer binding site-targeted silencing of murine leukemia virus in embryonic cells. Cell 131:46-57.

12. Rowe HM, Jakobsson J, Mesnard D, Rougemont J, Reynard S, Aktas T, Maillard PV, Layard-Liesching H, Verp S, Marquis J, Spitz F, Constam DB and Trono D. (2010) KAP1 controls endogenous retroviruses in embryonic stem cells. Nature 463:237-240.

13. Quenneville S, Verde G, Corsinotti A, Kapopoulou A, Jakobsson J, Offner S, Baglivo I, Pedone PV, Grimaldi G, Riccio A, Trono D. (2011) In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanu- cleotide to affect chromatin and DNA methylation of im- printing control regions. Mol Cell 44:361–72.

14. Zuo X, Sheng J, Lau HT, McDonald CM, Andrade M, Cullen DE, Bell FT, Iacovino M, Kyba M, Xu G, Li X. (2012) Zinc finger protein ZFP57

requires its co-factor to recruit DNA methyltransferases and maintains DNA methylation imprint in embryonic stem cells via its transcriptional repression domain. J Biol Chem 287:2107–18.

15. Chang et al. (2008) Phosphorylation at Ser473 regulates heterochromatin protein 1 binding and corepressor function of TIF1beta/KAP1. BMC Mol Biol 9:61.

VII. Appendices

A. Primers of α-, β-and γ-globin

1. α–Hemoglobin 2: 5’- ATGGTGCTGTCTCCTGCCGA -3’

   3’- TACCGAGGCTCCAGCTTAACG -5’

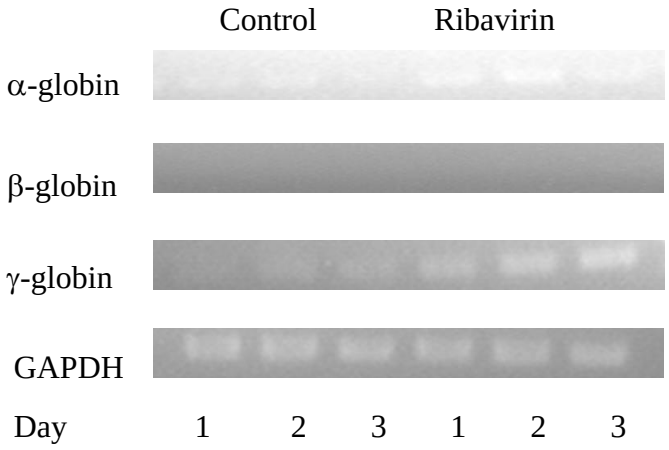
2. β-Hemoglobin : 5’- ATGGTGCATCTGACTCCTGAG -3’

   3’- CTTAGTGATACTTGTGGGCCA -5’

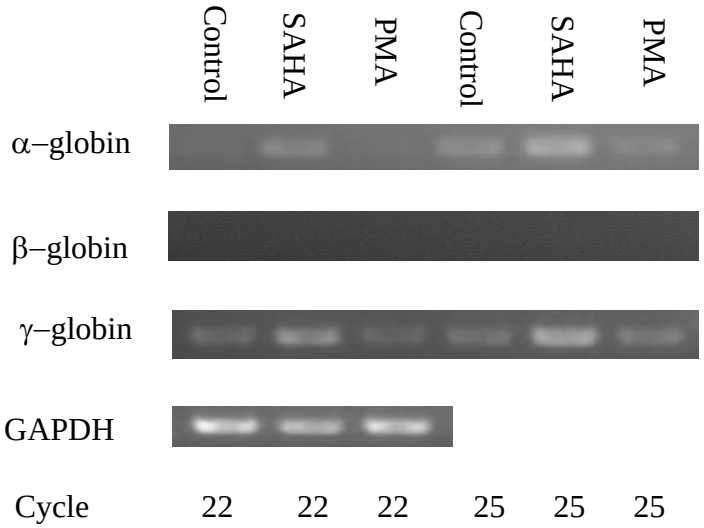
3. γ-Hemoglobin 1: 5’- ATGGGTCATTTCACAGAGGAG -3’

   3’- TCAGTGGTATCTGGAGGACAG -5’

B. The expression level of mRNA of α–, β–, γ-globin genes between Ribavirin-induced and control K562 cells



C. The expression levels of mRNA of α–, β–, γ-globin genes between SAHA, PMA-induced and control K562 cells



D. Primers for Site-directed mutagenesis

1. K774R : 5’- CAAACAGTTCAATAGGCTGACTGAGGAC -3’

   K774R : 3’- GTCCTCAGTCAGCCTATTGAACTGTTTG -5’

2. K774Q : 5’- CAAACAGTTCAACCAGCTGACTGAGGAC -3’

   K774Q-: 3’- GTCCTCAGTCAGCTGGTTGAACTGTTTG -5’

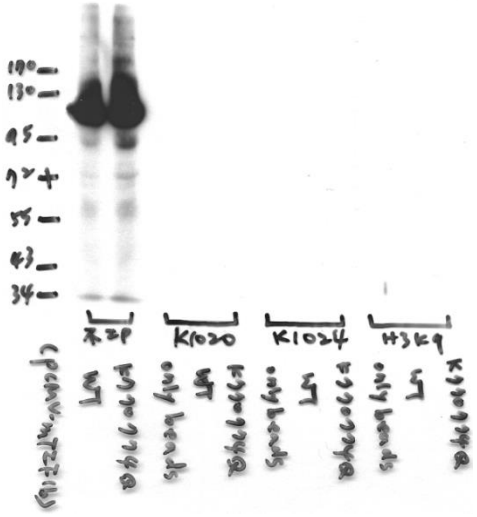
3. K770.774R : 5’- GCCGCATGTTCAGACAGTTCAATAGGC -3’

   K770.774R: 3’- GCCTATTGAACTGTCTGAACATGCGGC -5’

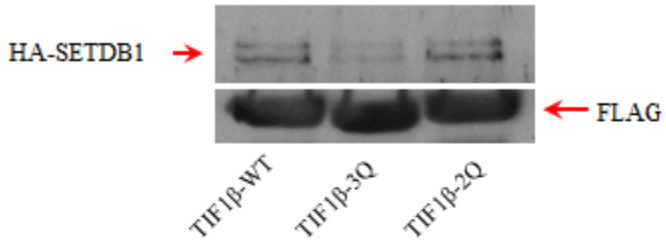
4. K770.774Q : 5’- GCCGCATGTTCCAACAGTTCAATCAG -3’

   K770.774Q : 3’- CTGATTGAACTGTTGGAACATGCGGC - 5’

E. The immunoprecipitation result of the interaction between acetylated peptides and acetylated bromodomain of TIF1β



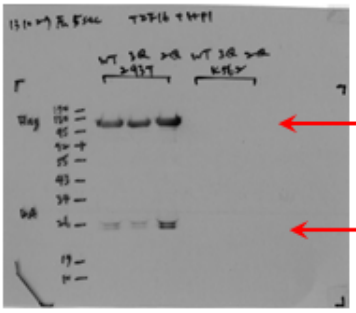
F. The interaction between TIF1β and SETDB1



	HA-SETDB1			FLAG			
293T	Area	%	WT為基準	Area	%	WT為基準	HA/FLAG
WT	6224.6	38.9	1.00	13095.7	29.9	1.00	1.00
3Q	2779.3	17.4	0.45	15926.0	36.3	1.22	0.37
2Q	7008.4	43.8	1.13	14849.7	33.8	1.13	0.99

TIF1β-3Q: K267.304.304Q
TIF1β-2Q: K770.774Q

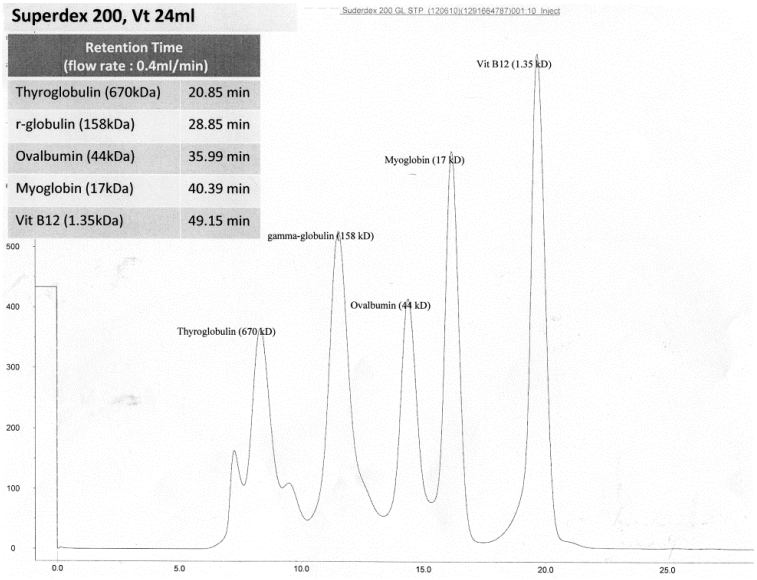
G. The interaction between TIF1β and HP1-γ



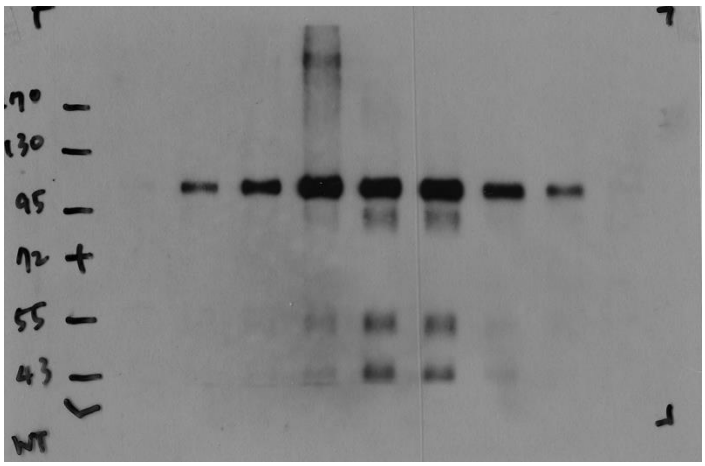
	HA-HP1			FLAG			
293T	Area	%	WT為基準	Area	%	WT為基準	HA/FLAG
WT	6078.4	19.6	1.00	17857.2	27.2	1.00	1.00
3Q	4575.9	14.7	0.75	19128.3	29.1	1.07	0.70
2Q	20424	65.7	3.36	28777.3	43.8	1.61	2.09

TIF1β-3Q: K267.304.304Q
TIF1β-2Q: K770.774Q

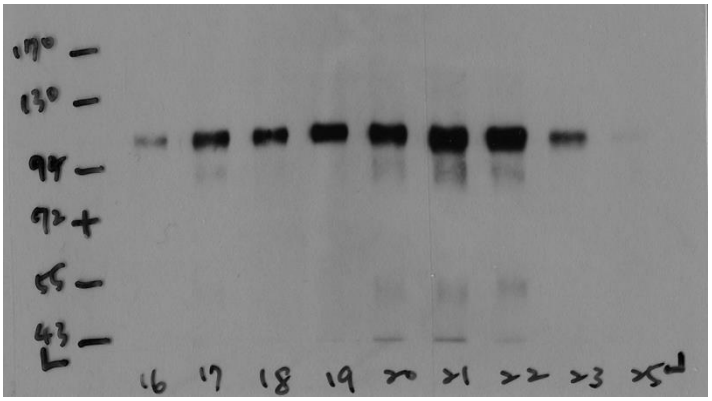
H. Superdex calibration



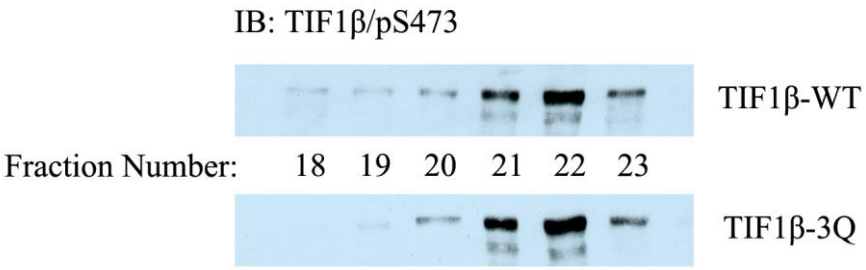
I. Elution profiles of recombinant TIF1β–WT, probed with anti-Flag antibody.



J. Elution profiles of recombinant TIF1β–3Q, probed with anti-Flag antibody.



K. The expression level of S473 phosphorylation of recombinant TIF1β–WT and TIF1β -3Q



VIII. Acknowledgements

Laboratory of Prof. Sheng-Chung Lee in the Institute of Molecular Medicine, National Taiwan University, Taipei, Taiwan:

Principal Investigator: Prof. Sheng-Chung Lee

Research assistant: Su-Hui Su

MS: Hsing-Yun Chang

Alumni: Chia-Wei Lee

Taipei First Girls High School, Taiwan:

Biology Teacher: Yi-Yi Hsu

And I would like to give thanks to all other teachers, friends, and indeed, my parents who have been supportive and caring.

評語

利用兩種臨床藥物 SAHA 及 Ribavirin 研究 K562 細胞分化成紅血球或巨核細胞的過程，並探討其可能之機制，具有應用性，可再深入探討。