HDAC6 promotes PMA-induced megakaryocyte differentiation of K562 cells by regulating ROS levels *via* NOX4 and repressing Glycophorin A

Thesis Submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that the thesis entitled **"HDAC6 promotes PMA-induced megakaryocyte differentiation of K562 cells by regulating ROS levels *via* NOX4 and repressing Glycophorin A"** submitted by **K Githavani** bearing registration number **13LAPH10** in partial fulfilment of the requirements for award of Doctor of Philosophy in the **School of Life Sciences** is a bonafide work carried out by her under my supervision and guidance.

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Further, the student has passed the following courses towards fulfillment of coursework requirement for Ph.D.

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DECLARATION

I hereby declare that the results of the study incorporated in the thesis entitled **"HDAC6 promotes PMA-induced megakaryocyte differentiation of K562 cells by regulating ROS levels *via* NOX4 and repressing Glycophorin A"** has been carried out by me under the supervision of **Dr. Arunasree MK** and this work has not been submitted for any degree or diploma of any other university earlier.

Dated:

K GITHAVANI

(Research Scholar)

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K Githavani

CONTENTS

ABBREVIATIONS

AFU	Arbitrary Fluorescence Units
APS	Ammonium per sulphate
ATP	Adenosine tri-phosphate
bp	Base pairs
CFU	Colony forming unit
ChIP-PCR	Chromatin Immuno precipitation-Polymerase chain reaction
CXCL	chemokine (C-X-C motif) ligand
EDTA	Ethylene diamine tetra acetic acid
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FACS	Fluorescence assisted cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOG-1	friend of GATA protein 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDAC6	Histone deacetylase 6
HDACs	Histone deacetylases
IL	Interleukin
K562	Chronic myelogenous leukemia cell line
kbp	Kilo base pair
m/z	Charge to mass ratio

MEP	Megakaryocyte-Erythroid Progenitor
MFI	Mean fluorescence intensity
MK	Megakaryocyte
nm	Nanometer
NuRD	Nucleosome Remodeling Deacetylase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PF	Platelet factor
PKC	Protein Kinase C
PMA	Phorbal 12-myristate 13-acetate
RIPA	Radio immunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RUNX-1	runt-related transcription factor 1
SDS-PAGE	Sodium dodecyl sulphate-Poly acrylamide gel electrophoresis
TAE	Tris acetate EDTA
TAF-9	TATA-binding protein–associated factor 9
TEMED	N, N, N, N - tetramethylethylene diamine
TFIID	Transcription factor IID
Tris	Tris-(Hydroxymethyl) aminoethane
Tub A	Tubastatin A
vWF	von Willebrand factor

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Introduction

1.1 Haematopoiesis

Haematopoiesis describes the process of differentiation of haematopoietic stem cells (HSCs) to all blood cell lineages with intermittent production of multipotent and bipotent progenitor cells (Fig. 1). The survival, self-renewal and differentiation of HSCs are tightly regulated by the presence of cytokines, lineage-specific transcription factors and micro RNAs (Iwasaki and Akashi 2007, Orkin and Zon 2008, Shaham, Binder et al. 2012). In mammals, yolk sac, liver and spleen during embryonic development and bone marrow in adults are the sequential sites of haematopoiesis (Orkin and Zon 2008). Recently, the placenta has been identified as an additional site of haematopoiesis during the transition from yolk sac to fetal liver (Mikkola, Gekas et al. 2005).

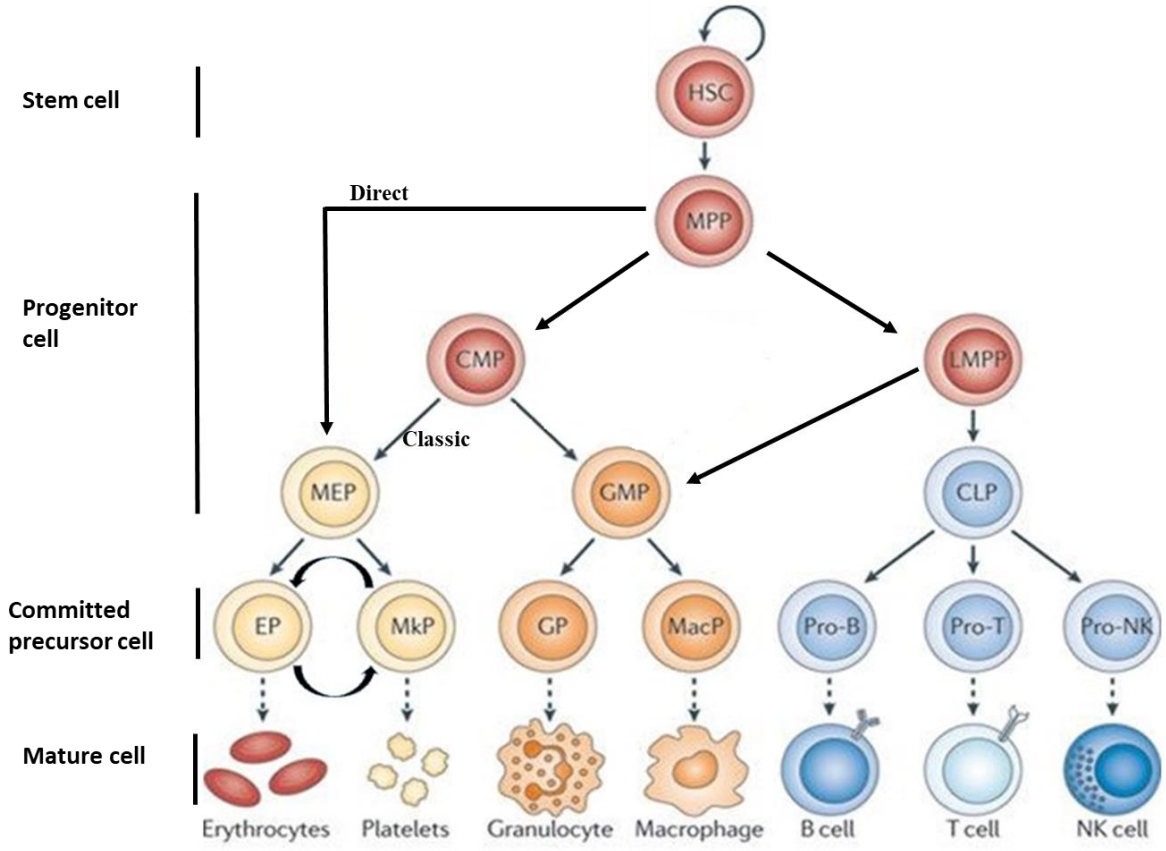

Fig 1: ***Schematic diagram depicting differentiation of stem cells to progenitor, precursor and mature blood cells.*** *MEP cells are derived from CMP cells in classic pathway and from MPP cells or HSCs in direct pathway. HSC, haematopoietic stem cell; GMP, granulocyte/monocyte progenitor; LMPP, lymphoid primed multipotent progenitor; CLP, common lymphoid progenitor; and MkP, megakaryocyte progenitor*. Source: *adopted and modified from Nature Reviews, Immunology.*

Megakaryocyte-Erythroid progenitors (MEP) are bipotent that can differentiate into erythrocytes and megakaryocytes and thus express the markers of megakaryocytes (MK) and erythrocytes (ER) at basal levels. MEP cells are derived from common myeloid progenitor (CMP) cells in classical model of differentiation or directly from multipotent progenitor (MPP) cells that are FLT3 negative. On the other hand, the FLT3 positive HSCs or MPPs have the potential to differentiate in to lymphoid and myeloid progenitors excluding MEP cells in an alternative pathway (Pang, Weiss et al. 2005). Huang H et al have shown that HSCs and MKs have some common features in expressing some receptors like c-MPL, CXCR4, CD150, CD41 and some transcription factors like RUNX1, GATA-2 etc. (Huang and Cantor 2009).

1.2 Megakaryocyte (MK), Thrombopoiesis and Thrombocytopenia

Megakaryocytes are referred as the largest cells of bone marrow with a diameter of ~50-100 μm. They account for 0.01% of total nucleated cells in bone marrow and increases to 10 fold during immune thrombocytopenia purpura (ITP) characterized by increased platelet destruction (Cajano and Polosa 1950). Each mature MK produces 1000 platelets, thus they release 10^{11} platelets in to the circulating blood. The entire cytoplasm of mature megakaryocyte starts distributing into pseudopods, which further expands into the long shaft like structures with multiple random bulges, called proplatelets. Microtubule assembly and sliding is required for proplatelet formation and elongation respectively (Patel, Richardson et al. 2005). Proplatelets are released as beaded cytoplasmic fragments in to circulating blood called platelets and this process is known as thrombopoiesis. Platelets are enucleated in mammals whereas they are diploid nucleated cells in fish (Jagadeeswaran, Sheehan et al. 1999). The final cell stages of both erythrocytes and MKs are circulating enucleated forms, which may be required for the flexibility in performing some specialized functions within the small capillaries. A healthy individual maintains 1,50,000-

4,50,000 platelets/μL of blood. The platelets are about 2-3 μm in diameter with a short half-life of 7-10 days approximately.

Thrombocytopenia refers to low platelet count, below lower limit of normal range. The platelet count of 50 – 70*10^3/μL is considered as mild thrombocytopenia and it becomes severe when the platelet count is less than 20,000–30,000/μL of blood (Buckley, James et al. 2000). Severe thrombocytopenia can be fatal due to internal bleeding. The susceptibility to trauma-induced bleeding increases in patients with platelet count less than 30,000/μL and count less than 10,000/μL results in spontaneous bleeding and considered as hematologic emergency. The bleeding severity is not same even though the patients have equally low platelet count due to variations in the degree of endothelial cells damage. Under non-traumatic conditions, micro vascular lesions caused by leukocyte infiltration during inflammation and damage of basement membrane in angiogenesis causes bleeding in thrombocytopenia. Acute megakaryoblastic leukemia in Down syndrome, thrombocytopenia and absent radius (TAR) syndrome and transient myeloproliferative disorder are the examples of neonatal thrombocytopenia causing severe morbidity of the neonates during their first few weeks of life (Hedberg and Lipton 1988). Idiopathic or immune thrombocytopenia purpura (ITP) is hematologic disorder, characterized by low platelet count (20,000/μL) with no known clinical cause. The reduction in platelet count during ITP due to decreased bone marrow production, production of antibodies against platelets (auto immune disorder) (Semple and Freedman 1991) and increased splenic sequestration (Kuwana, Okazaki et al. 2002). Easy bruising, petechiae, nosebleeds and bleeding gums are the clinical manifestations of acute ITP (Cines and McMillan 2005). Thus platelets play important role in blood clotting, thrombosis, angiogenesis, inflammation and immune response.

The causes for thrombocytopenia include bleeding-associated trauma or surgery, chemotherapy or radiation treatment of cancer, sepsis and dengue infection. The two reasons for thrombocytopenia are reduced production from MK cells or increased destruction of platelets. Platelet transfusion is the only treatment for thrombocytopenia. However, there are some critical challenges associated with the transfusion such as (i) the short half-life of the platelets (1.5-3 days) after infusion in to the patient, (ii) variation in platelet number and functionality, (iii) storage conditions that increase the risk of bacterial contamination, (iv) faster expiration dates of collected platelets create wastage etc. (Slichter, Davis et al. 2005). So, better understanding of the molecular mechanisms regulating MK differentiation and platelet production are required to identify the safe sources of platelet for therapeutic purposes and to cure the platelet diseases.

1.3 Megakaryocyte differentiation

Commitment of MKs from MEP cells requires the presence of thrombopoietin (TPO), transcription factors like GATA-2, FOG1, FLI-1 etc. (Fig. 2). Commitment and maturation of MKs from progenitor cells is marked by cell cycle arrest, suppression of erythroid lineage genes like glycophorin A (GYPA), upregulation of MK markers CD61 (GPIIIa, integrin β3) and CD41 (GPIIb, integrin αIIb,) and endomitosis, unique process of continuous replication of DNA without cytokinesis reaches the DNA content up to 128N (Odell, Jackson et al. 1965). It is difficult to differentiate “early-onset” MK-specific genes from “late-onset” ones as extent of ploidy is not correlated with MK maturation (Lefebvre, Winter et al. 2000). The early MKs known as CFU-Meg, are characterized by 2N ploidy and change in cell morphology whereas the mature MKs are larger in cell size with polyploid nucleus, a cytoplasmic demarcation membrane system (DMS) playing a role in platelet release and the accumulation of granules and proteins like platelet factor 4 (PF4 / CXCL4) and vWF for platelet function (Gewirtz, Calabretta et al. 1989, Gewirtz, Zhang

et al. 1995). Recent studies have shown that MKs play important role in maintenance of HSCs in quiescence stage by TGF-β signaling along with CXCL4. MKs also regulate the proliferation and expansion of HSCs by releasing FGF1 during the chemotherapy treatment of cancer (Zhao, Perry et al. 2014, Norozi, Shahrabi et al. 2016). Cytokines such as IL-3, IL-6, GM-CSF, IL-11, IL-12 are required for proliferation of megakaryocytes whereas megakaryocyte maturation and platelet release are modulated by IL-1 and LIF (leukemia inhibitory factor) (Yu and Cantor 2012).

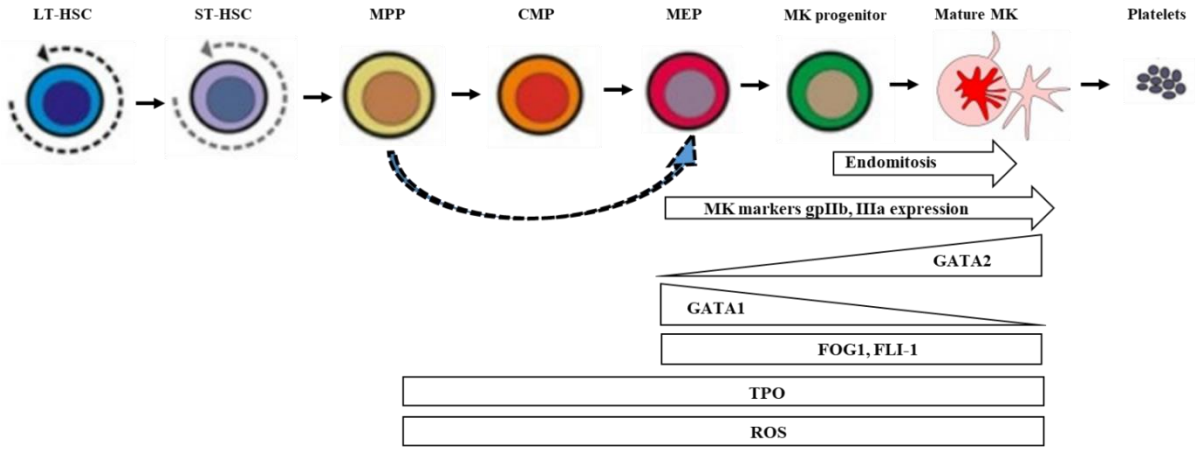

Fig 2: ***Megakaryopoiesis***. *Important cytokines and transcription factors affect megakaryopoiesis from MEP cells.*

These cytokines also affect other hematopoietic lineages. Thrombopoietin (TPO), a cytokine produced from the liver constitutively and to a lesser extent from kidney, spleen and bone marrow stromal cells, binds to the c-MPL receptor present on megakaryocyte progenitors as well as on HSCs and thus play a role in hematopoiesis and megakaryopoiesis (Young, Bruno et al. 1996). Better understanding of MK biology was possible with the discovery of TPO, which allows *in vitro* differentiation of CD34 (+) cells to pure megakaryocytes. It plays a role in proliferation, maturation and platelet production (de Sauvage, Hass et al. 1994). Platelets absorb TPO in the circulation and thus regulates its levels and availability to bone marrow HSCs (Kaushansky 1997). TPO regulates megakaryopoiesis and thrombopoiesis by activating PI-3 kinase-Akt, ERK1/2 and

MAPK signaling pathways and thus promote MK lineage gene expression (Kaushansky 2005). The disorders like congenital amegakaryocytic thrombocytopenia and aplastic anemia are associated with mutations in TPO-MPL protein (Ihara, Ishii et al. 1999, van den Oudenrijn, Bruin et al. 2000). Activating mutations in JAK2 gene or TPO / MPL results in familial essential thrombocythemia characterized by hyper aggregation of platelets (Ghilardi and Skoda 1999, Kaushansky 2005). SDF1 is also one of the important chemokine that acts independently from TPO in promoting megakaryopoiesis and migration of MKs to vascular niche. Studies have shown that $TPO^{-/-}$ and $Mpl^{-/-}$ mice were rescued from thrombocytopenia with the infusions of SDF-1 (Avecilla, Hattori et al. 2004). SDF-1 mimetic drugs cure thrombocytopenia during HIV infections by competitively binding to the CXCR4 receptors (Lee, Ratajczak et al. 1999).

Besides cytokines, lineage-specific transcription factors play an important role in the expression of MK lineage genes. GATA-2 play an important role in early megakaryopoiesis. It also has broader effects on other haematopoietic lineages. Studies have shown that continuous expression of GATA-2 rescued thrombocytopenia in GATA-1 knockdown mice (Huang, Dore et al. 2009). Targeted disruption of GATA-1 results in embryonic lethality with severe anaemia, immature and dysmorphic MKs and further severe thrombocytopenia indicating that GATA-1 acts at late stages of MK differentiation (Shivdasani, Fujiwara et al. 1997). FOG-1 (Zfpm1) function by associating with GATA-1 and GATA-2 and recruits NuRD repressor complex (Hong, Nakazawa et al. 2005). Since most or all the functions of GATA transcription factors involve FOG-1 interaction, disruption of FOG-1 results in embryonic lethality and impaired MK development (Tsang, Visvader et al. 1997). GATA-1 mutations that disrupts FOG-1 interaction are observed in patients with X-linked thrombocytopenia or variable anaemia (Yu, Niakan et al. 2002). FLI-1 is an Ets-family transcription factor and over expression of FLI-1 in progenitor cells promotes MK

differentiation indicating that it is specific to MK lineage. It binds to the GGAA core sequences present on most of the proximal promoters of MK lineage genes (Wang, Crispino et al. 2002). TAL-1, NF-E2 and RUNX1 are also required for MK development from progenitor cells (Shivdasani, Rosenblatt et al. 1995, Elefanty, Begley et al. 1998, Song, Sullivan et al. 1999, Ichikawa, Asai et al. 2004).

Apart from cytokines and transcription factors, Reactive oxygen species (ROS) is also an important factor in MK differentiation and maturation. ROS are reactive chemical molecules or free radicals, derived from molecular oxygen. The chemical species includes superoxide anion (O_2^-), peroxides (H_2O_2) and hydroxyl radicals (OH^-). NADPH oxidases (NOX) are responsible for intracellular ROS production. NOX family proteins are membrane associated flavoproteins with multi subunit complexes. The action of NOX involves the electron transfer from NADPH to oxygen resulting in superoxide formation. NOX family consists of 7 homologs named as NOX1, NOX2, NOX3, NOX4, NOX5 and Duox proteins (DUOX1 and DUOX2), that differ in their composition of subunits, activation and tissue distribution. NOX1, NOX2, NOX3 and NOX4 are p22-phox dependent whereas NOX5 and Duox proteins are Ca^{+2} regulated enzymes (Panday, Sahoo et al. 2015).

Other than NOX proteins, xanthine oxidoreductases, uncoupled NO synthases, cytochrome P450 monooxygenases, cyclooxygenases and lipoxygenases also play significant role in ROS production. These enzymes are located in mitochondria, endoplasmic reticulum, peroxisomes and plasma membrane and thus they are the sites of ROS generation in animal cells. Low levels of ROS is implicated in various biological and physiological processes by regulating signaling pathways (PI3K and MAPK). Oxidative stress or high levels of ROS is associated with various pathological conditions like cancer, neurodegenerative disorders, diabetes and aging by damaging

the macromolecules, DNA, protein and lipids. The antioxidant enzymes glutathione peroxidase (GPx), superoxide dismutase (SD), thioredoxins, catalase, and peroxiredoxins play important role in safeguarding the cell from oxidative damage (Ray, Huang et al. 2012).

Low levels of ROS plays a role in stem cell maintenance whereas slight increase in ROS levels is necessary for stem cell differentiation. In mice, AKT1 and AKT2 deficient HSCs have shown poor differentiation due to reduced ROS levels (Juntilla, Patil et al. 2010). Moderately high ROS levels trigger differentiation of stem and progenitor cells in Drososphila (Owusu-Ansah and Banerjee 2009). But what levels of ROS is required for physiological processes and pathology is not fully understood. Several lines of evidence indicate that ROS plays an important role in the differentiation of progenitor cells to MKs and further platelet release (Fig. 3). Which NOX is required for MK differentiation remains unclear. NOX2 is well studied among the NOX family and is known as phagocytic oxidase due to its role in antimicrobial host defense (El-Benna, Dang et al. 2009). Chronic granulomatous disease (CGD) is a genetic disorder characterized by frequent bacterial and fungal infections with inefficient clearance of ingested pathogens due to defect in gp91 subunit of NOX2 (Deffert, Carnesecchi et al. 2012). However, high expression of NOX1 and moderate expression of NOX4 was observed in bone marrow derived MKs with significant reduction in ploidy in the presence of NOX inhibitors whereas NOX1 knockout mice showed no effect on ploidy levels (McCrann, Eliades et al. 2009). NOX4 was shown to play a role in polyploidization of VSMC during aging by decreasing chromosome passenger protein, survivin (McCrann, Yang et al. 2009). Sardina JL et al have shown that ROS is produced by p22 (phox)-dependent NADPH oxidase during the differentiation of progenitor or CD34 (+) cells to MKs. Treatment with antioxidants like Trolox and NAC prevented the acquisition of MK morphological features with reduced expression of CD61 and CD41 (Sardina, Lopez-Ruano et al. 2010). In

addition, the differentiation of CD34 (+) cells under 20% O(2) promoted MK maturation and proplatelet formation compared to 5% O(2) (Mostafa, Miller et al. 2000) by upregulating the MK markers (CD41, CD42a), and late MK transcription factors (GATA-1 and NF-E2) (Mostafa, Papoutsakis et al. 2001). The mechanism through which ROS promotes MK differentiation remain elusive.

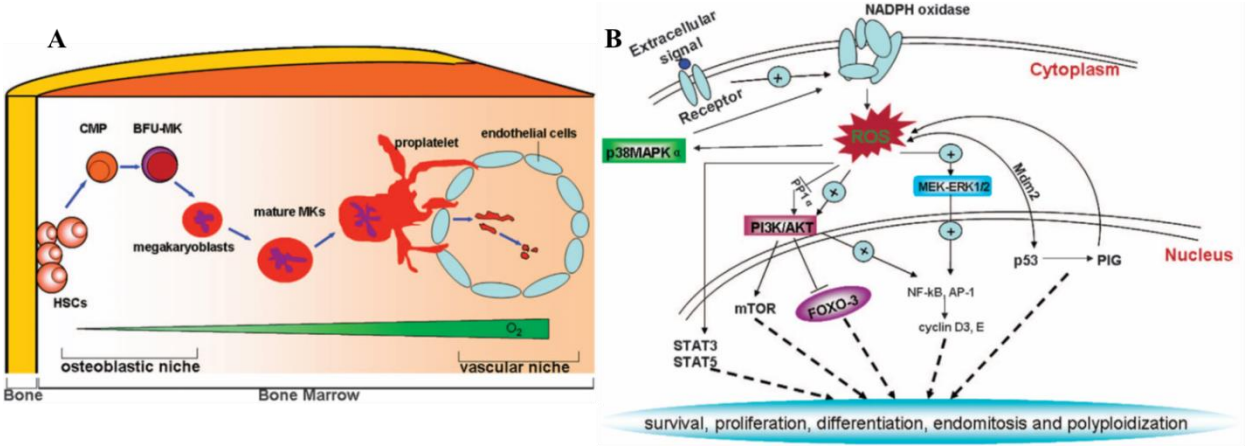

Fig 3: ***Significance of ROS production during MK development and platelet production.*** *MK differentiation from HSCs takes place in osteoblastic niche and vascular niche (A). ROS mediated activation of signaling pathways during megakaryopoiesis (B).* Source: *Cell Death and Disease (2013) 4, e722.*

1.4 Epigenetics of haematopoiesis

Most of the studies have shown that epigenetic modifications play an important role in the HSCs self-renewal and differentiation into various cell types (Raghuwanshi, Dahariya et al. 2018) . The term “epigenetic” means that “in addition to changes in genetic sequence”. It is defined as any process that changes the gene activity without altering the DNA sequence (Jenuwein and Allis 2001). Histone code hypothesis is defined as the regulatory system of controlling the chromatin accessibility and gene expression by combinatorial or sequential fashion of histone tail modifications including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation. Post-translational modifications (PTMs) of histone proteins as act docking

sites for reader proteins, directing the recruitment of protein complexes, which modify chromatin structure and further target gene expression (Bannister and Kouzarides 2011). The positively charged ε- amino group of the lysine residues present on N-terminal tails of histone proteins undergoes PTMs. Among these PTMs, methylation and acetylation of lysine residues are dominant and well-studied in regulating gene expression. Acetylation and deacetylation of histone proteins is carried out by histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively (Ito, P et al. 2000) (Fig. 4).

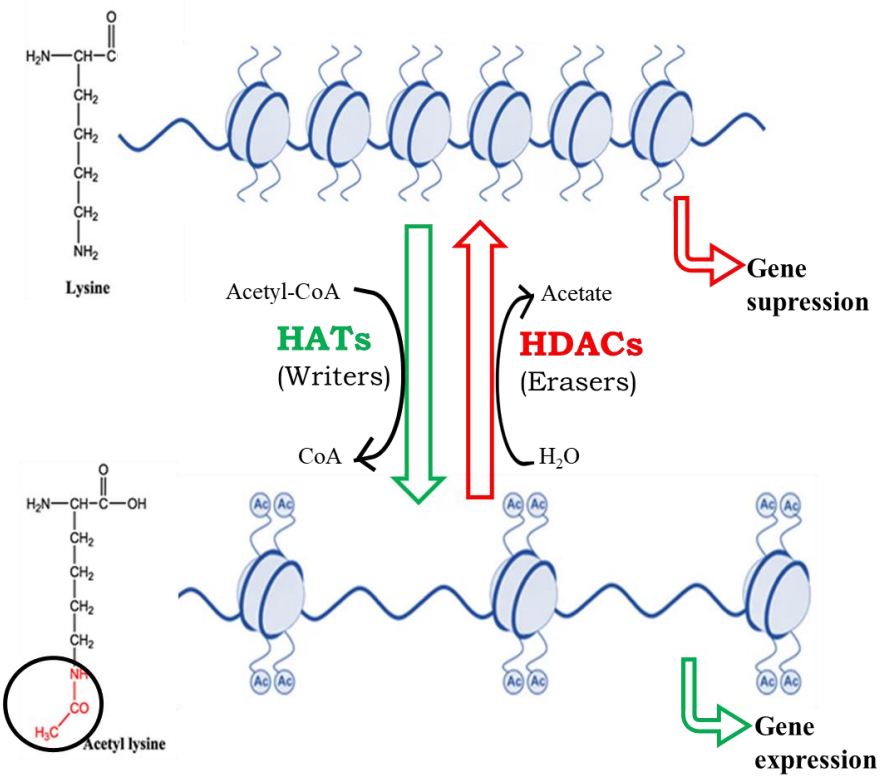

Fig 4: ***Regulation of gene expression by reversible acetylation mediated by HATs and HDACs.*** *Active state of genes correlates with increased acetyl groups on lysine residues present on N-terminal tails of histone proteins in contrast to inactive state of genes.* Source: *Adopted and modified from Expert Rev Anticancer Ther. 2008 Future Drugs Ltd.*

Acetylation of amino group neutralizes the positive charge on histone proteins and reduces electrostatic interaction between DNA and histone protein, leading to relaxed conformation which

allows the binding of transcriptional machinery and further gene expression. Hence HATs are called transcriptional co-activators. In contrast to HATs, HDACs are named as transcriptional co-repressors as they remove acetyl group results in condensed chromatin formation and further gene suppression. Acetylation has shown to affect transcription of approximately 2-10% of genes (Mariadason, Corner et al. 2000). Acetylation is one of the important post-translational modification known to regulate the activity or function of the non-histone proteins besides histone proteins. Similar to histone proteins, non-histone protein acetylation and deacetylation is carried out by HATs and HDACs and thus regulate the function of several proteins such as nuclear receptors, signaling mediators, DNA-binding transcription factors, chromatin remodeling proteins, DNA repair enzymes, transcription co-regulators, structural proteins etc (Fig. 5). Due to their involvement in gene repression and protein function regulation *via* deacetylation, HDACs play important role in various cellular processes and pathologies (Seto and Yoshida 2014).

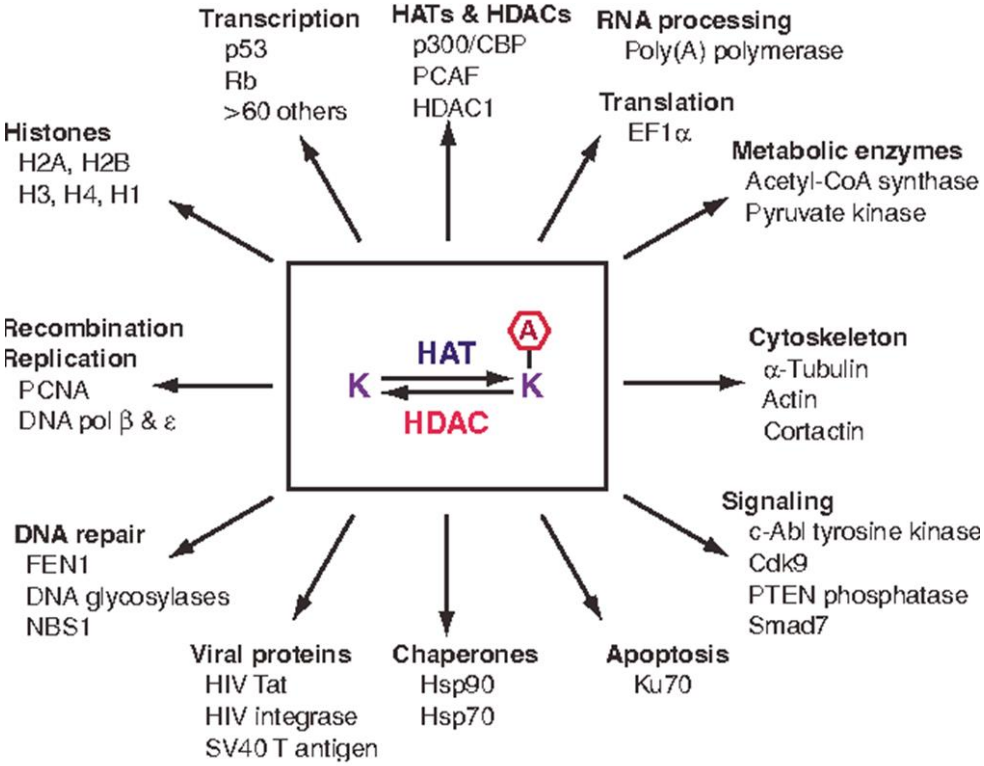

Fig 5: ***Role of reversible acetylation of lysine residue in diverse cellular processes.*** *Some well-studied proteins are listed in each process.* Source: *Oncogene* 26 (2007): 5310-5318.

1.5 HDACs: Classification, pathology & inhibitors

In humans, 4 families (GNAT, MYST, p300/CBP and Rtt109 family) of HATs are implicated in histone protein acetylation whereas PCAF and p300/CBP are the two HATs, responsible for non-histone protein acetylation (Das and Kundu 2005). On the contrary, there are a total of 18 HDACs involved in both histone and non-histone protein deacetylation indicating their importance in cell physiology (Yang and Seto 2007).

The 18 HDACs have been divided in to four classes based on their homology to yeast histone deacetylases (Fig. 6). Class I HDACs are homologous to yeast rpd3 gene; Class II are homologous

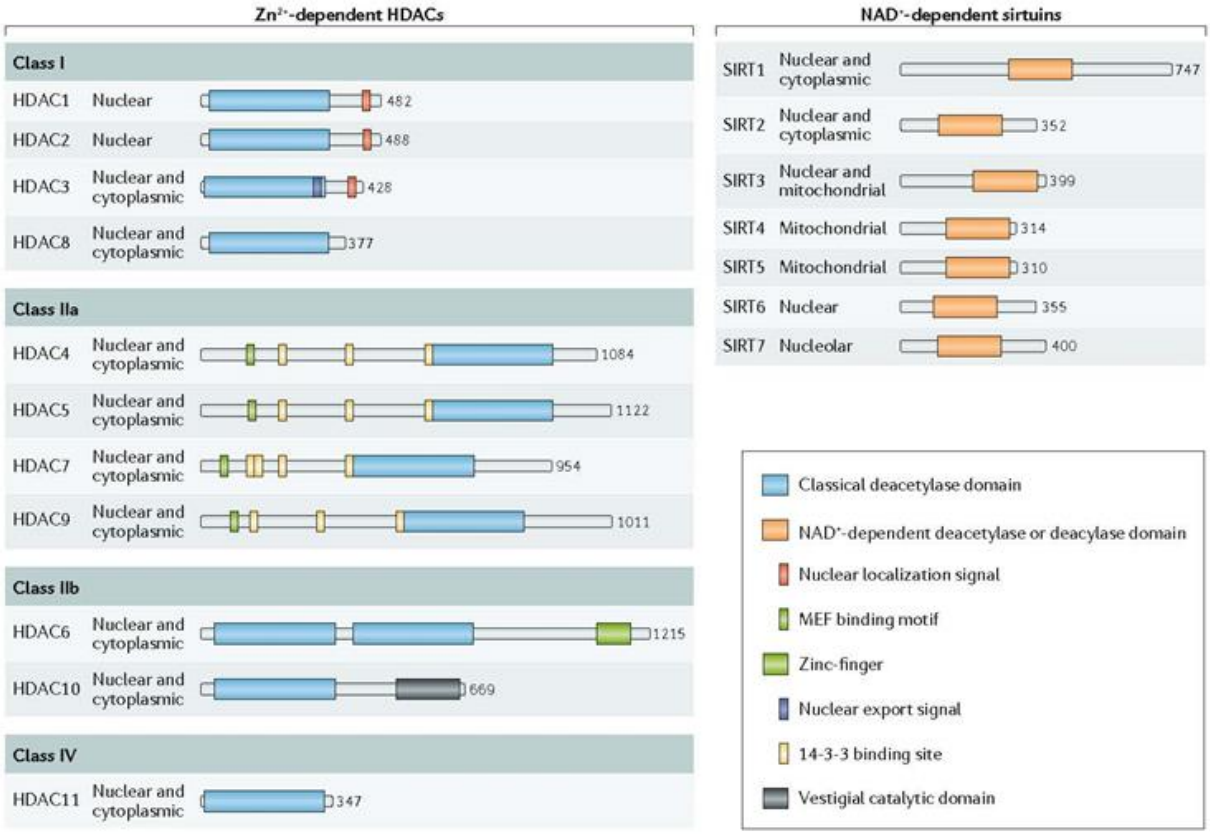

Fig 6: ***Classification of HDACs.*** *Different classes of HDACs with their structural organization and localization.* Source**:** *Nat Rev Immunol.2018 Oct; 18(10):617-634.*

to yeast hdac1; class III HDACs (Sirtuins) are homologous to yeast sir2 gene and class IV is homologous to both class I and II HDACs. Class I, II and IV are called as classical HDACs, use

acetyl-CoA as co-substrate and Zn^{+2} as cofactor whereas class III HDACs requires NAD as cofactor for their activity (de Ruijter, van Gennip et al. 2003, Gregoretti, Lee et al. 2004, Walkinshaw and Yang 2008).

HDACs lack DNA binding domain and hence generally function in multiprotein complexes (Fischle, Dequiedt et al. 2002). HDAC1 and HDAC2 are shown to form homo- and hetero-dimers and are associated with complexes of transcription factors, co repressors known as SIN3, nucleosome remodeling and deacetylase (NuRD), REST corepressor 1(CoREST), mitotic HDAC (MiDAC), SHIP168 and NODE169. HDAC3 is part of nuclear receptor co- repressor 1 (NCoR1) and retinoic acid and thyroid hormone receptor (SMRT) multiprotein complex and also interacts with HDAC4, HDAC5, HDAC7 and HDAC10. HDAC1 and HDAC2 are localized in the nucleus, HDAC3 and HDAC 8 of class I, class II and class IV HDACs shuttle between nucleus and cytoplasm and class III Sirtuins are present in nucleus, nucleolus, cytoplasm and mitochondria of the cell (Yao and Yang 2011). It is very well established that HDACs activity is regulated by several posttranslational modifications (Yang and Seto 2007). Depending upon the location and post-translational modifications they undergo, HDACs are involved in a wide variety of physiological processes including cell differentiation, cell cycle regulation, cellular metabolism DNA repair etc. (Haberland, Montgomery et al. 2009).

1.6 HDACs in stem cell differentiation

Several studies have identified the role of HDACs in stem cell differentiation into skeletal muscle cells (Arnold, Kim et al. 2007), neuronal cells (Castelo-Branco, Lilja et al. 2014), HSCs to progenitor cells etc. (Fujieda, Katayama et al. 2005, Obier, Uhlemann et al. 2010). The sustained expression of HDAC1 by GATA-1 transcription factor allows the differentiation of myeloid progenitor cells to erythrocyte and megakaryocytic lineages (Wada, Kikuchi et al. 2009).

Knockout of HDAC1 and HDAC2 results in anaemia and thrombocytopenia indicating their involvement in erythrocyte and megakaryocyte differentiation (Wilting, Yanover et al. 2010). HDAC1 was shown to repress the CDK inhibitor, p21/CIP1/WAF1 and thus positively regulates the proliferation of mouse embryonic stem cells (mESC) and fibroblasts (mEFs). Thus, loss of HDAC1 was shown to upregulate p21 expression and further ES cell differentiation (Lagger, O'Carroll et al. 2002, Zupkovitz, Grausenburger et al. 2010). Although, HDAC1 and HDAC2 have redundant functions, germ-line deletion of mouse HDAC1 results in early embryonic lethality due to reduced HDAC activity of corepressor complexes and significant increase in histone H3K56 acetylation (H3K56Ac) (Dovey, Foster et al. 2010). During embryonic development, the first haematopoietic progenitor cells are generated by transdifferentiation of hemogenic endothelium (HE), named due to their localization and expression of both hematopoietic and endothelial markers (Zovein, Hofmann et al. 2008, Bertrand, Chi et al. 2010). Recent studies have shown that modulation of TGF-β signaling by HDAC1 and HDAC2 is critical in generation of blood cells through endothelial-to-hematopoietic transition (EHT) (Thambyrajah, Fadlullah et al. 2018). It has been known that, HDACs are recruited to the promoter regions for gene repression. However, studies by Wei Jian et al shown that HDAC1 acts as coactivator for PU.1 gene transcription, an important master regulator of haematopoietic self-renewal and differentiation. HDAC1 dependent deacetylation of TAF9 protein, a component of TFIID complex is required for binding to the PU.1 promoter and further gene expression (Jian, Yan et al. 2017). Several studies have indicated role of HDAC1 and HDAC2 in hematopoiesis but the specific role of HDACs in MK lineage commitment and differentiation remains elusive.

1.7 HDAC inhibitors (HDACi)

Aberrant HDAC activity or expression is observed in many pathological conditions such as

neurodegenerative diseases, cancer, metabolic disorders etc. where they affect transcription of oncogenes and tumor suppressor genes or activity of non-histone proteins and thus influence normal cellular behavior (Grunstein 1997, Marks, Rifkind et al. 2001, Adcock, Tsaprouni et al. 2007, Colussi, Mozzetta et al. 2008, Grabiec, Tak et al. 2008, Weichert, Denkert et al. 2008, Weichert, Roske et al. 2008, Kilgore, Miller et al. 2010). Due to the involvement of HDACs in various cellular physiological processes, their role in different pathologies is also well established making them good drug targets. Currently, there are numerous natural and synthetic HDACi under clinical development, which can be divided into three groups based on their specificity: (1) nonselective HDACi, (2) selective HDACi, and (3) multipharmacological HDACi. Food Administration and development (FDA) has approved four HDACi as anticancer agents, namely Vorinostat (Mann, Johnson et al. 2007), Romidepsin (Grant, Rahman et al. 2010) for therapy of CTCL (cutaneous T- cell lymphoma), Belinostat (Sawas, Radeski et al. 2015) used for peripheral T-cell lymphoma (PTCL) treatment and Panobinostat (Moore 2016) for the treatment of multiple myeloma. HDACi induce cell cycle arrest, differentiation, apoptosis, autophagy, angiogenesis and regulate immune response, signaling pathways and ROS production. But the mechanism of action of HDACi changes depending on the type of tumor, dose, and specificity to target HDACs (Kretsovali, Hadjimichael et al. 2012). For example, Valproic acid (VPA) does not show effect on prostate cancer but inhibits invasiveness in bladder cancer (Chen, Sung et al. 2006). Vomiting, diarrhea, thrombocytopenia, neutropenia, nausea and fatigue, cardiac toxicity, including ventricular arrhythmia are the side effects of HDACi (Shah, Binkley et al. 2006). These studies indicate the importance of HDACs in MK differentiation and thrombopoiesis.

1.8 HDAC inhibitors in thrombocytopenia

Most of the studies have shown that HDACi used in cancer treatment induce moderate to severe

thrombocytopenia**.** Matsuoka H et al have shown that HDACi inhibits the expression of GATA-1 which is involved in MK maturation in human megakaryocytic HEL cells and in rat model (Matsuoka, Unami et al. 2007). It is also reported that, HDACi downregulates CDC42 and Rac1 *via* Rho/Rac/CDC42 pathway resulting in net increase in pMLC (myosin light chain) which is associated with cytoskeleton abnormalities and further reduced proplatelet formation from mature

Table 1. Overview of different classes and clinical trial status of HDACi.

Class	HDAC Inhibitor	Target HDAC Class	Clinical Status
hydroxamic acids	Trichostatin A	pan	preclinical
	SAHA	pan	approved for cutaneous T-cell lymphoma
	Belinostat	pan	approved for peripheral T-cell lymphoma
	Panabiostat	pan	approved for multiple myeloma
	Givinostat	pan	phase II clinical trials—relapsed leukemia and multiple myeloma
	Resminostat	pan	phase I and II clinical trials—hepatocellular carcinoma
	Abexinostat	pan	phase II clinical trial—B-cell lymphoma
	Quisinostat	pan	phase I clinical trial—multiple myeloma
	Rocilinostat	II	phase I clinical trial—multiple myeloma
	Practinostat	I, II and IV	phase II clinical trial—prostate cancer
	CHR-3996	I	phase I clinical trial—advanced/metastatic solid tumors refractory to standard therapy
short chain fatty acids	Valproic acid	I, IIa	approved for epilepsia, bipolar disorders and migraine, phase II clinical trials—several studies
	Butyric acid	I, II	phase II clinical trials—several studies
	Phenylbutyric acid	I, II	phase I clinical trials—several studies
benzamides	Entinostat	I	phase II clinical trials—breast cancer, Hodgkin´s lymphoma, non-small cell lung cancer, phase III clinical trial—hormone receptor positive breast cancer
	Tacedinaline	I	phase III clinical trial—non-small cell lung cancer and pancreatic cancer
	4SC202	I	phase I clinical trial—advanced hematological malignancies
	Mocetinostat	I, IV	phase II clinical trials—Hodgkin´s lymphoma
cyclic tetrapeptides	Romidepsin	I	approved for cutaneous T-cell lymphoma
sirtuins inhibitors	Nicotinamide	all class III	phase III clinical trial—laryngeal cancer
	Sirtinol	SIRT 1 and 2	Preclinical
	Cambinol	SIRT 1 and 2	Preclinical
	EX-527	SIRT 1 and 2	cancer preclinical, phase I and II clinical trials—Huntington disease, glaucoma

Source: *Int. J. Mol. Sci. 2017, 18, 1414.*

MKs (Bishton, Harrison et al. 2011). HDACi-induced thrombocytopenia can be prevented by the use of AMP-4, a mouse-specific TPO-mimetic. Mice administered with AMP-4 during the ongoing treatment with HDACi like Panobinostat or Romidepsin shown consistent improvement in platelet numbers similar to untreated controls (Bishton, Harrison et al. 2011, Ali, Bluteau et al. 2013). In addition, A Ali *et al*, found that pan HDACi, Abexinostat or PCI-24781 induce DNA

damage and apoptosis of MKs in p53 dependent pathway and reduces proplatelet (PPT) formation independently of p53 (Ali, Bluteau et al. 2013).

1.9 Rational of the study

The commitment of the bipotent progenitor cells to a particular lineage requires suppression of the opposite lineage genes which involves lot of changes in the acetylation status of DNA. Since HDACs are involved in gene repression *via* compact chromatin formation, we hypothesize that one or few of the HDACs might be involved in lineage commitment of progenitor cells. Although the role of HDACs in other biological processes is well known, the role of HDACs and the underlying molecular mechanisms of lineage commitment remains elusive. Also it becomes extremely important to identify HDACs involved in MK differentiation so as to increase HDACi-based treatment strategy by reducing HDACi-induced thrombocytopenia. We therefore, in the present study, aimed at identifying the role of HDACs in MK lineage commitment of K562 cells.

Objectives:

Objective 1

HDACs profiling and identification of HDAC(s) upregulated during MK differentiation

Objective 2

Functional studies of HDAC6

(A) Role of HDAC6 in regulating MK and erythroid marker expression

(B) Role of HDAC6 in sustainable ROS production during MK differentiation.

Materials and Methods

2.1 Materials:

RPMI 1640 (A10491-01), FBS (10270), Antibiotic-antimycotic (15240062), Dynabeads® Protein G (10004D) are purchased from Invitrogen. HDAC6 clone was obtained from Addgene (#30482), USA. Antibodies; HDAC6 (H-300) from Santacruz, GAPDH (MA5-15738) from Thermo Scientific, USA, HDAC1,2,3,5 sample kit (#9928) from Cell signaling, CD61-PerCP (347408) BD Biosciences. Tri-reagent procured from Ambion. cDNA synthesis kit (G234) was from abm, CANADA. HDAC activity assay kit was purchased from Enzo Life Sciences, Inc. USA. SYBR Green procured from KAPA Biosystems, USA. Polyethylenimine (PEI), Linear (MW 25,000, 1gm, 23966-1) for transfections is purchased from Poly sciences, Inc.

Methods

2.2 Cell culture

K562 cells were obtained from the National Centre for Cell Science (NCCS), India cell repository. The cells were cultured in RPMI 1640 medium mixed with 10% FBS and 1% penicillin and streptomycin and grown at 37 ^{o}C, 5% CO_2. The exponentially growing K562 cells were seeded at a density of 2.5 x10^5 cells / ml and treated with 20 nM PMA for 24 h and 48 h. In combination treatments, the cells were treated with 5 µM Tubastatin A or 100 µM Quercetin or 10 µM Apigenin 1 h prior to PMA addition and the control cells received DMSO alone.

2.3 RNA extraction, RT-PCR and Real-time PCR

Total RNA was extracted by TRI reagent according to the manufacturer protocol. Briefly, cells (5*10^6) were washed with PBS and resuspended in 1 ml of TRI reagent. 200 µl of chloroform was added to 1 ml of TRI reagent and mixed the components thoroughly. Aqueous layer was collected

Table 2: List of primers used for expression analysis

S.No	Gene Name	Primer	Sequence	Length	Tm	Amplicon size
1	HDAC1	FP	TTC TTG CGC TCC ATC CGT CCA G	22	61	142
		RP	CAG CAC TTG CCA CAG AAC CAC C	22	61	
2	HDAC2	FP	GAT GCT TGG AGG AGG TGG CTA C	22	59.7	157
		RP	TGG CAA CTC ATT GGG AAT CTC AC	23	57.5	
3	HDAC3	FP	CAT TAA CTG GGC TGG TGG TCT GC	23	60.3	110
		RP	GAG GGT GGT ACT TGA GCA GCT C	22	59.5	
4	HDAC4	FP	ATGAGCTCCCAAAGCCATCC	20	60	351
		RP	CTCCTGTTGTTGCTTGATGTGC	21	58	
5	HDAC5	FP	AAC TCA CAC CTC ACT GCC TCC	21	59.2	127
		RP	AGC CAG GAA TAG AGG ATG TGC TC	23	58.5	
6	HDAC6	FP	TGT CTC TGG AGG GTG GCT ACA AC	23	60.5	137
		RP	GGA AAC TGA AGC CTG GGC ACT C	22	60.5	
7	HDAC8	FP	GGC TAG GTT ATG ACT GCC CAG C	22	59.9	141
		RP	CAT GAT GCC ACC CTC CAG ACC	21	61.9	
8	HDAC10	FP	GTC ATC CAA CAG GAA GCG TCA GC	23	60.3	159
		RP	GGC TGC TAT ACC ACT GTT CAC CTG	24	59.4	
9	GAPDH	FP	GAG AAG GCT GGG GCT CAT TTG C	22	60.9	145
		RP	TGG TGC AGG AGG CAT TGC TGA TG	22	61.1	
10	CD61	FP	GGA GAC ACG GTG AGC TTC AG	20	62.4	196
		RP	ACT CAA AGG TCC CAT TGC CA	20	62.5	
11	ITGA2B	FP	AAC GCC CAG ATA GGA ATC GC	20	60.5	186
		RP	GGC TGG AAA GGA GTT CCC TC	20	60.5	
12	GYPA	FP	ATA CGC ACA AAC GGG ACA CA	20	58.4	171
		RP	ATA ACA CCA GCC ATC ACC CC	20	60.5	
13	NOX2	FP	ATT GCA ATA ACG CCA CCA AT	20	60	116
		RP	CAT CTG GCT CTC CAG CAG TT	20	60	
14	NOX4	FP	CTGGCTCTCCATGAATGTCC	20	60	123
		RP	ACCCCAAATGTTGCTTTGGT	20	61	
15	BIRC5	FP	TGGACAGAGAAAGAGCCAAGAA	22	61	89
		RP	CTTCCAGTCCCTCCCTGAAT	20	60	
16	RUNX1	FP	TCCTTCGTACCCACAGTGCT	20	60.7	124
		RP	GCGGTAGCATTTCTCAGCTC	20	60.1	
17	GATA1	FP	TGGAGACTTTGAAGACAGAGCGGCTGAG	28	63	146
		RP	GAAGCTTGGGAGAGGAATAGGCTGCTGA	28	64	
18	GATA2	FP	ACGACAACCACCACCTTATG	20	62	157
		RP	TCTTGGACTTGTTGGACATCTT	22	62	
19	FLI-1	FP	CAGAACATGGATGGCAAGGA	20	62	148
		RP	CGGTGTGGGAGGTTGTATTATAG	23	62	
20	FOG-1	FP	CCTTCGTGTGCCTGATCT	18	61	146
		RP	CAAGTGGCTGTAGAGGATGT	20	61	
21	EKLF-1	FP	CAGGATGACTTCCTCAAGTGG	21	62	178
		RP	AGAAGTTGGTGAGGAGGAGA	20	62	

after centrifuging at 12000 g, 4 ºC for 20 min. 500 µl of isopropanol for 1 ml of TRI reagent was added to aqueous layer to precipitate RNA and incubated for 10 min at room temperature. The samples were centrifuged at 12000 g, 4 ºC for 30 min and then the pellet was washed with 70% ethanol to get rid of salts. Finally the pellet was air dried and dissolved in DEPC treated water. Quantification and purity of RNA were determined by Nanodrop and the integrity of RNA was examined by agarose gel electrophoresis. 2 µg of RNA was reverse transcribed with Oligo(dT)15, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 200 µM each deoxy nucleotide triphosphate [dNTP]) using 200 U of M-MLV Reverse Transcriptase (Invitrogen) in 20 µl of a reaction volume. The reaction mixture was incubated at 65ºC for 5 min, 42ºC for 1 h. Initially, amplification of target genes were confirmed with semi quantitative PCR using EmeraldAmp® MAX PCR Master Mix and the reaction conditions are as follows, initial denaturation 95 ºC: 2 min, and is followed by 35 cycles at 95 ºC for 30 sec, 60 ºC for 30 sec, and 72 ºC for 30 sec with the final extension of 72 ºC. Diluted cDNA (1:6) was used to amplify target genes with gene specific primers and were quantified using SYBR green chemistry. The expression levels were calculated after normalizing with control cells using $2^{-\Delta\Delta CT}$ method. The GAPDH was used as reference gene to calculate the changes in target gene expression levels. primers (Table2) were designed using UCSC Genome browser software.

2.4 Western blotting

Following different treatments for the times indicated in figures, the cells were first washed with PBS and then resuspended, lysed in RIPA buffer (Abcam) on ice for 30 min with intermittent vortexing. The cell lysates were collected at 13000 g, 4 ºC for 30 min. The quantity of protein was measured by BCA (bicinchoninic acid, Santacruz, SC-202389) assay as per the manufacturer instructions. Reagent A and B were mixed in 1:50 ratio. 1 µl of protein lysate was mixed with 200

µl of reagent mix in 96 well plate. The plate was incubated at 37 ºC for 30 min and then the OD (Optical density) values were recorded at 562 nm. BSA (1 mg/ml) was used as standard to calculate the protein concentration in test samples. Equal amounts of protein was separated on SDS-PAGE and transferred on to PVDF or nitrocellulose membrane. The membrane was stained with ponceau to confirm the protein transfer and then blocked with 3% nonfat skimmed milk solution prepared in TBS-T for 45 min. Specific proteins were probed with primary antibodies-HDAC6, HDAC10, p-ERK, ac-NF-κB, H3, acetyl tubulin, tubulin, Ac-H3K9, Ac-H3K56 and GAPDH for overnight at 4 ºC . After washing the membrane with TBS-T for 3 times, each wash for 10 min; secondary antibody conjugated with horseradish peroxidase was added and incubated for an hour. After two washes with TBS-T for 10 min each, detection was performed by enhanced chemi luminescence reagent (ECL, Amersham) using Bio-Rad imaging system.

2.5 Isolation of histone proteins

Histone proteins were isolated from treated and control cells as described in Nature protocols (Shechter, Dormann et al. 2007). In brief, the cells were washed with ice cold PBS and resuspended in 5-10 volumes of hypotonic solution (10 mM Tris pH 8, 10 mM KCl, 1.5 mM $MgCl_2$ and 1 mM DTT) and incubated on ice for 1 h. Intact nuclei were pelleted at 10000 g, 4 ºC for 10 min and then resuspended in acidic solution (10 mM HEPES pH7.9, 0.4 N H_2SO_4, 10 mM KCl, 1.5 mM PMSF, 1.5 mM $MgCl_2$, 0.5 mM DTT and protease inhibitor cocktail) with mixing on rotospin for 30 min. The supernatant containing histone proteins collected at 16000 g at 4 ºC for 10 min and precipitated by adding 132 µl of 100% TCA (Tri chloro acetic acid). After 30 min incubation on ice, the histone proteins were pelleted and washed with acetone twice to remove the acid. Finally the protein was air dried and dissolved in water. Concentration of isolated histone proteins was measured as

mentioned before. The different acetylation levels of lysine residues of histone protein (H3) were detected by Western blotting as mentioned above.

2.6 HDAC activity assay

HDAC activity was determined using fluorescent substrate FLUOR DE LYS® (ALX-260-137-M005) purchased from Enzo Life Sciences. The HDAC6 protein was immunoprecipitated from total protein lysates (0.5-1 mg) of cells treated with different compounds using protein A agarose beads (Sigma). The HDAC6 protein bound beads were used as enzyme source and was incubated with the HDAC assay buffer (50 mM Tris pH 8.0, 1 mM $MgCl_2$, 2.7 mM KCl, 137 mM NaCl and 1 mg/ml BSA) and fluorescent substrate (60 µM final) in a final volume of 50 µl at 37 ºC for 15 min. Reaction was stopped with the addition of 50 µl developer solution (Enzo Life Sciences) followed by incubation at 37 ºC for 30 min. The Arbitrary Fluorescent Units (AFU) were recorded at Excitation wavelength of 360 nm and Emission wavelength of 460 nm.

2.7 Transfection

K562 cells (1 $x10^6$) were transfected with plasmid pcDNA-HDAC6-FLAG and pLKO-HDAC6 using PEI reagent as per the manufacturer protocol. Briefly, the plasmid DNA was mixed with PEI (1 mg/ml) reagent in the ratio of 1:1 in serum free media and incubated for 15 min before adding to the cells. After 3 h incubation, incomplete medium was replaced with complete medium and allowed to grow for different time points. Overexpression and knockdown was confirmed by qPCR and / or HDAC activity assay. pcDNA-HDAC6-FLAG was a gift from Dr.Tso-Pang Yao (Addgene plasmid # 30482).

2.8 Nuclear and cytoplasmic extractions

Briefly, the cells were lysed in cytosolic extraction buffer (30 mM Tris-HCl, [pH 7.5], 0.5% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 10 mM $MgCl_2$ and protease inhibitor cocktail),

incubated on ice for 3 min and centrifuged at 10000 g for 3 min. The pelleted nuclei were washed with cytosolic extraction buffer and spun at 16000 g for 5 min. Nuclear extraction buffer (10 mM HEPES [pH 7.9], 1 mM PMSF, 1 mM EDTA, 420 mM NaCl, 25% glycerol, 10 mM $MgCl_2$ and protease inhibitor cocktail) was used for the lysis of nuclei for 30 min on ice with intermittent vortexing. The nuclear extract was collected at 16000 g for 30 min at 4^{o}C.

2.9 Immuno Fluorescence

The cells were fixed in 4% formaldehyde at room temperature for 15-20 min after PBS wash. Formaldehyde was added slowly along the walls of the tube. Then, the cells were washed twice with PBS and permeabilized with 0.25% Triton X-100 prepared in PBS for 10-15 min. The cells were washed twice with PBS and blocked with 3% bovine serum albumin in PBS for 1 h. The cells were spun at 2500 rpm for 5 min and incubated with primary HDAC6 antibody (1:100) prepared in 3% BSA for 1 h at room temperature. This is followed by washes with 1% BSA in PBS-T, twice with PBS and then the cells were incubated with fluorophore-conjugated (Alexa 647) secondary antibody (1:200, 3% BSA in PBS) at room temperature under dark conditions for 1 h. After the sequential washes with PBS-T (in 1% BSA) and twice with PBS, cells were resuspended in mounting media consists of 4, 6-diamidino-2-phenylindole (DAPI) and placed on cleaned glass slide. Mounted samples were viewed under a Carl Zeiss, NLO 710 laser scanning confocal microscope and images were captured with ZEN 2010 acquisition software.

2.10 Measurement of ROS by Flow cytometry

2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), an oxidation sensitive dye, was used to measure the ROS production in cells treated with PMA and / or Tubastatin A. The cells were washed with PBS and then incubated with 5 μM H_2DCFDA in PBS for 30 min at 37 °C. Minimum

of 20,000 events were counted for each sample and analyzed using BD LSR Fortessa flow cytometry system.

2.11 Chromatin Immuno precipitation (ChIP)

Day-1

Cells (2.5 x 10^6) were fixed in 1% formaldehyde solution in 5 ml of serum free media for 10 min at RT. The cells were quenched with 0.125 M glycine for 5 min with gentle agitation followed by wash twice with PBS at 1200 rpm for 5 min at 4 ^oC and then resuspended in 100 µl of cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM sodium butyrate, 10 mM NaCl, 50 µg/ml PMSF, 0.2% NP40 and 1 µg/ml leupeptin) for 10 min on ice. Nuclear pellet was collected at 2500 rpm for 5 min at 4 ^oC and then the nuclei were lysed in 300 µl of nuclear lysis buffer (NLB-50 mM Tris-HCl pH 8.1, 10 mM sodium butyrate, 1% SDS, 10 mM EDTA, 50 µg/ml PMSF and 1 µg/ml leupeptin) for 10 min on ice. 180 µl of immuno precipitation dilution buffer (IPDB-20 mM Tris-HCl pH 8.1, 10 mM sodium butyrate, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, 0.01% SDS, 50 µg/ml PMSF and 1 µg/ml leupeptin) was added and subjected to sonication (Sonics, VCX 750) at an amplitude of 36%, for 25-30 cycles, each cycle with 20 sec on and 40 sec off for shearing the DNA into fragments of 300-1000 bp. Cell debris is removed by centrifugation at 14000 rpm for 5 min at 4^oC. The chromatin sample was diluted with 1 ml of IPDB to bring the ratio of IPDB: NLB to 4:1. 5 µl of rabbit IgG was used for the preclearance of sample for 1 h followed by incubation with 15 µl of Protein G Dynabeads® (Invitrogen 10004D) for 3 h at 4 ^oC on rotating wheel. The beads were collected using DynaMag^{TM}-2. Then 60 µl of chromatin sample was taken in separate tube as input and stored at -20 ^oC. The remaining sample was divided in to parts (450-700 µl) depending on the number of antibodies used for ChIP. In our experiments, we have divided the sample in two different tubes (each 700 µl) and diluted with equal volume of IPDB^{mod} (mix NLB and IPDB in

1:4 ratio) followed by incubation with 5 µl of HDAC6 (ab47181), 3 µl of H3K9 (9649) at 4 ºC on rotating wheel for overnight.

Day-2

The samples were then centrifuged at 13000 rpm for 5 min at 4ºC. 20 µl of Protein G-Dynabeads® were added to the sample and incubated for 3 h at 4ºC with rotation. The beads were collected using DynaMag™-2 and washed with 1 ml of IP wash buffer 1 (IPWB 1- 20 mM Tris-HCl pH 8.1, 1% Triton X-100, 0.1%SDS, 50 mM NaCl and 2 mM EDTA) followed by 1 ml of IPWB 2 (IPWB 2-10 mM Tris-HCl pH 8.1, 1 mM EDTA, 1% deoxycholic acid, 250 mM NaCl and 1% NP-40) and 1 ml of TE pH 8.0. 50 µl of IP elution buffer (IPEB—100 mM NaHCO3, 1% SDS) was added to elute the immune complexes (DNA-protein-antibody) from the beads. Elution was repeated again with another 50 µl of IP elution buffer and both elutions were combined in one tube. 0.5 µl of RNase A (5 mg/ml stock) and 6 µl of 5 M NaCl (final concentration of 0.3 M) were added to each sample. Also, 0.35 µl of RNase A (5 mg/ml stock), 0.36 µl of 5 M NaCl were added to the input sample. All the samples were incubated at 65 ºC for 6 h followed by proteinase K treatment (2 µl of 10 mg/ ml stock) at 45 ºC for overnight.

Day-3

2 µl of tRNA was added (5 mg/ml) to the samples before adding 200 µl of phenol/chloroform, mixed well by vortexing and then centrifuged at 13200 rpm for 5 min at room temperature. The aqueous layer was collected in a new tube and mixed with 500 µl of chloroform and spun at the same speed as mentioned above. 18 µl of sodium acetate (3 M, pH 5.2) and 0.5 ml of 100 % ethanol was added to precipitate DNA at -70 ºC for 30 min. Then, the samples were centrifuged at 14000 rpm for 20 min at 4 ºC. 70 % ethanol was used to wash the DNA pellet and then allowed to air dry. The DNA was dissolved in 50 µl of nuclease free water and 20 µl for input samples. The DNA

from input and IP samples was quantified by using Nanodrop and the samples were stored at -20 ^{o}C. PCR was performed to amplify GYPA promoter with 5 sets of primers (Table 3).

Table 3: ChIP primers designed for GYPA promoter

	primer	5' ⟶ 3'
Set1	FP	CTT GAG CAC AAT TCC TGC AA
	RP	CTG AGC AGC AGG ACA AGA A
Set2	FP	ATT GAG CTT CCT CGC ATT TT
	RP	CGC AGC TAT GAA ACC AGT GA
Set3	FP	GGC TCC ACA ACA GCT ACC TC
	RP	TCT TGG GGC TAT GAA AGT GG
Set4	FP	AAA TGC CTC CCC TGC CTA T
	RP	CCT GAG ATC ATG AGC TGG TTC
Set5	FP	CAA GGG AGC CCA GTA TTT ATG
	RP	GCC ATT TGG CAG AAA TAG GA
GAPDH	FP	TAC TAG CGG TTT TAC GGG CG
	RP	TCG AAC AGG AGG AGC AGA GAG CGA

2.12 shRNA cloning

The shRNA oligos (Table 4) were designed, cloned in pLKO.1 puro vector and confirmed as per the protocol from addgene (Addgene Plasmid 10878. Protocol Version 1.0. December 2006.) shRNA sequences targeting different regions of HDAC6, were chosen from Block it design tool and Origene company. The sense and antisense sequences of target gene were incorporated with stem and loop sequences along with EcoR1 and Age-1 restriction enzyme sites as mentioned in the table 4 and got them synthesized from Integrated DNA Technologies. 5 µl of each forward and reverse oligo of 20 µM concentration were annealed in 50 µl reaction in the presence of 10X NEB buffer 2 at 95 ºC for 4 min followed by 70 ºC for 10 min in Eppendorf® Mastercycler and then slowly cool down to room temperature. Meanwhile, pLKO vector was digested with EcoRI and Age-1 (BshT1) restriction enzymes in a 30 µl reaction volume consists of 3 µl of 10X Buffer O (Fermentas, USA), 3 µg of vector, 1 µl of EcoR1, 0.5 of Age-1 and nuclease free water. The reaction was carried out in a water bath (EQUITRON) at 37 ºC for 12 h. The digested vector was

separated on 1% agarose gel, and extracted by gel extraction method (Thermo scientific, USA) according to standard protocol. The digested vector (60 ng) was mixed with 2 µl of annealed oligo for ligation using 1 µl of T4 DNA ligase (Fermentas, USA) in the presence of 2 µl of 10X ligase buffer in total reaction volume of 20 µl and kept at 4 ºC for overnight. XL-Blue cells / DH5 alpha cells were employed for plasmid transformation. Briefly, 10 µl of ligation sample was added to the cells, mixed gently and the sample was incubated for 10 min on ice. After heat pulse of 90 sec at 42 ºC, the cells were transferred on to ice immediately. 1ml of Luria-Bertani (LB) broth was added to the cells and incubated in an orbital shaker (ORBITEK) for 1 h at 180 rpm. The culture was spun down for 8 min at 5000 rpm and plated on ampicillin LB agar plate. Colonies were screened for the insert presence by performing PCR reaction with vector primers and confirmed by double digestion and sequencing. pLKO.1 puro vector is a generous gift from Dr. Kishore Parsa from DRILS, Hyderabad. Transfection was done as mentioned above.

Table 4: shRNA HDAC6 oligo nucleotide sequences

Primer	Sequence 5'-----3'
ShRNA-pLKO1 FP	CCGGT GGAGGACAATGTAGAGGAGAG ACTCGAGA CTCTCCTCTACATTGTCCTCC TTTTTT G
ShRNA-pLKO1 RP	AATTC AAAAAA GGAGGACAATGTAGAGGAGAG ACTCGAGA CTCTCCTCTACATTGTCCTCC A
ShRNA-pLKO2 FP	CCGGT AGGTCTACTGTGGTCGTTACATCAATGGC ACTCGAGA GCCATTGATGTAACGACCACAGTAGACCT TTTTTT G
ShRNA-pLKO2 RP	AATTC AAAAAA AGGTCTACTGTGGTCGTTACATCAATGGC ACTCGAGA GCCATTGATGTAACGACCACAGTAGACCT A

2.13 HDAC10 cloning

The complete sequence of 2.1 kb of human HDAC10 was amplified from clone purchased from MGC clones using gene specific primers (FP 5'GTCGGAATTCATGGGGACCGCGCTTGTG TAC 3' and RP 5' GTCG CTCGAG TCAAGCCACCAGGTGAGGATG 3'). The total reaction volume of 50 µl consisting of final concentrations of 10 pmol primers, 0.3 mM dNTP each, buffer, 1 µl of Pfu DNA polymerase enzyme (KAPA HiFi) and nuclease free water. PCR amplification

was carried out in Eppendorf® Master cycler. The reaction conditions include initial denaturation 95 ºC: 2 min followed by 35 cycles of 95 ºC for 45 sec, 70 ºC: 45 sec, 72 ºC: 2 min 10 sec. and final extension of 72 ºC: 10 min. The amplified product was run on 1 % agarose gel and the bands were visualized by UV transilluminator (Major science, USA). The separated amplified products were extracted using Gel extraction kit (Thermo scientific, USA) as per standard protocol. Vector (pcDNA3.1+) and insert were double digested with EcoR1 and Xho1, gel extracted, ligated and transformed in DH5α competent cells as mentioned above. The colonies were screened for HDAC10 insert and confirmed the presence of insert with double digestion and sequencing of clone.

2.14 Statistical analysis

Results of at least three independent experiments with duplicates were shown as the means of ±standard deviation (SD). Analysis was carried out using one-way ANOVA, two-way ANOVA, followed by Tukey's multiple comparison tests by Graphpad Prism 6.01. *p-value < 0.05; **p-value < 0.01 and ***p-value < 0.001.

Results and Discussion

Objective 1

HDACs profiling and identification of HDAC(s) upregulated during MK differentiation

All types of blood cells develop from hematopoietic stem cells (HSCs) by a process of haematopoiesis. Several factors regulate the process of lineage commitment such as cytokines, lineage-specific transcription factors and microRNAs etc. The development of megakaryocytes (MK) from bipotent erythrocyte-megakaryocyte progenitor (MEPs) cells depends upon lineage commitment of MEPs *via* the suppression of erythrocyte (ER) lineage (Corral-Fernandez, Cortes-Garcia et al. 2017). At molecular level, several studies have identified the involvement of transcription factors, miRNAs and cytokines for MK differentiation from MEPs (Chang, Bluteau et al. 2007). Recently, epigenetic mechanisms involving gene repression by histone deacetylases (HDACs) has been identified (Nan, Ng et al. 1998). However, a detailed study on the underlying molecular mechanisms of MK differentiation *via* ER lineage suppression by HDACs remains elusive. With an aim to identify the role of HDACs in MK lineage commitment and differentaiton of MEPs to MK, the Phorbal 12-myristate 13-acetate (PMA) induced MK differentaiton of K562 cells was used as model system.

3.1 Validation of PMA induced differentiation of K562 cells to megakaryocytes

K562 cells are chronic myeloid leukemia cells, well studied model system to find out the molecular mechanisms regulating the erythroid and megakaryocyte lineages in the presence of different chemical inducers (Lozzio and Lozzio 1977). PMA induces the differentiation of K562 cells towards megakaryocyte lineage whereas hemin, hydroxyurea and Ara-C (Arabinosyl cytosine) induce erythroid differentiation of these cells (Villeval, Pelicci et al. 1983, Shelly, Petruzzelli et al. 1998). Furthermore, Sardina JL et al. clearly demonstrated that the results obtained in their study using K562 cells as megakaryocyte progenitor cells and results obtained using CD34+ cells were similar. This indicates that whatever observations they got in K562 cells are biologically

meaningful (Sardina, Lopez-Ruano et al. 2010). The differentiation of K562 cells to megakaryocytes is monitored by cell growth arrest, endomitosis, changes in morphology and adhesive properties due to expression of integrins CD61 and CD41 (MK markers) and reduced expression of erythroid genes Glycophorin A (Butler, Ziemiecki et al. 1990). PMA activates PKC leading to further activation of ERK1/2, and transcription factors like c-JUN, c-FOS, NF-κB (Murray, Baumgardner et al. 1993, Franklin and Kraft 1995, Kang, Lee et al. 1996, Kim, Kim et al. 2001).

To validate the model system, we have treated cells with 20 nM PMA for 24 h and 48 h and studied morphological features and expression analysis of MK markers, CD61, CD41 and erythroid lineage gene marker, Glycophorin A (GYPA). Phase contrast microscopy showed change in morphology and size of cells treated with 20 nM PMA compared to control cells which are spherical and same size (Fig: 7A). We have observed upregulation of MK markers (CD61 and CD41) along with downregulation of an erythroid lineage gene GYPA time dependantly in PMA treated cells compared to control cells (Fig: 7B).

3.2 Differential expression of class IIb HDACs during MK differentiation of K562 cells

Earlier studies have shown that sustained expression of HDAC1 by GATA-1 transcription factor allows the differentiation of myeloid progenitor cells to erythrocyte and megakaryocytic lineages (Wada, Kikuchi et al. 2009). Hence after the validation of the model system, we did HDAC expression profiling (mRNA and protein expression) to find out which HDAC(s) are involved in MK differentiation. We have isolated RNA and protein from control and PMA treated cells for different time points and did qPCR and Western blotting. The RNA and protein expression levels of HDAC1, 2 and 8 belongs to class I and HDAC4 from class IIa did not change significantly in

control and PMA-treated cells (Fig 8A & B). However, RNA and protein levels of class IIb HDACs, HDAC6 and 10, were significantly upregulated during MK differentiation (Fig 8A & B).

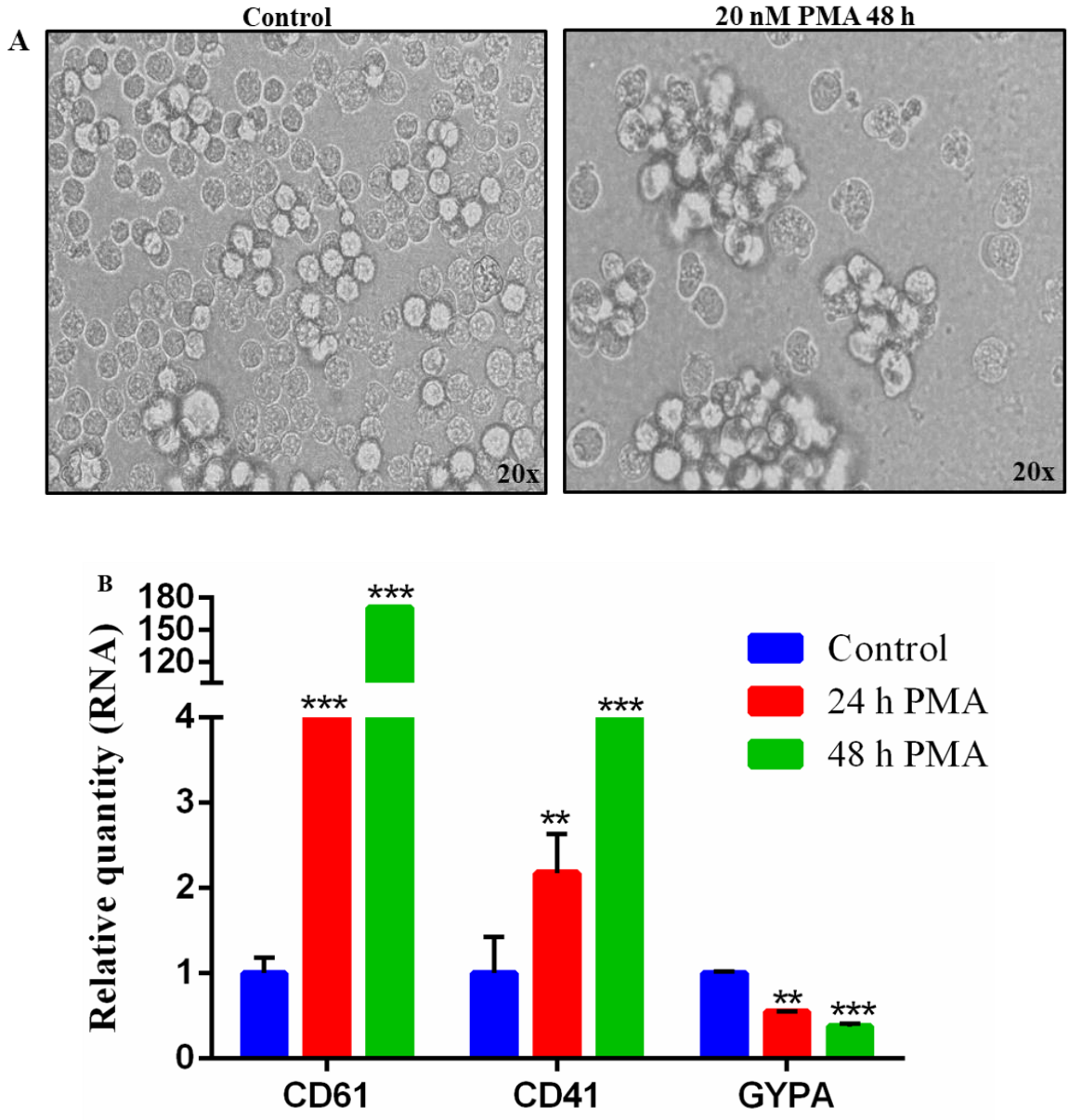

Fig 7*: Validation of PMA induced differentiation of K562 cells to megakaryocytes* *morphology of K562 cells treated with PMA by Phase contrast microscopy (A) and RNA expression levels of MK lineage markers CD61 and CD41 and (GYPA) in PMA treated cells at different time points (B).*

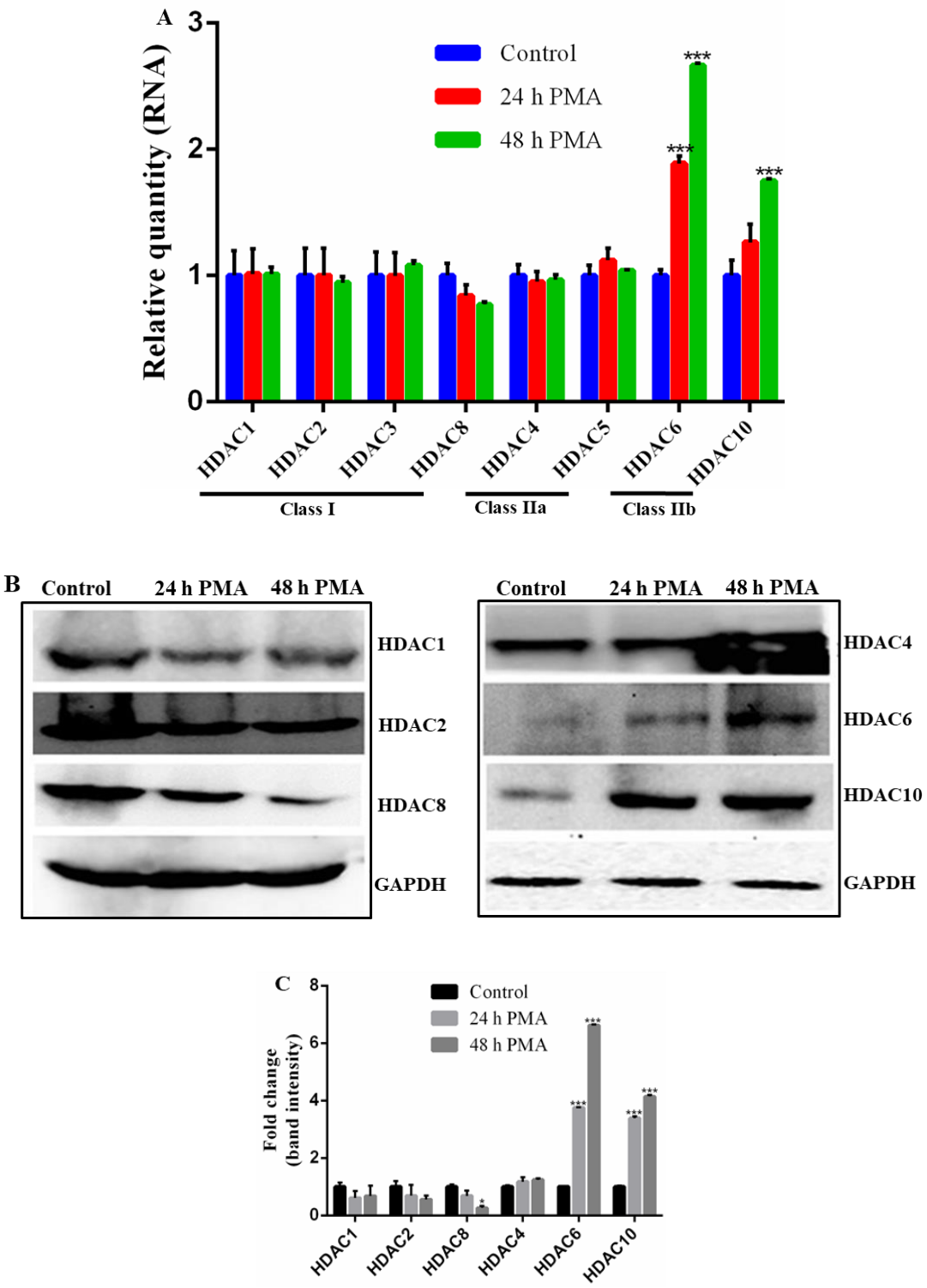

Fig 8: ***Differential expression of classIIb HDACs during MK differentiation of K562 cells*** *RNA expression levels of class I and II HDACs (A), protein expression of HDAC2, 4, 6, 8 and 10 during MK differentiation (B) and densitometry by Image J software (C).*

3.3 Upregulation of HDAC6 throughout MK differentiation

Class IIb HDACs, particularly HDAC6 plays an important role in maintaining cell morphology by regulating the dynamics of cytoskeletal proteins including tubulin and cortactin (Hubbert, Guardiola et al. 2002, Zhang, Yuan et al. 2007). Since mature MKs undergo endomitosis and form pseudopodia which involves cytoskeletal changes in the cell, we wanted to find out upregulation of class IIb HDACs is limited to early stages of MK commitment or throughout MK differentiation. To address this, we have done time-course mRNA expression analysis up to 5 days of PMA treatment. We have observed upregulation of HDAC10 till 48 h whereas HDAC6 was upregulated along with MK markers (CD41 and CD61) till 4 days of PMA treatment. We also observed gradual downregulation of GYPA significantly till 5 days during differentiation (Fig. 9).

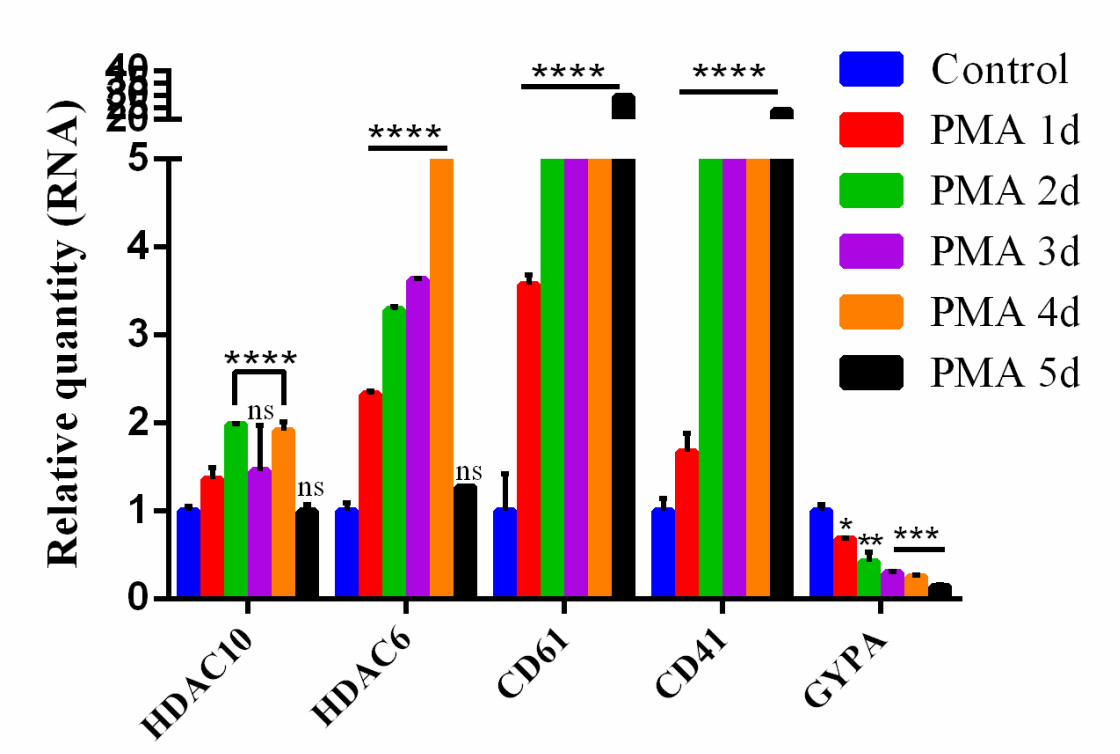

Fig 9: ***Upregulation of HDAC6 throughout MK differentiation*** *RNA expression of HDAC10, 6, MK markers and GYPA in PMA treated cells for 5 days.*

These results suggest that HDAC6 might play a role in both early and late differentiation of MK cells. In line of our results, Messaoudi et al have shown that HDAC6 is required for formation of demarcation membrane system (DMS) in mature MKs derived from CD34+ cells by regulating the acetylation levels of cortactin protein (Messaoudi, Ali et al. 2017). Other studies have also shown the involvement of HDAC6 in regulating the function of microtubules by deacetylation, which is indispensable for platelet synthesis, structure and function (Kile 2012, Sadoul, Wang et al. 2012).

HDAC6 is a class IIb cytoplasmic deacetylase with 1215 amino acid residues (131kDa) and largest of HDAC family. The gene for HDAC6 is localized on X chromosome at sub band border of Xp11.22-23 in humans. It possess two homologous catalytic domains (CD1 and CD2) making it unique among all other HDACs (Grozinger, Hassig et al. 1999, Zou, Wu et al. 2006). The well-studied cytoplasmic substrates of HDAC6 include tubulin, heat shock protein (HSP90), cortactin, peroxiredoxins (Hubbert, Guardiola et al. 2002, Bali, Pranpat et al. 2005, Zhang, Yuan et al. 2007, Choi, Kim et al. 2017). Studies have shown role of HDAC6 in variety of biological processes including transcription, cell signaling, migration, inflammation, cell survival, angiogenesis, cell motility, autophagy, viral infection and various diseases as shown in fig 10 (Lee, Lim et al. 2008, Lee, Koga et al. 2010) (Valenzuela-Fernandez, Cabrero et al. 2008, Zhang, Ogden et al. 2016, Bitler, Wu et al. 2017, Lin, Chen et al. 2017).

3.4 Decreased acetylation levels of H3K9 and H3K56 during MK differentiation

Since histones are the primary substrates for HDACs, we have purified histone proteins by acid extraction method and did Western blotting for global acetylation levels of lysine residues of histone H3. We have observed a significant decrease in acetylation levels of H3K9 and H3K56

during differentiation whereas the acetylation levels of H3K18, H3K14 and H3K27 did not alter in control and PMA treated cells (Fig 11). These results suggest that H3K9 and H3K56 might be

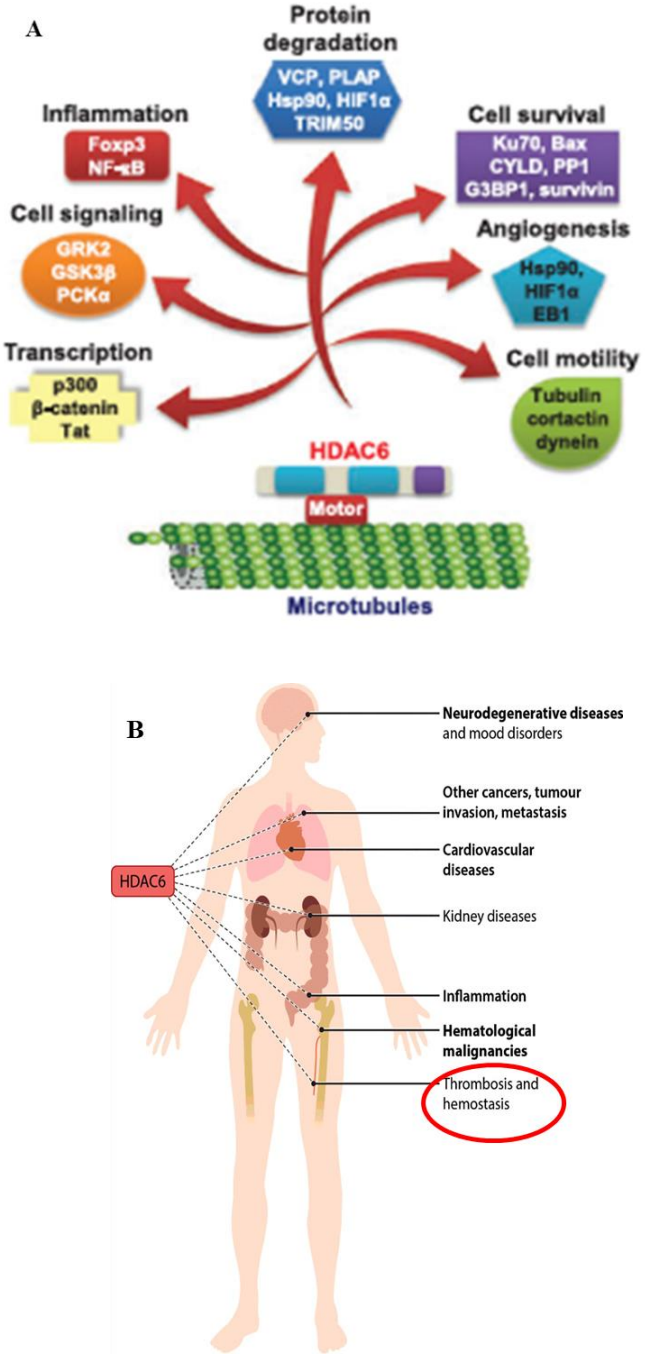

Fig 10: ***Physiological and pathological functions of HDAC6*** *different substrates and interacting partners of HDAC6 involved in various biological processes (A) and altered expression or activity of HDAC6 in pathological conditions (B)* Source *: FEBS Journal 280 (2013) 775–793; Sri N. Batchu, et al. Clinical Science May 06, 2016,130(12)987-1003.*

the targets of over expressed and increased activity of HDAC6 and HDAC10, similar observations were made in earlier studies supporting our data (Dovey et al. 2010).

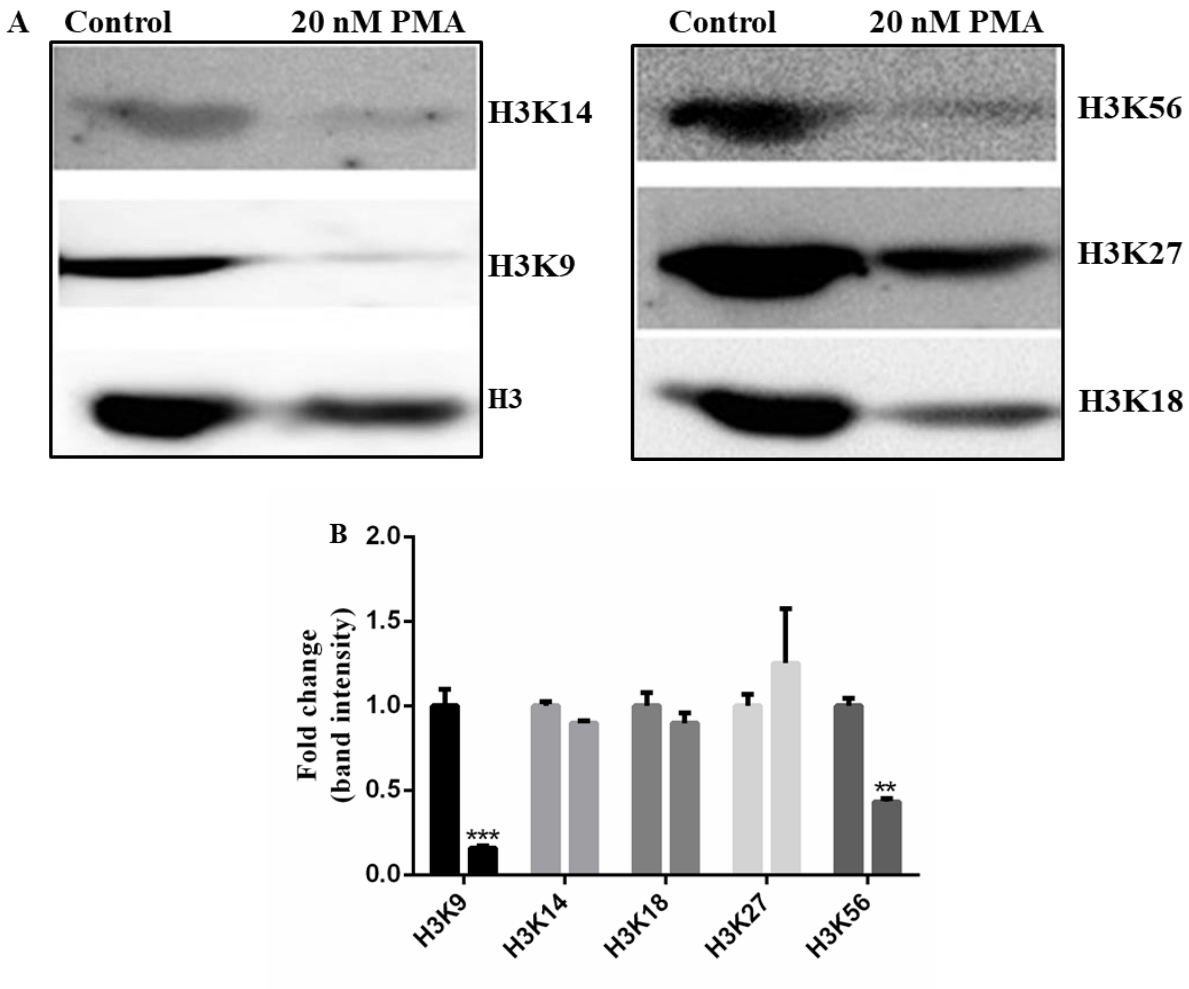

Fig 11: ***Decreased acetylation levels of H3K9 and H3K56 during MK differentiation*** *Western blotting of acetylation levels of different lysine residues of H3 protein in control and PMA treated cells (A) and densitometry by Image J software (B).*

Objective-2

Functional studies of HDAC6

3.5 HDAC6 upregulation during MK differentiation might be *via* PKC-p-ERK1/2-NF-κB signaling pathway

We next investigated the mechanism by which HDAC6 is upregulated during MK differentiation of K562 cells. PMA is known to activate PKC-ERK1/2 signaling pathway leading to activation of downstream targets NF-κB and FosB which enter into the nucleus to regulate gene expression during MK differentiation (Kang, Lee et al. 1996, Whalen, Galasinski et al. 1997, Kang, Han et al. 1998). Hence we have analyzed the PKC pathway activated by PMA in K562 cells during MK differentiation. We determined the levels of phospho-ERK1/2 and Ac-NF-κB (K310) in control and PMA-treated K562 cells. In the immunoblots, we found increased p-ERK1/2 levels, Ac-NF-κB (K310) and HDAC6 during PMA treatment (Fig 12A).

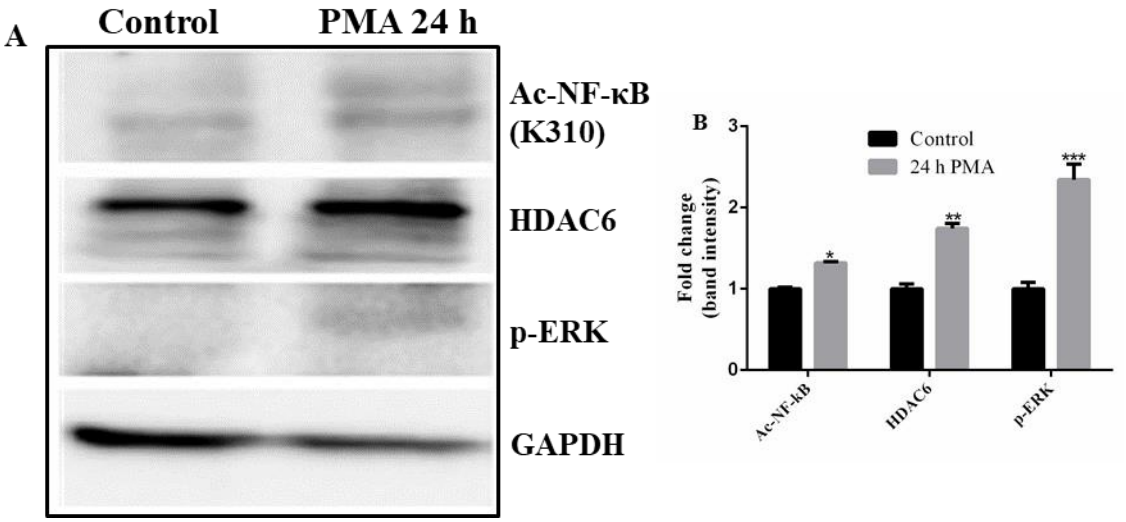

Fig 12: ***HDAC6 upregulation during MK differentiation is via PKC-p-ERK1/2 signaling pathway*** *Western blotting of HDAC6, ac-NF-κB, p-ERK1/2 in control and PMA treated cells (A) and densitometry of blots by Image J software (B).*

To confirm HDAC6 upregulation *via* PKC-p-ERK1/2-NF-κB, we have treated the cells with ERK1/2 inhibitor, Apigenin during PMA-induced differentiation of K562 cells and then analyzed RNA expression of HDAC6 and CD61. We found reduced HDAC6 and MK marker expression when ERK1/2 activation was inhibited with Apigenin in PMA-induced K562 cells (Fig 13A) suggesting that HDAC6 upregulation during MK differentiation is indeed *via* PKC-p-ERK1/2 axis. In addition, HDAC6 promoter analysis showed the presence of NF-κB binding sites, indicating that ac-NF-κB (K310) might be positively regulating HDAC6 expression during MK differentiation (Fig 13B).

Previous studies showing the increased expression of HDAC6 by binding of NF-κB to the HDAC6 gene promoter in hepatocellular carcinoma (Ding, Liu et al. 2013) further reinforced our *in silico* results. A significant reduction in the CD61 expression was observed in Apigenin-treated PMA-induced K562 cells. However, we did not observe significant change in the expression levels of CD41 and GYPA in the presence of ERK1/2 inhibitor during MK differentiation.

3.6 PMA induced upregulation of HDAC6 is specific to MK cells

PMA is known to activate several signaling pathways including PKC and PI3K in other cell lines (Dufourny, Alblas et al. 1997). So we asked the question whether HDAC6 upregulation is due to PMA signaling or is MK lineage specific. To address this, we have used another myeloid progenitor cell line, HL-60 (promyelocytic leukemia cell line). Some chemical reagents cause the differentiation of HL60 cells to granulocyte-like cells, or to monocyte/macrophage-like cells (Birnie 1988). PMA activates PKC during HL-60 cells differentiation to monocytic/macrophage as like K562 cells (Slosberg, Yao et al. 2000). We have treated K562 and HL-60 cells with PMA and then assessed the RNA expression levels of HDAC6. The results showed no change in HDAC6 expression levels in PMA treated HL-60 cells indicating that upregulation of HDAC6 in PMA-

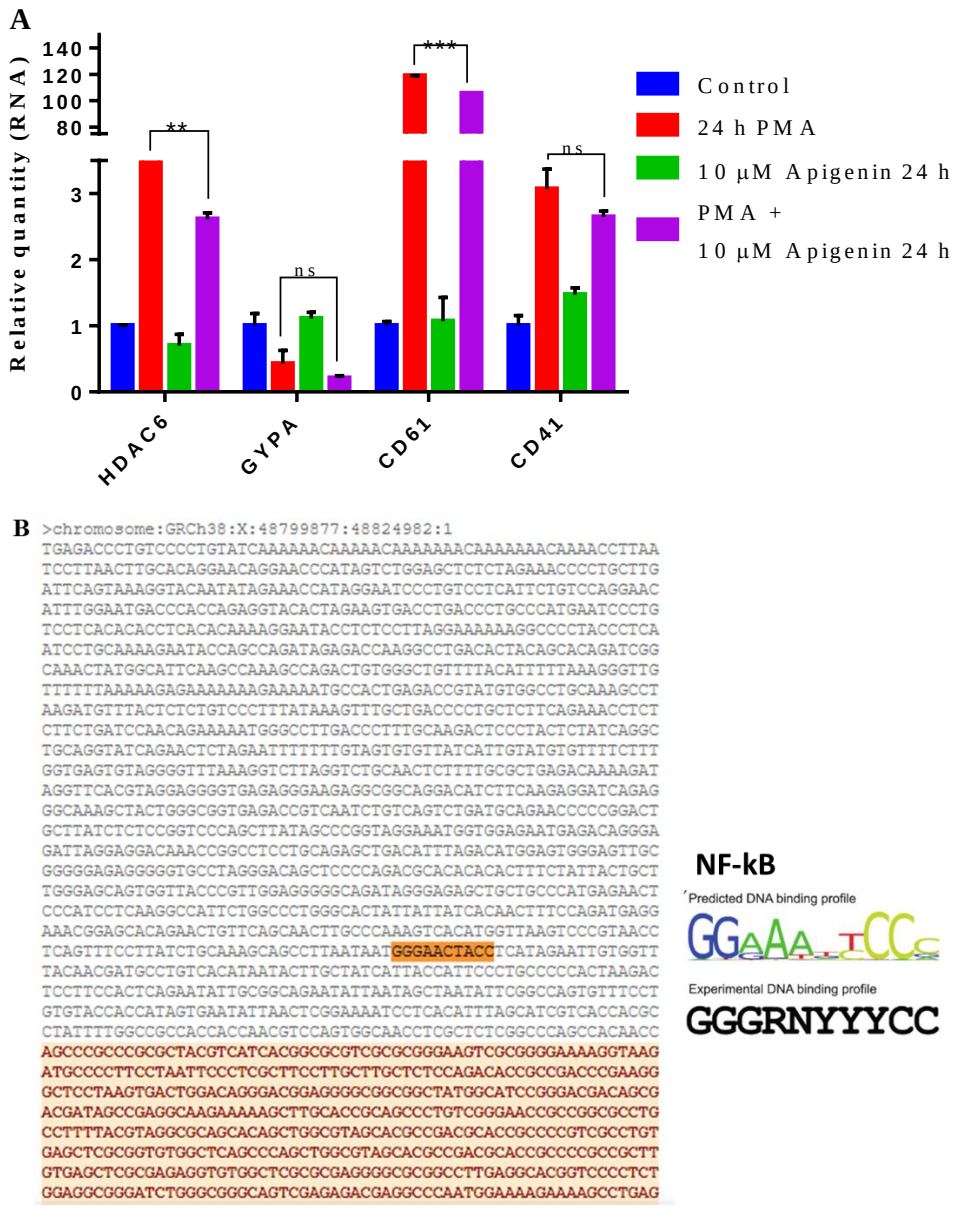

Fig 13: ***Confirmation of HDAC6 upregulation during MK differentiation via PKC-p-ERK1/2-NF-κB signaling pathway*** *RNA expression analysis of HDAC6, MK markers and GYPA in the presence of Apigenin and /or PMA (A) and NF-κB binding site on HDAC6 promoter using Ensemble genome browser (B).(Brown color marking indicates first exon in HDAC6 promoter analysis).*

treated K562 cells is MK lineage-specific but not due to PMA treatment (Fig 14A). Further, we have confirmed upregulation of HDAC6 along with MK marker (CD61) and downregulation of

GYPA in another MEP cell line, Dami, which differentiates into megakaryocyte upon PMA treatment (Fig 14B).

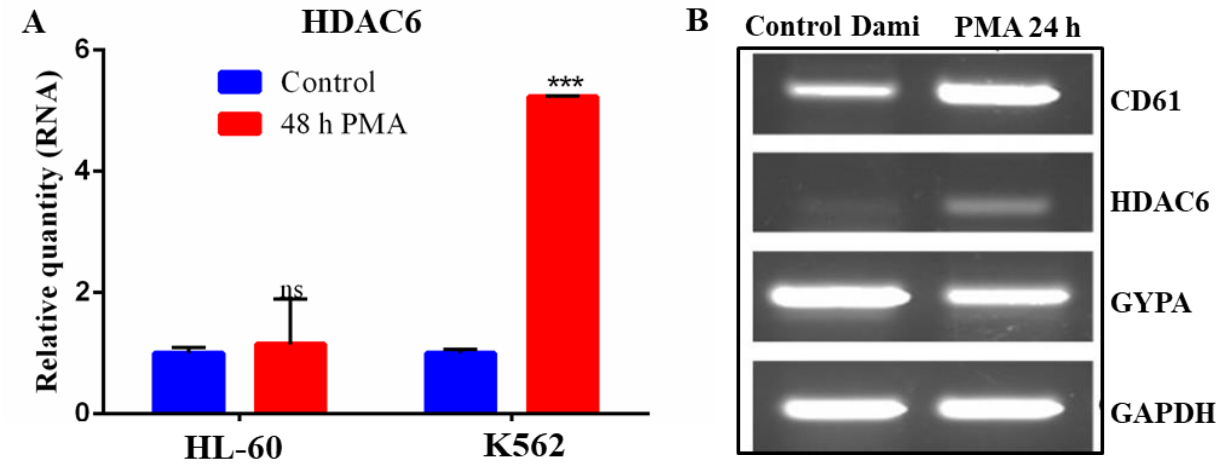

Fig 14: ***PMA induced upregulation of HDAC6 is specific to MK cells*** *HDAC6 expression in K562 and HL-60 cells treated with 20 nM PMA for 48 h (A) and semi quantitative-PCR of HDAC6, GYPA and CD61 in Dami cells (B).*

3.7 Localization of HDAC6 in the nucleus during MK differentiation

It is very well known that class II HDACs shuttle between cytoplasm and nucleus based on their phosphorylation status (Kao, Verdel et al. 2001). HDAC6 possess nuclear localization signal (NLS) at N-terminus and two nuclear export signals (NES) at N and C terminals of the protein, plays a role in cytoplasmic and nuclear shuttling. Besides NES, it harbors Ser-Glu-containing tetra peptide (SE14) motif, also required for anchoring the protein in cytoplasm (Bertos, Gilquin et al. 2004). In the nucleus, it regulates transcription by interacting with nuclear proteins such as HDAC11, sumoylated p300, transcriptional repressors such as LCoR, ETO-2 (Fernandes,

Fig 15: ***Functional domains of the human HDAC6 protein*** *SE14,Serine Glutamine rich tetrapeptide; BUZ-ubiquitin binding domain; NES, Nuclear export signal; NLS, Nuclear localization signal.* Source: *volume 35, issue 10, p501–509, 2014, Trends in pharmacological sciences.*

Bastien et al. 2003) and transcription factors, RUNX2 and NF-κB (Zhang and Kone 2002) (Westendorf, Zaidi et al. 2002). It has been shown that in differentiating osteoblasts, HDAC6 interacts with RUNX2 and suppress p21Cip1/Waf1 gene expression (Westendorf, Zaidi et al. 2002).

To delineate the role of upregulated HDAC6 in MK differentiation as a repressor of gene or as a protein activity regulator in cytoplasm, we first investigated HDAC6 localization by confocal microscopy. As shown in Fig 16, HDAC6 is predominantly localized to the nucleus during MK

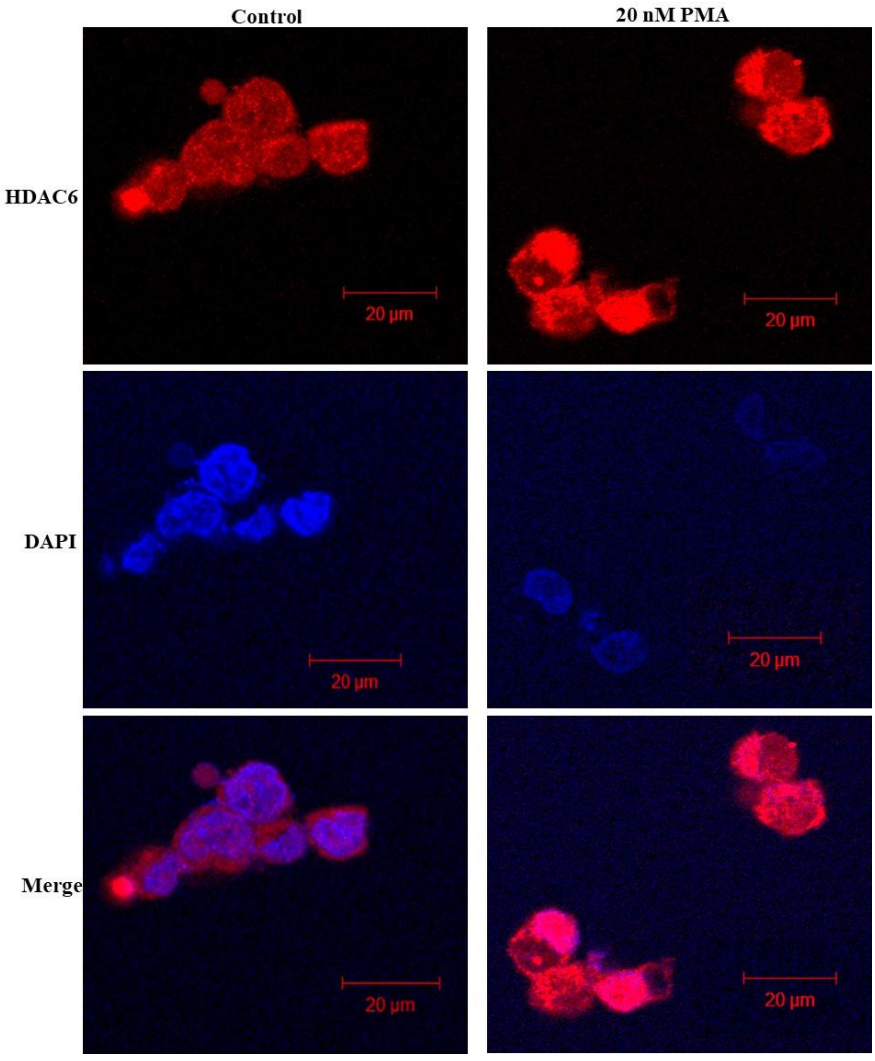

Fig 16: ***localization of HDAC6 in the nucleus during MK differentiation*** *localization of HDAC6 in control and PMA treated cells by confocal microscopy.*

differentiation when compared to pan-cellular distribution in control cells. These results indicate that nuclear localization of HDAC6 during MK differentiation might plays a role in regulation of gene expression.

3.8 Pharmacological inhibition of HDAC6 by Tubastatin A

Tubastatin A (Tub A) is a specific inhibitor of HDAC6. Most of the studies showed that 1-10 µM concentration of Tub A inhibits HDAC6 activity in different cell lines (Cheng, Lienlaf et al. 2014, Wang, Rao et al. 2014). To dissect the role of HDAC6 in MK differentiation, we employed inhibitor studies using Tub A. To standardize Tub A concentration in our model system, we have treated the cells with different concentrations (2.5-10 µM) of Tub A and analyzed the acetylation levels of tubulin, a well-known substrate of HDAC6 (Fig 17A). As shown in the fig 17A, we have observed gradual increase in the acetyl tubulin levels in concentration dependent manner. Based on these results, we have chosen 5 µM concentration of Tub A as an optimal concentration to inhibit HDAC6 during MK differentiation. Further, we also evaluated its inhibitory effect on other HDACs (HDAC1, 2 and 8) by qPCR (Fig. 17B) and found no significant effect on HDAC1 and HDAC2 expression whereas HDAC8 expression is altered in PMA and Tub A treated cells.

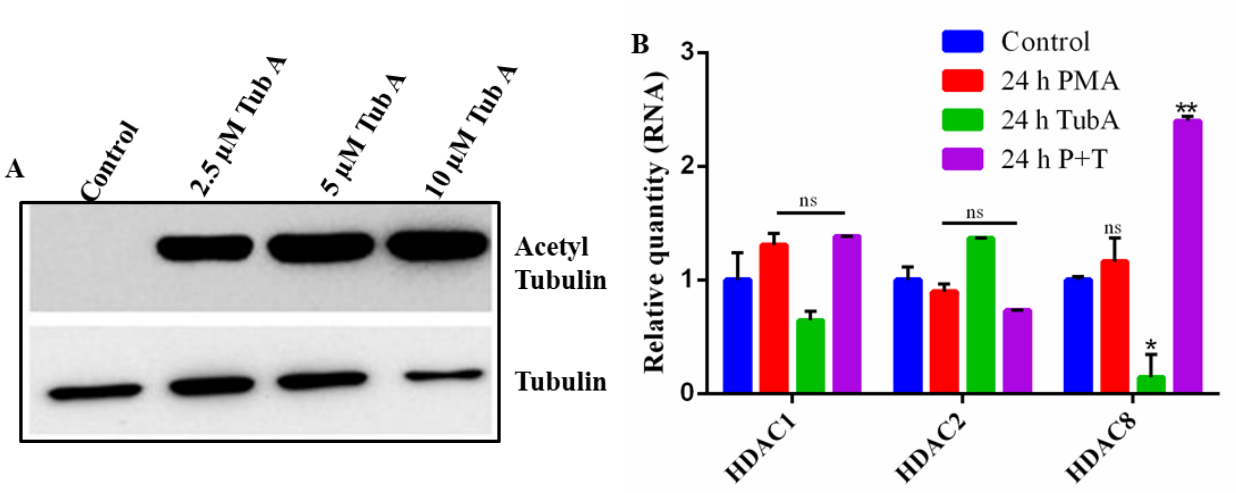

Fig 17: *Pharmacological inhibition of HDAC6 by Tubastatin A* *Western blotting of acetyl tubulin in cells treated with different concentrations of tub A (A) and RNA expression levels of HDAC1, 2 and 8 in the presence of PMA and / or Tub A for 24 h (B).*

3.9 HDAC6 inhibition by Tub A results in downregulation of CD61 and upregulation of GYPA

To get an insight into the molecular function of HDAC6 during MK differentiation, we have treated the cells with Tub A and analyzed the expression levels of MK lineage genes. The qPCR results clearly indicated that when HDAC6 is inhibited, MK markers were downregulated significantly along with increased erythroid marker expression (Fig 18).

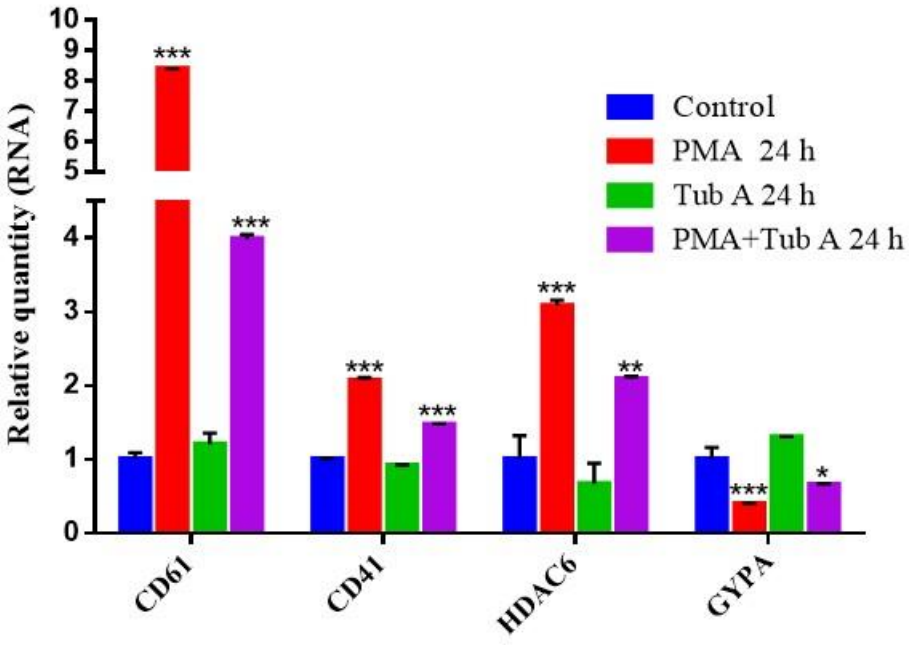

Fig 18: ***Pharmacological inhibition of HDAC6 results in downregulation of MK markers and upregulation of erythroid marker*** *RNA expression levels of MK markers (CD61, CD41), HDAC6 and GYPA in cells treated with PMA and / or Tub A for 24 h.*

Our results are further supported by Rubin et al who demonstrated that the intensity of GPIIb / IIIa (CD61 and CD41) staining in individual MKs within the various colonies was notably reduced in pan HDAC inhibitor (LBH589), inhibiting several HDACs including HDAC6, treated cultures as compared to control cultures (Iancu-Rubin, Gajzer et al. 2012).

To confirm the qPCR results, we have measured the protein levels of CD61 by flow cytometry during MK differentiation in the presence of Tub A. In agreement with qPCR data, we have observed 15% reduction in the number of differentiated cells expressing CD61 in the presence of HDAC6 inhibitor indicating that HDAC6 positively regulates CD61 expression during MK differentiation (Fig 19).

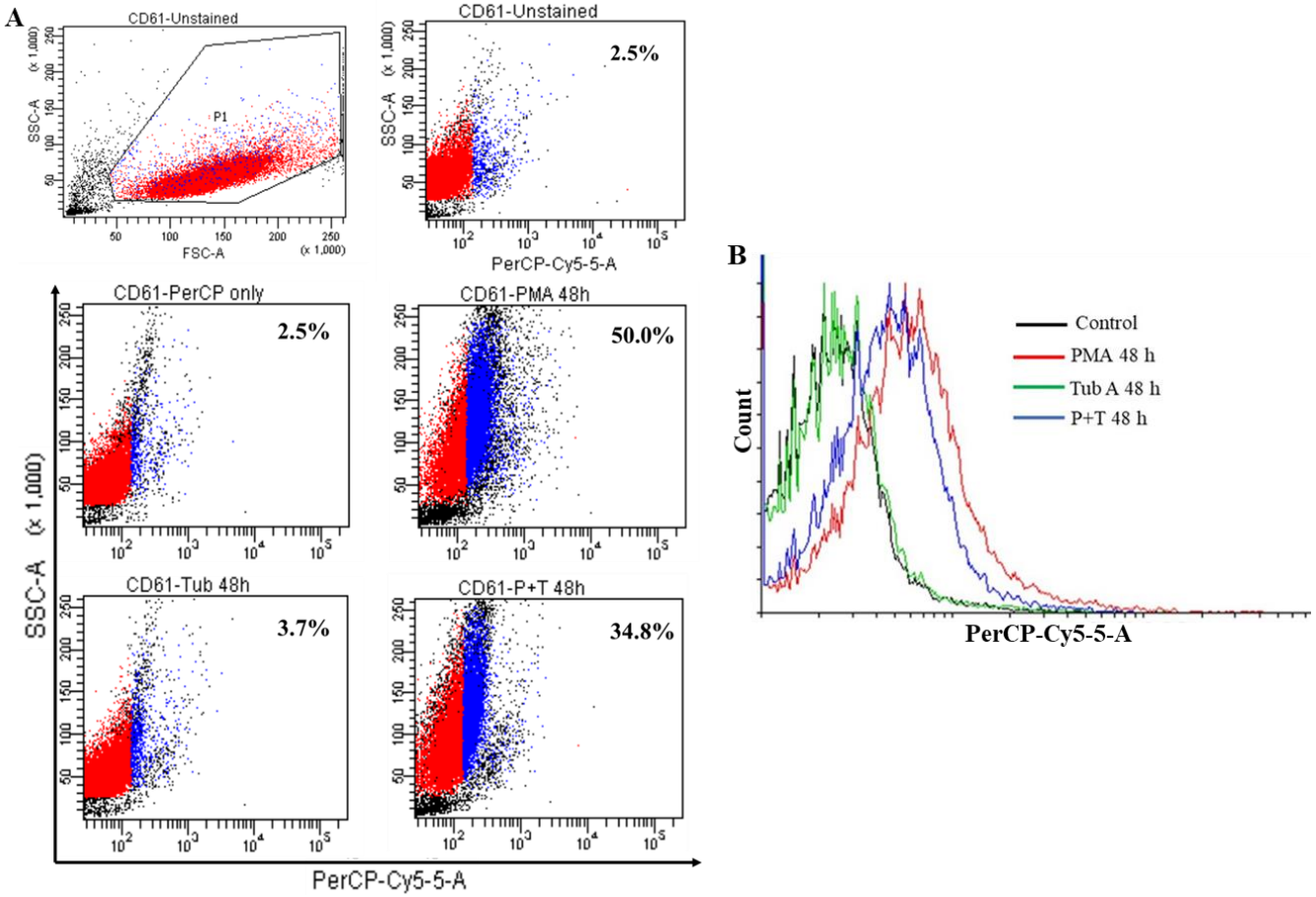

Fig 19: ***Downregulation of CD61 protein levels in the presence of HDAC6 inhibitor during MK differentiation*** *CD61 protein expression by flow cytometry in PMA and or Tub A treated cells for 48 h (A) and overlay of dotplots using flowing software (B).*

It is established that HDAC6 plays a role in ubiquitination and studies by ChonghuaLi, *et al*, have shown that SnoN, an inhibitor of Smad Pathway, is a repressor of CD61, undergoes ubiquitination during PMA signaling of MK differentiation (Li, Peart et al. 2014). Based on these results, we presume that HDAC6 might be promoting SnoN degradation and thus positively

regulating CD61 expression. Recently, Messaoudi et al have shown significant decrease in the no. of CFU when they have treated CD34+ cells with 5 µM Tub A and this supports that our data is biologically meaningful as we also observed that 5 µM Tub A inhibited the acquisition of MK marker, CD61.

Tub A is a specific inhibitor of HDAC6 and inhibits HDAC6 activity at protein level. To further confirm the previous results, we wanted to inhibit HDAC6 at mRNA level by genetic knockdown using shRNA. We have cloned HDAC6 shRNA into the EcoR1 and Age-1 sites of pLKO.1 puro vector (Fig 20A). The colonies were screened with vector specific primer by semi quantitative PCR (Fig 20B). The presence of insert was further confirmed by double digestion (Fig 20C) followed by sequencing.

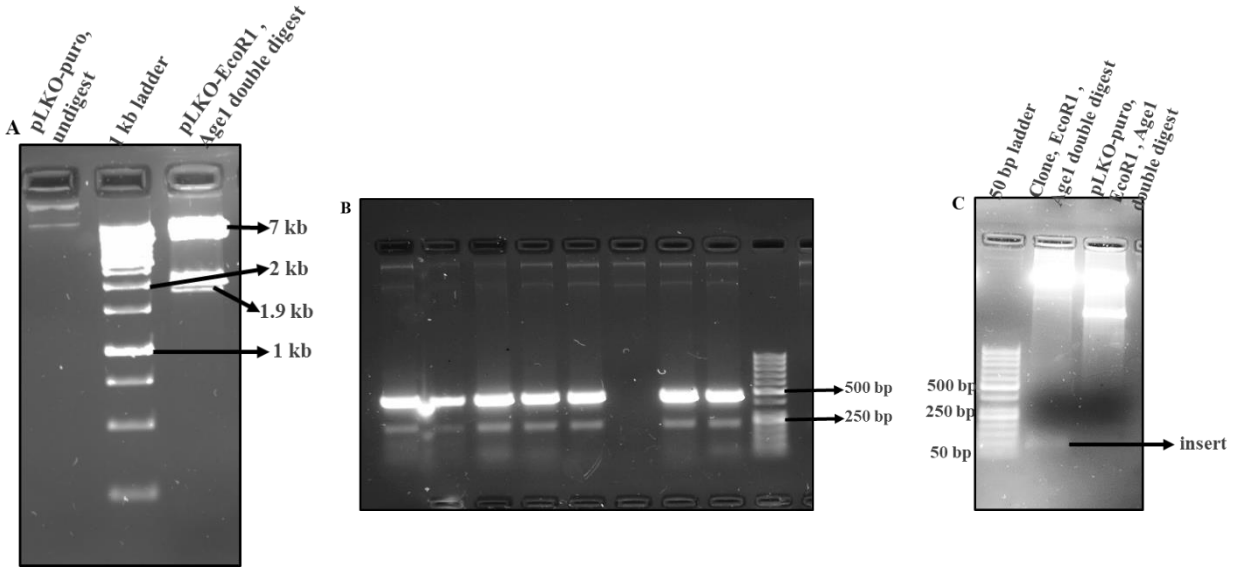

Fig 20: ***Cloning of shRNA-HDAC6 in pLKO.1puro vector*** *Double digestion of the pLKO.1-puro plasmid with EcoR1 and Age-1 (A), PCR for plasmids isolated from different colonies after transformation with vector primers(B), positive clone and pLKO.1puro vector double digestion with EcoR1 and Age-1 for confirming the presence of insert (C).*

We have performed HDAC6 knockdown using pLKO.1-shRNA-HDAC6 during MK differentiation. HDAC6 knockdown was confirmed at RNA level by qPCR for 24 h and 48 h (Fig 21A) and the expression of MK markers and GYPA was analyzed by qPCR. When HDAC6 is knockdown during MK differentiation (PMA + shRNA), the expression levels of MK marker (CD61) were reduced significantly at 24 h and 48 h compared to PMA treated cells (Fig 21B). Whereas, the erythroid marker (GYPA) showed significant upregulation during MK differentiation when HDAC6 is inhibited (Fig 21C). Importantly GYPA levels were 1.5 fold higher

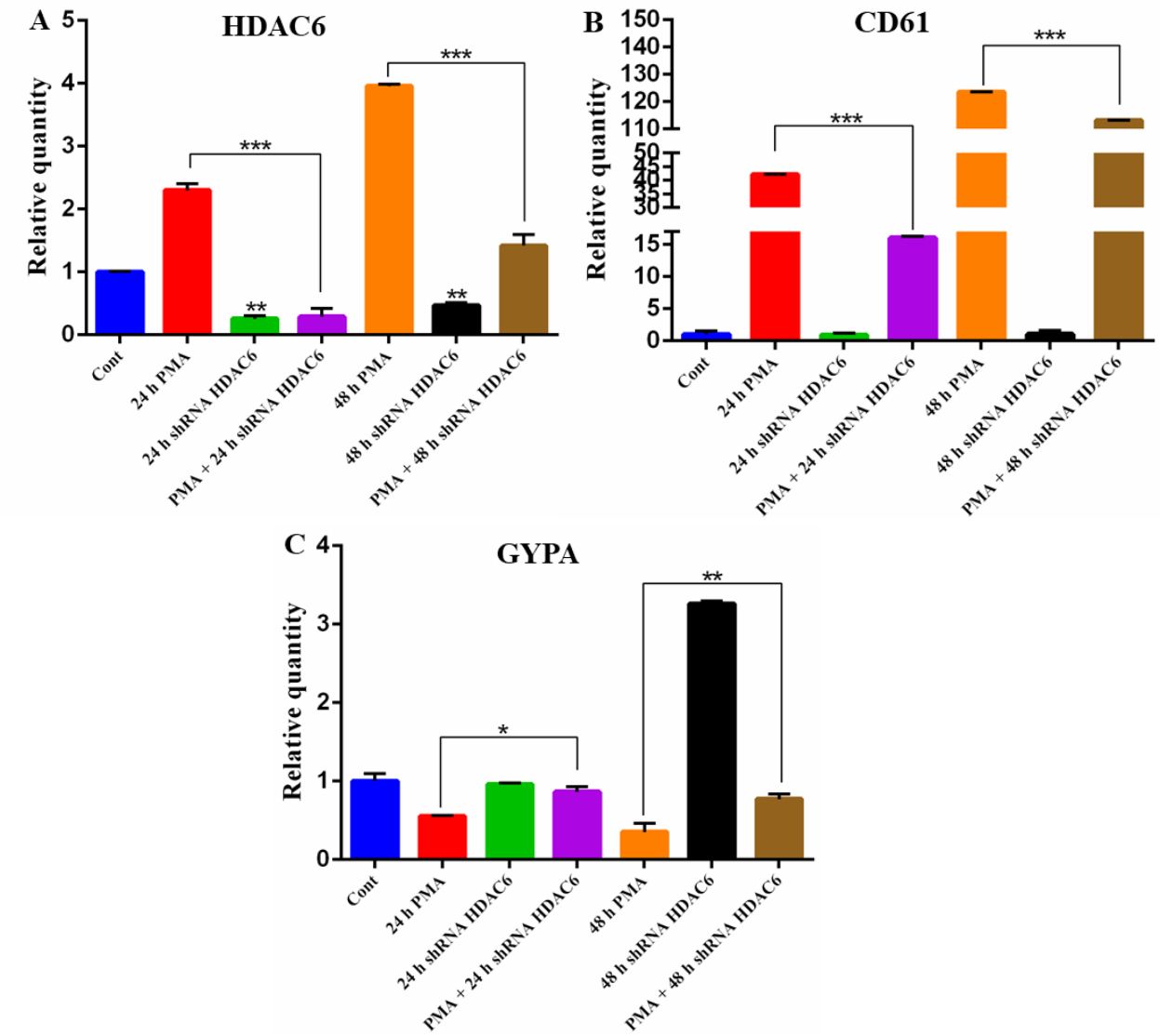

Fig 21: ***Genetic knockdown of HDAC6 during MK differentiation mimicking effects obtained with Tub A*** *RNA expression analysis of HDAC6 (A), CD61 (B), and GYPA (C) in HDAC6 knockdown cells for 24 h and 48 h in the presence or absence of PMA.*

in control cells transfected with shRNA-HDAC6 at 48 h, confirming that HDAC6 plays an important role in repressing GYPA expression levels during MK differentiation.

3.10 HDAC6 and HDAC10 overexpression positively regulates MK marker expression

To further confirm that HDAC6 plays a role in MK lineage commitment, we have overexpressed HDAC6 in K562 cells and studied MK lineage commitment. Overexpression of HDAC6 in K562 cells was confirmed at RNA level by semi quantitative PCR (Fig 22A) and protein level by Immunoblot (Fig 22B) and HDAC activity assay (Fig 22C). Overexpression of HDAC6 resulted in significant upregulation of CD61, but not CD41 and downregulation of erythroid lineage gene, GYPA (Fig 22D).

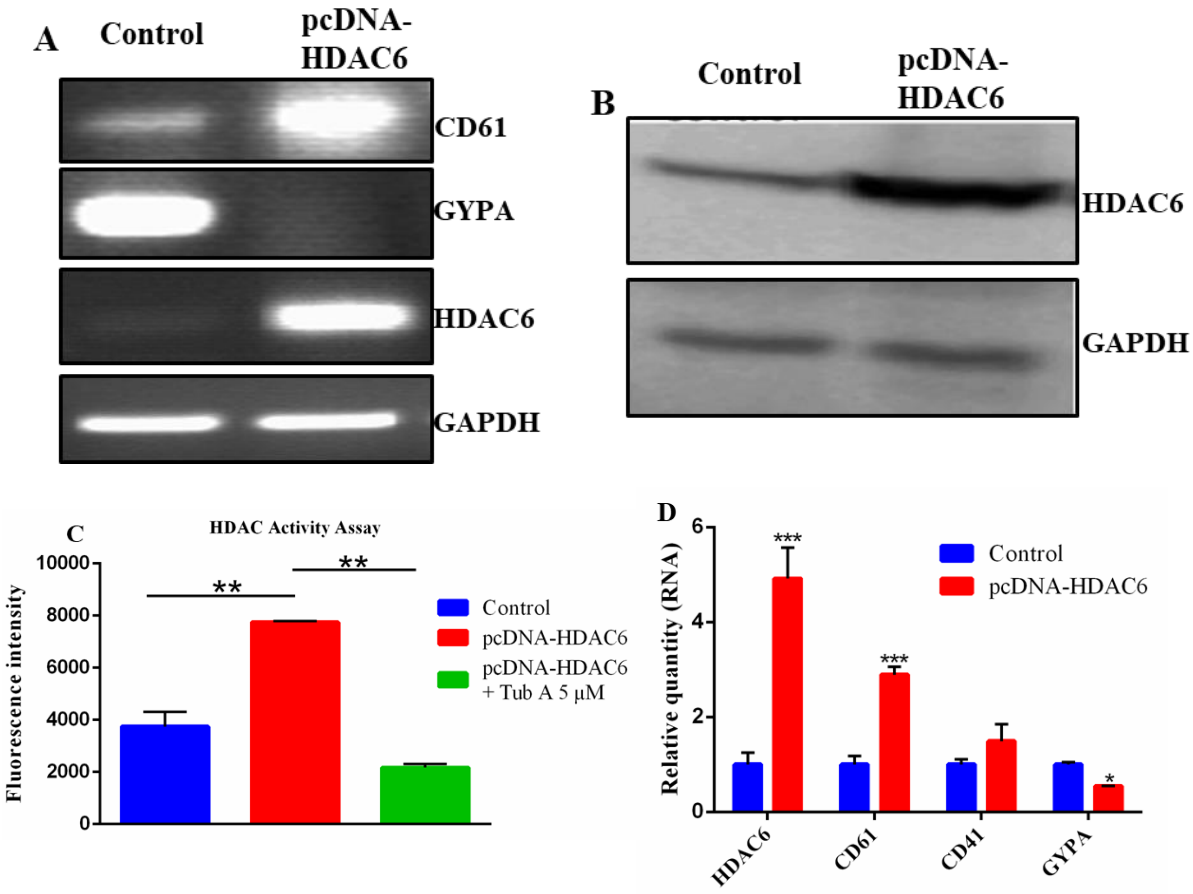

Fig 22: *HDAC6 and HDAC10 overexpression positively regulate MK marker expression* *HDAC6 overexpression analyzed by semi quantitative PCR (A) and Western blotting (B), HDAC activity assay (C) and RNA expression analysis of CD61, CD41 and GYPA in HDAC6 transfected cells (D).*

Since CD41 levels did not change upon HDAC6 overexpression, we thought HDAC10 that is upregulated during MK differentiation of K562 cells treated with PMA might be playing a role in CD41 expression. Hence we have cloned HDAC10 into the EcoR1 and Xho1 restriction sites of pcDNA3.1 (Fig. 23A&B), confirmed by double digestion (Fig. 23C) followed by sequencing. Overexpression of HDAC10 in K562 cells showed upregulation of CD41 with no change in CD61 levels (Fig: 23D) indicating that HDAC10 positively regulates expression of CD41 whereas HDAC6 regulates CD61 expression.

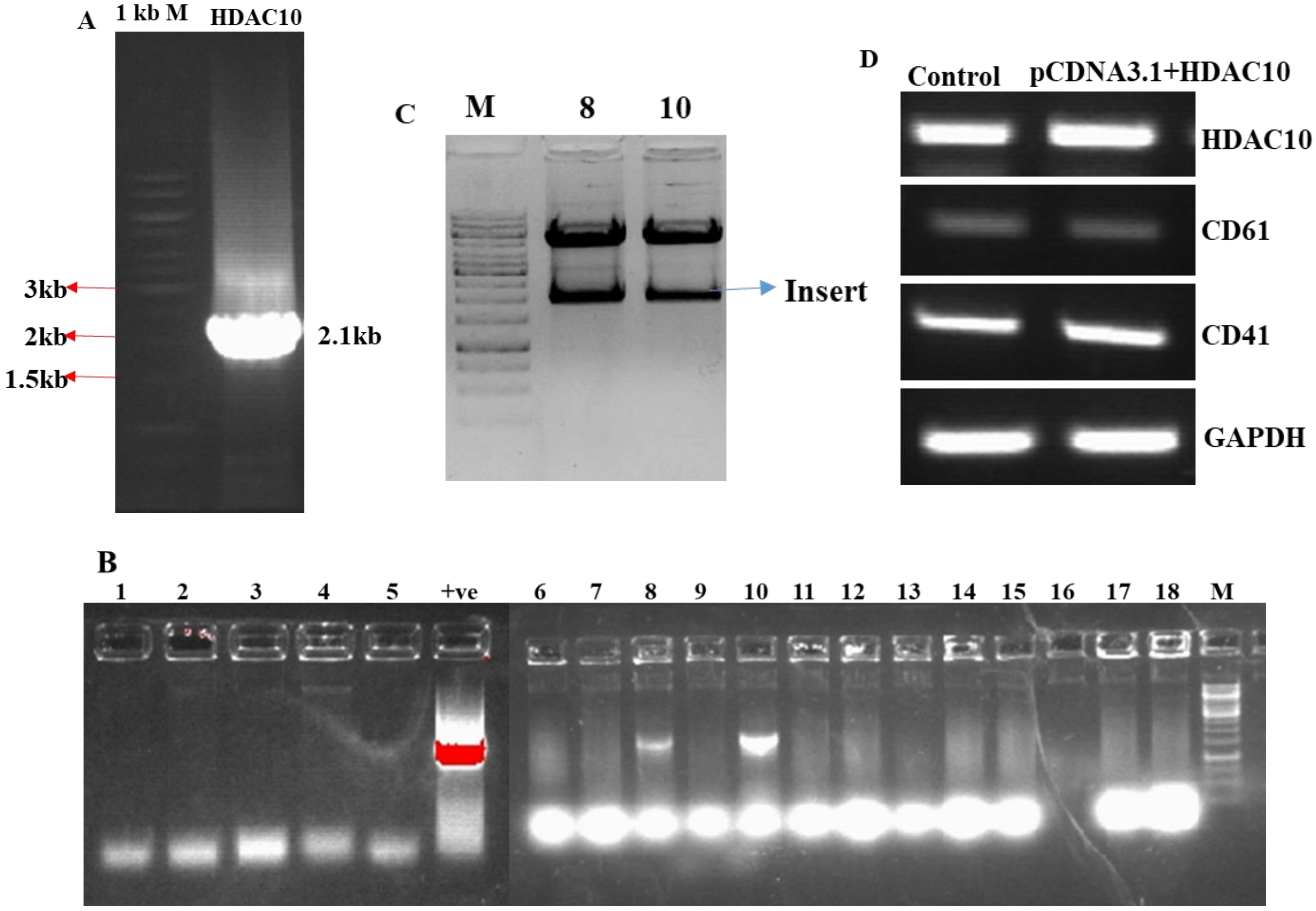

Fig 23: ***HDAC10 cloning and overexpression in K562 cells positively regulate MK marker expression Amplification*** *of HDAC10 by PCR (A), colony PCR for screening the presence of HDAC10 insert (B), double digestion of positive clones (8 and 10) with EcoR1 and Xho1 (C) and RNA expression levels of MK markers (CD61, CD41), GYPA and HDAC10 in HDAC10 overexpressed K562 cells (D).*

3.11 HDAC6 might regulates MK lineage transcription factor expression

Next, we sought to determine how HDAC6 is positively regulating the expression of MK marker (CD61). To explore this, we have analysed the expression of MK transcription factors in HDAC6 inhibited and overexpression condition. We observed reduced expression of FOG-1 and GATA-2 in Tub A treated cells alone but not in HDAC6 inhibited cells treated with PMA (Fig. 24A &B). GATA-1 expression levels did not change significantly in the presence of Tub A during differentiation (Fig. 24C). On the contrary, we observed upregulation of GATA-2 and FOG-1 in HDAC6 overexpressed cells without PMA treatment (Fig. 24D). These results indicate that HDAC6 might be regulating the expression levels of GATA-2 and FOG-1 indirectly that in turn regulate CD61 expression. Further studies are required to confirm these presumptions.

The function of GATA-2 is not only limited to early MK development from MEP cells but also for proliferation of other haematopoietic progenitor cell lineages. Overexpression of GATA-2 in K562 cells results in activation of MK markers and MK lineage commitment at the expense of erythroid lineage (Ikonomi, Rivera et al. 2000). GATA-1 plays role in terminal differentiation of megakaryocytes and for platelet production (Kuhl, Atzberger et al. 2005). GATA-1 deficient megakaryocytes are small with low ploidy and significant reduction in the expression levels of MK associated genes, platelet factor 4 (PF4), c-MPL, and p45 NF-E2 (Vyas, Ault et al. 1999). GATA transcription factors activities are modulated by the interaction with multiple zinc finger proteins like FOG-1. Thus, FOG-1 plays important role in commitment and maturation of megakaryocytes by interacting with GATA-2 at early stages and GATA-1 at late stages of MK development (Tsang, Fujiwara et al. 1998). Thus our results are in line with earlier results demonstrating that HDAC6 might be regulating GATA-2 and FOG-1 expression and thus MK differentiation.

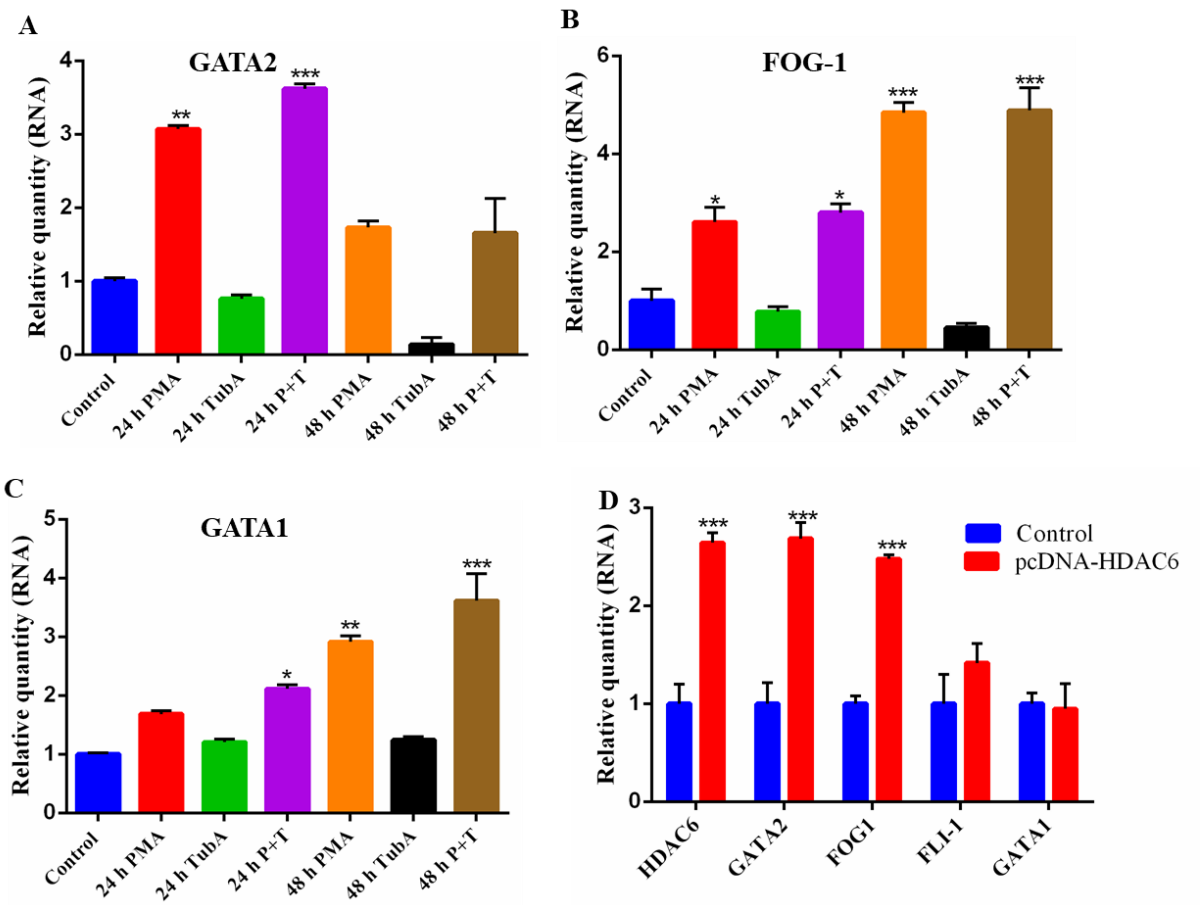

Fig 24: *HDAC6 might regulates MK lineage transcription factor expression* *Transcription factor expression in the presence of PMA and / or Tub A for 24 h and 48 h (A, B,C) and in HDAC6 overexpressed cells without PMA treatment (B).*

3.12 HDAC6 represses GYPA promoter

Most of the studies mentioned HDAC6 function in various cellular processes *via* regulating non histone targets as it is predominantly localized to cytoplasm (Gao, Hubbert et al. 2007). Earlier studies have shown that the nuclear HDAC6 promotes epithelial-mesenchymal transition (EMT) by suppressing the genes involved in formation of tight junctions (Ding, Liu et al. 2013). The nuclear localization of HDAC6 in our studies led us to hypothesize that HDAC6 might be regulating gene expression or regulating the activity or function of transcription factors during MK differentiation. In addition, when HDAC6 was inhibited with Tub A, we have observed upregulation of GYPA not only in PMA-treated cells, but also in Tub A treated control cells (Fig

25A). We did not observe similar significant effect on other erythroid lineage genes like γ-globin and EKLF (Fig 25B) indicating that HDAC6 is involved in repression of GYPA alone. To confirm, we have analyzed the acetylation levels of the two histone H3 lysine residues, H3K9 and H3K56 that were actually downregulated during MK differentiation. The immunoblot for Ac- H3K9 and Ac-H3K56 showed a significant increase in the acetylation levels of H3K9 and H3K56 in presence of HDAC6-specific inhibitor, Tub A. (Fig. 25C).

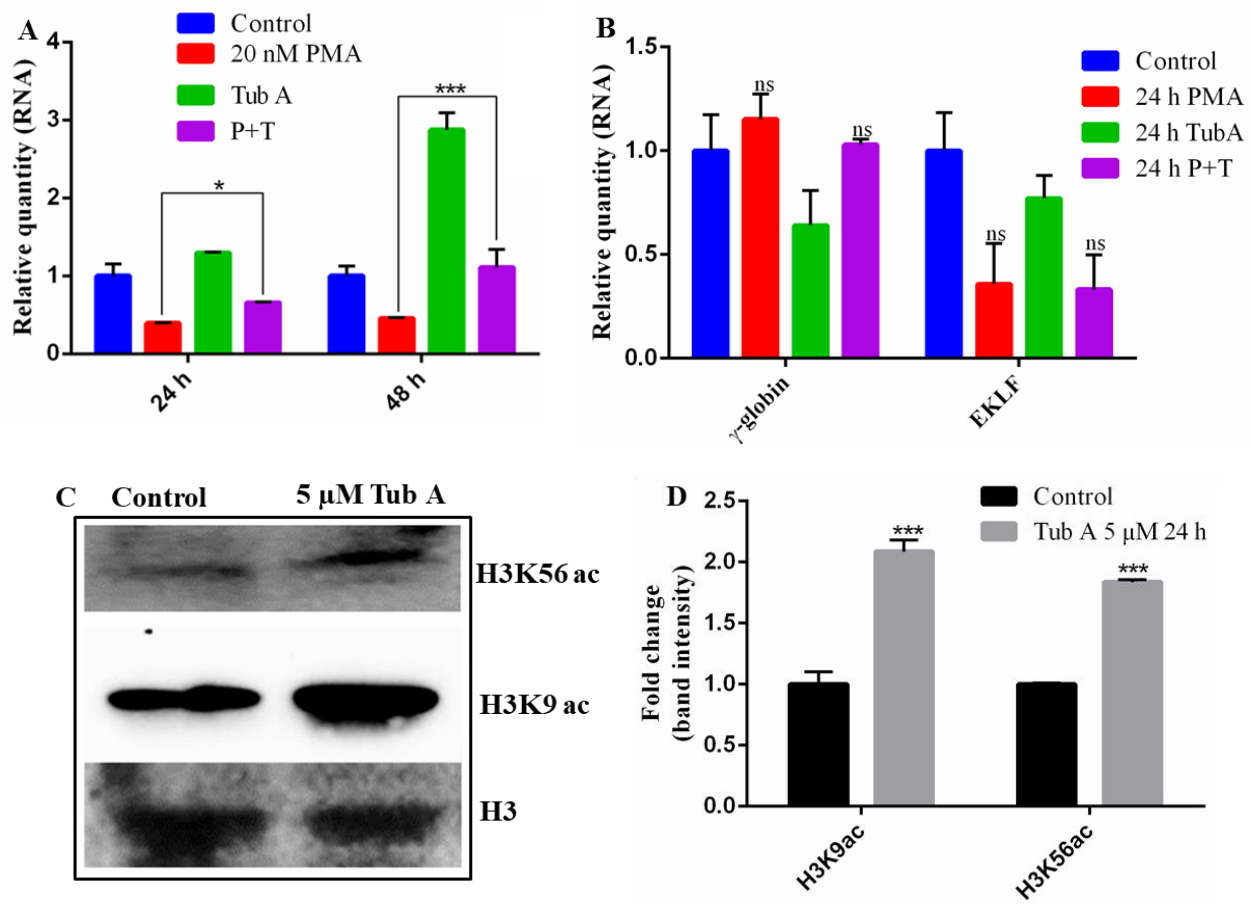

Fig 25: ***HDAC6 represses GYPA promoter*** *RNA expression levels of GYPA in PMA and / or Tub A treated cells for 24 h and 48 h (A), other erythroid lineage genes EKLF and globin in PMA and / or TubA treated cells for 24 h (B), Western blotting showing the acetylation levels of H3K9 and H3K56 in the presence of Tub A (C) and densitometry by image J software (D).*

This prompted us to look into the role of HDAC6 in GYPA transcriptional repression. We therefore designed 5 sets of primers from first intron to the 1400 bp upstream to the transcription start site (TSS) of GYPA promoter (Fig. 26A). After sonication of nuclear fraction, we have

checked DNA fragmentation on agarose gel electrophoresis. As shown in Fig 26B, most of the DNA was fragmented into 500-1000bp and then proceeded with ChIP-PCR using HDAC6 antibody. We observed a 2-fold enrichment of HDAC6 over the GYPA promoter at 4 out of 5 sites studied in control cells (Fig 26C). However, there was a significant enrichment at site 1 (-1363 bp to 1183 bp) in PMA-treated cells. The HDAC6 binding was decreased significantly when treated with Tub A (Fig 26C) compared to control and PMA-treated cells. Interestingly, we have observed the enrichment of HDAC6 to GYPA promoter in control cells (progenitor cells) also suggesting biasedness of MEP cells towards MK lineage presumably due to the short half-life of MK cells (5-7 days) compared to longevity of erythrocytes (120 days). But, further studies are required to get a deeper insight into the MEP-MK biasedness. HDACs are recruited to the target promoter regions by transcription factors and repressor proteins, as they do not contain DNA binding domain (Shahbazian and Grunstein 2007). Further studies are required to find out the interacting partner of HDAC6 in repressing GYPA. Thus in progenitor cells, GYPA is expressed at basal level due to the repressors bound to its promoter and repressed completely in the presence of lineage-specific cytokines or overexpression of lineage specific transcription factors. These results clearly indicated that HDAC6 plays an important role in repressing GYPA.

3.13 HDAC6 is involved in ROS production *via* NOX4

The strong nuclear localization of HDAC6 in the present study correlated partly with the GYPA gene repressing function of HDAC6. However, the immunofluorescence data indicated cytoplasmic presence of HDAC6. So we also focused on other probable functions that HDAC6 might be having during MK differentiation. Most of the studies have shown that ROS is required for MK cell commitment, maturation and for the release of platelets (Mostafa, Miller et al. 2000,

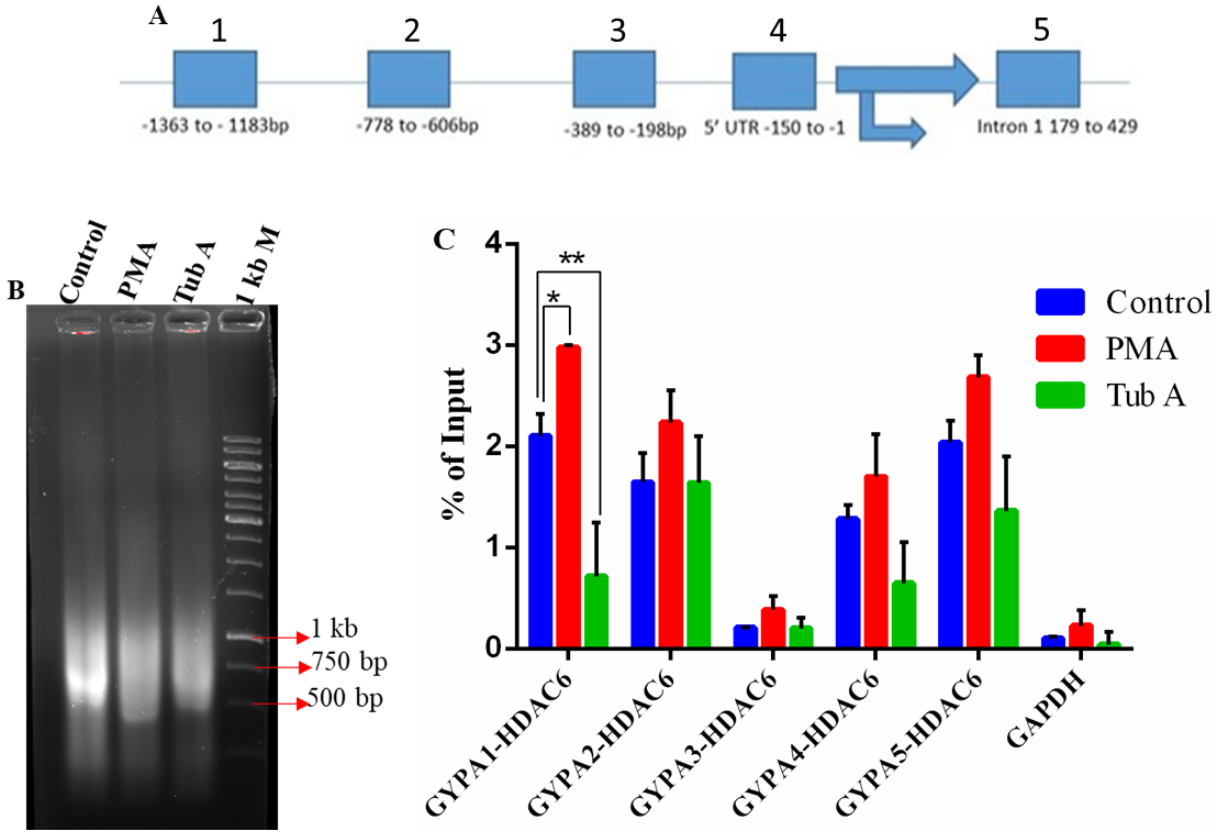

Fig 26: ***HDAC6 represses GYPA promoter*** *5 sets of primers designed from first intron to the 1400 bp upstream to the transcription start site (TSS) of GYPA promoter (A), ChIP fragmentation in control, PMA and Tub A treated cells on 1% agarose gel (B),ChIP-PCR with HDAC6 for different sites of GYPA promoter using specific primers in PMA or Tub A treated cells (C).*

Sardina, Lopez-Ruano et al. 2010). ROS is the upstream regulator in activating the cascade of signaling proteins and finally leads to the activation of downstream targets like NF-κB, c-JUN, c-FOS which further regulate lineage specific gene expression. Studies have shown that treatment of cells with antioxidants like Trolox, NAC prevented the acquisition of MK morphological features and reduction in MK marker expression (Sardina, Lopez-Ruano et al. 2010). Youn GS *et al,* have shown that in macrophages, HDAC6 overexpression induces ROS production by upregulating NADPH Oxidase (Youn, Lee et al. 2016). Since the role of ROS in MK biology and the role of HDAC6 in ROS production is well known, we tried to find a link between ROS and

HDAC6 in MK differentiation. We measured ROS levels using H2DCF-DA by flow cytometry and observed increased ROS levels in PMA treated cells that were reduced upon HDAC6 inhibition (Fig 27A). The primary source of ROS production is p22phox-dependent NADPH oxidase (Sardina, Lopez-Ruano et al. 2010) but it is not clear, out of the four NOX homologues (NOX1-4), which NOX is responsible for the production of ROS. Further, McCrann et al. have shown the high expression levels of NOX1, weak expression of NOX4 with no significant expression levels in NOX2 expression during the TPO-induced differentiation of bone marrow derived CD34+ cells. In addition, NOX1 knockout mice showed no effect on ploidy levels (McCrann, Eliades et al. 2009). So, we next examined the effect of Tub A on NOX2 and NOX4 expression and found that NOX4 was upregulated during MK differentiation and significantly downregulated in the presence of HDAC6 inhibitor (Fig 27D) with no change in NOX2 expression (Fig 27C) suggesting for the first time that PMA-induced ROS production in MK differentiation of K562 cells is *via* NOX4. Further, we have confirmed downregulation of NOX4 in HDAC6 knockdown cells (Fig 27E). These results indicate that HDAC6 is required for sustainable ROS production *via* NOX4 during MK differentiation.

3.14 Crosstalk between PMA signaling, ROS and HDAC6 during MK differentiation

Studies have shown a positive feedback circuit between HDAC6, NOX and ROS (Youn, Lee et al. 2016). To further understand the relation between HDAC6 and NOX4 in our studies, we analysed the effect of an antioxidant, Quercetin, on the expression of HDAC6, NOX2, NOX4 and MK markers during PMA-induced K562 cell differentiation. During MK differentiation, treatment with antioxidant shown significant decrease in the HDAC6, NOX4 along with MK markers expression was observed (Fig 28A). In astrocytes, crosstalk between HDAC6 and NOX2 regulates the expression of pro-inflammatory mediators during HIV-1 infection (Youn et al. 2017). In line

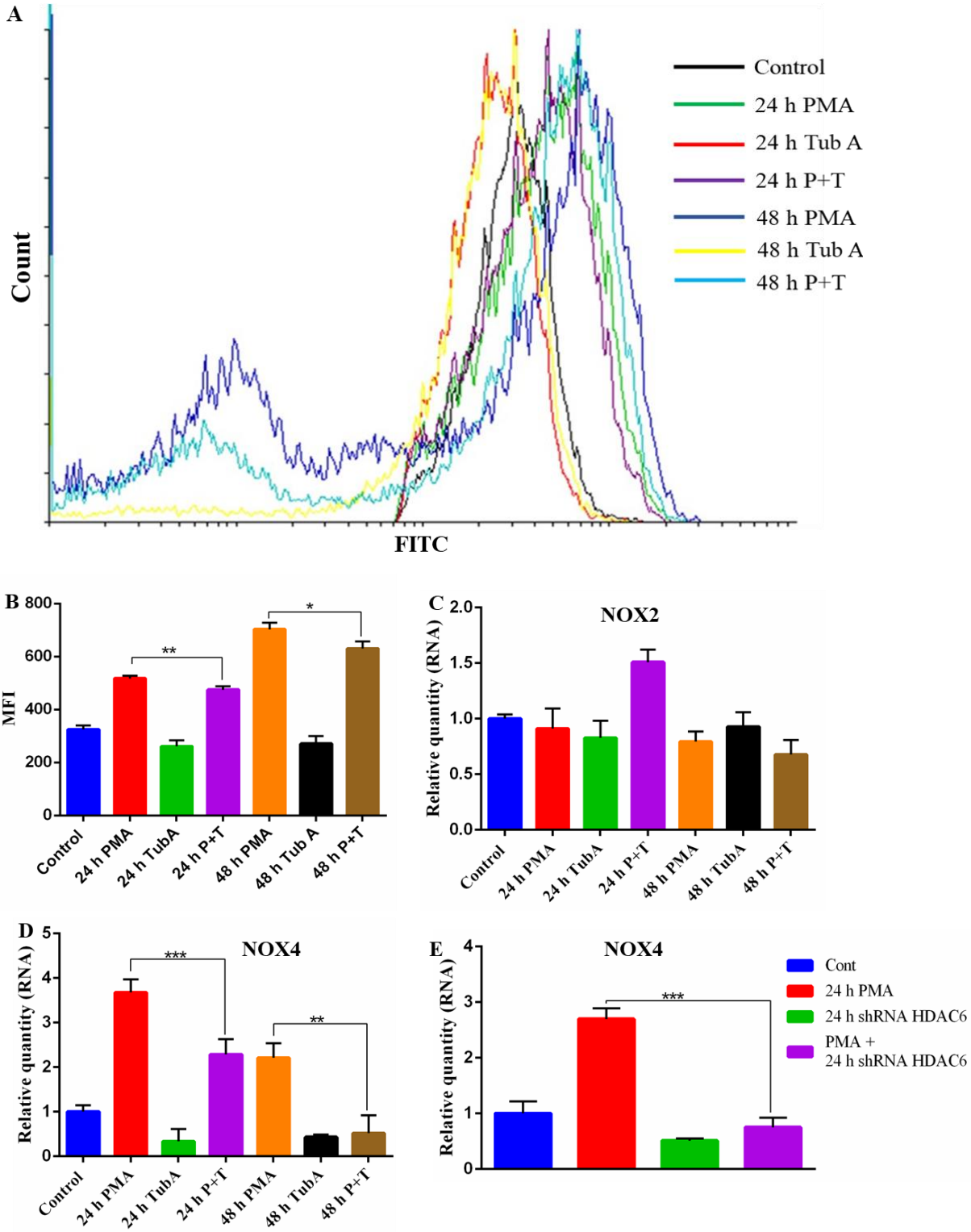

Fig 27: ***HDAC6 is involved in ROS production via NOX4*** *ROS measurement by FACS in PMA and / or Tub A treated cells for 24 h and 48 h represented as overlay of histogram (A) and expressed as MFI (B), RNA expression analysis of NOX2 (C), NOX4 (D) during MK differentiation in the presence of PMA and / or Tub A for 24 h and 48 h,, RNA expression analysis of NOX4 in HDAC6 knockdown cells (E).*

of these results we found downregulation of HDAC6 and NOX4 when ROS is inhibited with an antioxidant, Quercetin during MK differentiation indicating the positive feedback loop between HDAC6, NOX4 and ROS production.

Zhang Y et al found that Aurora-B/AIM-1 and survivin, the 2 critical mitotic regulators were mislocalized or absent during endomitosis of mouse MKs (Zhang, Nagata et al. 2004). This was further supported by findings by D J. McCrann et al, who have shown that NOX4 plays role in polyploidazation of vascular smooth muscle cells (VSMC) by down regulating survivin (McCrann, Yang et al. 2009). Next, we confirmed the role of HDAC6 in ROS production *via* NOX4 in MK biology, by analysing the mRNA levels of survivin gene, a chromosome passenger protein involved in cell division. During MK differentiation, survivin is downregulated so as to promote polyploidy in MK cell. We observed that survivin is upregulated when HDAC6 is inhibited (Fig 28B) suggesting that ROS produced by NOX4 is important for polyploidy of MK cell and thus HDAC6 is involved in ROS production. Taken together, these results confirm the interplay between ROS, NOX4 and HDAC6 during differentiation of K562 cells to MKs.

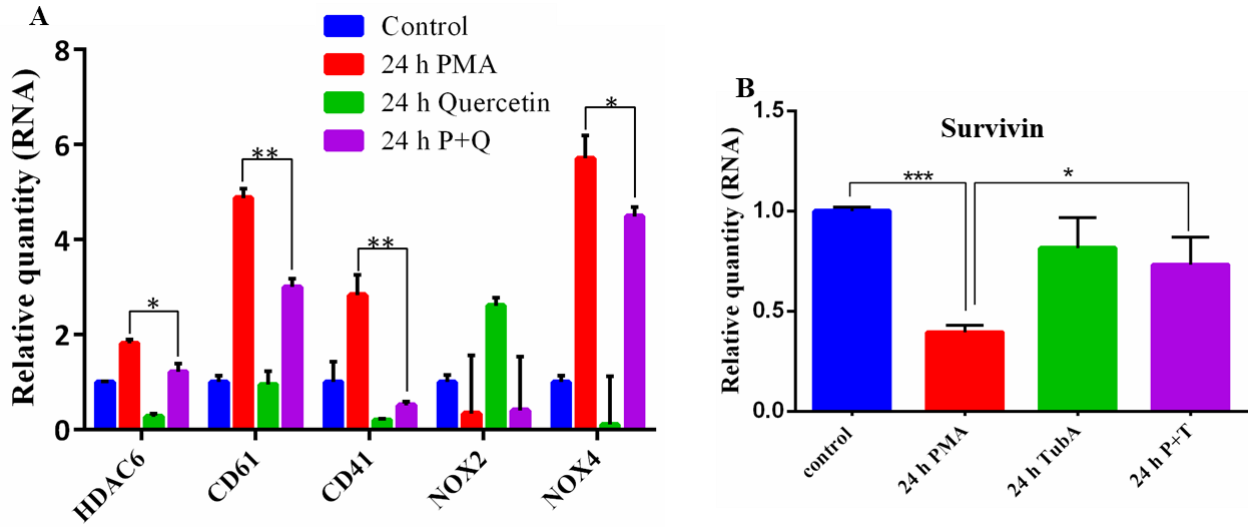

Fig 28: ***Crosstalk between PMA signaling, ROS and HDAC6 during MK differentiation*** *RNA expression analysis of HDAC6, MK markers, NOX2 and NOX4 in the presence of antioxidant, Quercetin and or PMA for 24 h (A) and RNA expression analysis of survivin in PMA and / or Tub A treated cells for 24 h (B).*

Conclusions

Conclusions

- Class IIb HDACs, HDAC6 and HDAC10 were upregulated during megakaryocyte differentiation.
- HDAC6 upregulation is specific to MK lineage but not due to PMA signaling.
- Upregulation of HDAC6 during MK differentiation is *via* PKC-pERK1/2-NF-κB axis
- During MK differentiation, HDAC6 is predominantly localized to the nucleus.
- Pharmacological and genetic knockdown of HDAC6 promotes MK differentiation by positively regulating MK marker, CD61 and by suppressing GYPA, an erythroid lineage gene.
- HDAC6 positively regulates the expression of MK lineage transcription factors, GATA-2 and FOG-1 indirectly.
- HDAC6 inhibits erythroid lineage gene expression (GYPA) by binding to the GYPA promoter.
- HDAC6 is involved in sustained ROS production by upregulating NOX4 and negatively regulating survivin in promoting polyploidy.
- HDAC10 overexpression in K562 cells upregulates CD41.

Summary

Summary

Thrombocytopenia, reduced platelet count, is a clinical problem in many disease conditions such as cancer therapy, trauma, sepsis and viral infections such as dengue, etc. (Suharti et al. 2002). The two reasons for thrombocytopenia are reduced production from megakaryocytes (MK) or increased destruction of platelets (Ballem et al. 1987). Understanding the molecular mechanisms regulating MK differentiation and platelet production are required to identify the safe sources of platelets for therapeutic purposes and to cure the platelet diseases.

K562 cells are chronic myeloid leukemia cells, well studied model system to find out the molecular mechanisms regulating the erythroid and megakaryocyte lineages in the presence of different chemical inducers (Lozzio and Lozzio 1977). PMA (Phorbal 12-myristate 13-acetate) induces the K562 cell differentiation towards megakaryocyte lineage whereas hemin, hydroxyurea, Ara-C (Arabinosyl cytosine) induces erythroid differentiation of these cells (Shelly et al. 1998; Villeval et al. 1983). The differentiation of K562 cells to megakaryocytes is monitored by cell growth arrest, changes in morphology, adhesive properties due to expression of integrins CD61 and CD41 (MK markers) (Butler et al. 1990) and reduced expression of erythroid genes Glycophorin A (GYPA) and endomitosis. PMA activates PKC leading to further ERK1/2, NF-κB, c-FOS and c-JUN transcription factor activation (Franklin and Kraft 1995; Kang et al. 1996; Kim et al. 2001; Murray et al. 1993).

Acetylation and deacetylation of histone proteins is controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively. Deacetylation of histone proteins by HDACs leads to the formation of compact chromatin which prevents the binding of transcriptional machinery and thus gene suppression (Cress and Seto 2000). HDACs also play an important role in various physiological and pathological processes by regulating the activity of non-histone

proteins by deacetylation (Eom and Kook 2014). In humans, total of 18 HDACs have been identified and grouped into four classes based on their homology to yeast orthologs. Class I (HDAC1,2,3 and 8), Class II (HDAC4,5,7,9 & 6,10) and class IV (HDAC11) are classical zinc dependent enzymes whereas class III HDACs are named as sirtuins from SIRT1-7 which requires NAD^+ as cofactor for their activity (de Ruijter et al. 2003; Gregoretti et al. 2004; Walkinshaw and Yang 2008). Recent studies indicate that the epigenetic gene regulation governs the stem cell differentiation and the role of HDAC inhibitors (HDACi) in reprogramming of somatic cells to pluripotency is well established (Kretsovali et al. 2012).

The commitment of the bipotent progenitor cells to a particular lineage requires suppression of the opposite lineage genes. Since HDACs are involved in gene repression *via* compact chromatin formation, we hypothesize that one or few of the HDACs might be involved in lineage commitment of progenitor cells. Although the role of HDACs in other biological processes is well studied, the role of HDACs and the underlying molecular mechanisms of lineage commitment remains elusive. Recently, Messaoudi et al have shown that HDAC6 is required for proplatelet release from MKs by deacetylating cortactin, an important cytoskeletal protein (Messaoudi et al. 2017). Therefore, in the present study, we aimed at identifying the role of HDACs in MK lineage commitment of K562 cells with the following objectives.

1 HDACs profiling and identification of HDAC(s) upregulated during MK differentiation

2 Functional studies of HDAC6

(A) Role of HDAC6 in regulating MK and erythroid marker expression

(B) Role of HDAC6 in sustainable ROS production during MK differentiation

PMA-induced MK differentiation of K562 cells was confirmed by change in morphology of cells, upregulation of MK markers (CD41 and CD61) and downregulation of an erythroid marker glycophorin A (GYPA). We next did HDAC expression profiling (mRNA and protein expression). The expression levels of class I and class IIa HDACs (HDAC4) did not change significantly in control and PMA-treated cells. However, RNA and protein levels of class IIb HDACs, HDAC6 and HDAC10, were significantly upregulated during MK differentiation. Further, time-course mRNA expression analysis indicated upregulation of HDAC6 along with MK markers till 4 days of PMA treatment and gradual downregulation of GYPA significantly. Since histones are the primary substrates for HDACs, we have analyzed the global acetylation levels of lysine residues of histone H3. We have observed a significant decrease in acetyl levels of H3K9 and H3K56 during differentiation of K562 cells, suggesting these might be the targets of upregulated class IIb HDACs. The acetylation levels of H3K18, H3K14 and H3K27 did not alter in control and PMA treated cells.

Next, we assessed HDAC6 expression in PMA-induced HL-60 cells that differentiate into monocytes or macrophages to find out HDAC6 upregulation is due to PMA signaling or is MK lineage specific. The results showed no change in HDAC6 expression levels in PMA-induced HL-60 cells indicating that HDAC6 upregulation is MK lineage specific. To delineate the role of HDAC6 in MK differentiation as a repressor of gene or as a protein activity regulator, we first investigated HDAC6 localization by confocal microscopy. We have observed HDAC6 is predominantly localized to the nucleus during MK differentiation when compared to pan-cellular distribution in control cells. To find out the mechanism by which HDAC6 is upregulated, we analyzed the PKC pathway activated by PMA in K562 cells during MK differentiation. We found increased p-ERK1/2 levels, Ac-NF-κB (K310) along with increased HDAC6 during PMA

treatment. Further, we found reduced HDAC6 expression when ERK1/2 activation was inhibited with Apigenin during differentiation. *In silico* analysis of HDAC6 promoter shown the presence of NF-κB binding sites suggesting that HDAC6 upregulation during MK differentiation is *via* PKC-p-ERK1/2-NF-κB axis.

To further delineate the role of HDCA6 during MK differentiation, we have treated the cells with HDAC6 specific inhibitor, Tub A, and analysed the expression levels of MK lineage genes. The qPCR results clearly indicated that MK markers were downregulated when HDAC6 is inhibited in PMA treated cells along with significant upregulation of erythroid marker. We also measured the protein levels of CD61 by flow cytometry and the results are in agreement with qPCR data. We have further confirmed these observations by HDAC6 knockdown. Overexpression of HDAC6 in K562 cells resulted in significant upregulation of CD61, but not CD41 and downregulation of erythroid lineage gene, GYPA. These results suggest that HDAC6 might be negatively regulating GYPA expression and positively regulating the MK lineage gene, CD61. CD41 is upregulated when HDAC10 is overexpressed in K562 cells. We then sought to determine how HDAC6 is positively regulating the expression of MK marker (CD61). We observed reduced expression of MK transcription factors, GATA-2 and FOG-1 in Tub A treated cells alone but not in HDAC6 inhibited cells treated with PMA. On the contrary, we observed upregulation of GATA-2 and FOG-1 in HDAC6 overexpressed cells without PMA treatment. These results indicate that HDAC6 might be regulating the GATA-2 and FOG-1 expression indirectly that in turn regulate CD61 expression.

When HDAC6 was inhibited with Tub A, we have observed upregulation of GYPA not only in PMA-treated K562 cells, but also in Tub A treated cells. But we did not observe significant effect on other erythroid lineage genes like γglobin and EKLF. This prompted us to look into the

role of HDAC6 in GYPA transcriptional repression. We therefore designed 5 sets of primers from first intron to the 1400 bp upstream to the transcription start site (TSS) of GYPA promoter and did ChIP-PCR with HDAC6 antibody. We observed a 2-fold enrichment of HDAC6 over the GYPA promoter at 4 out of 5 sites studied in control cells. However, there was a significant enrichment at site 1 (-1363 bp to 1183 bp) in PMA-treated cells. The HDAC6 binding decreased significantly at site 1 when treated with Tub A compared to control and PMA-treated cells. These results clearly indicated that HDAC6 plays an important role in repressing erythroid-specific lineage gene, GYPA.

Since the role of ROS in MK biology and the role of HDAC6 in ROS production is well known, we tried to find a link between ROS and HDAC6 in MK differentiation. We observed increased ROS levels in PMA treated cells that were reduced upon HDAC6 inhibition. Since NADPH oxidases are involved in ROS production, we next examined the effect of Tub A on NOX2 and NOX4 expression and found that NOX4 was upregulated during MK differentiation and significantly downregulated in the presence of HDAC6 inhibitor with no change in NOX2 expression suggesting for the first time that PMA-induced ROS production in MK differentiation of K562 cells is *via* NOX4. In addition, we have observed downregulation of NOX4 in HDAC6 knockdown cells. Treatment of cells with antioxidant during MK differentiation shown significant decrease in HDAC6, NOX4 and MK markers expression levels. This result implicated a clear crosstalk between HDAC6, NOX4 and ROS during MK differentiation. Next, we confirmed the role of HDAC6 in ROS production *via* NOX4 in MK biology, by analysing the mRNA levels of survivin gene, a chromosome passenger protein involved in cell division. During MK differentiation, survivin is downregulated so as to promote polyploidy in MK cell. We observed

that survivin is upregulated when HDAC6 is inhibited suggesting that ROS produced by NOX4 is important for polyploidy of MK cell and thus HDAC6 is involved in ROS production.

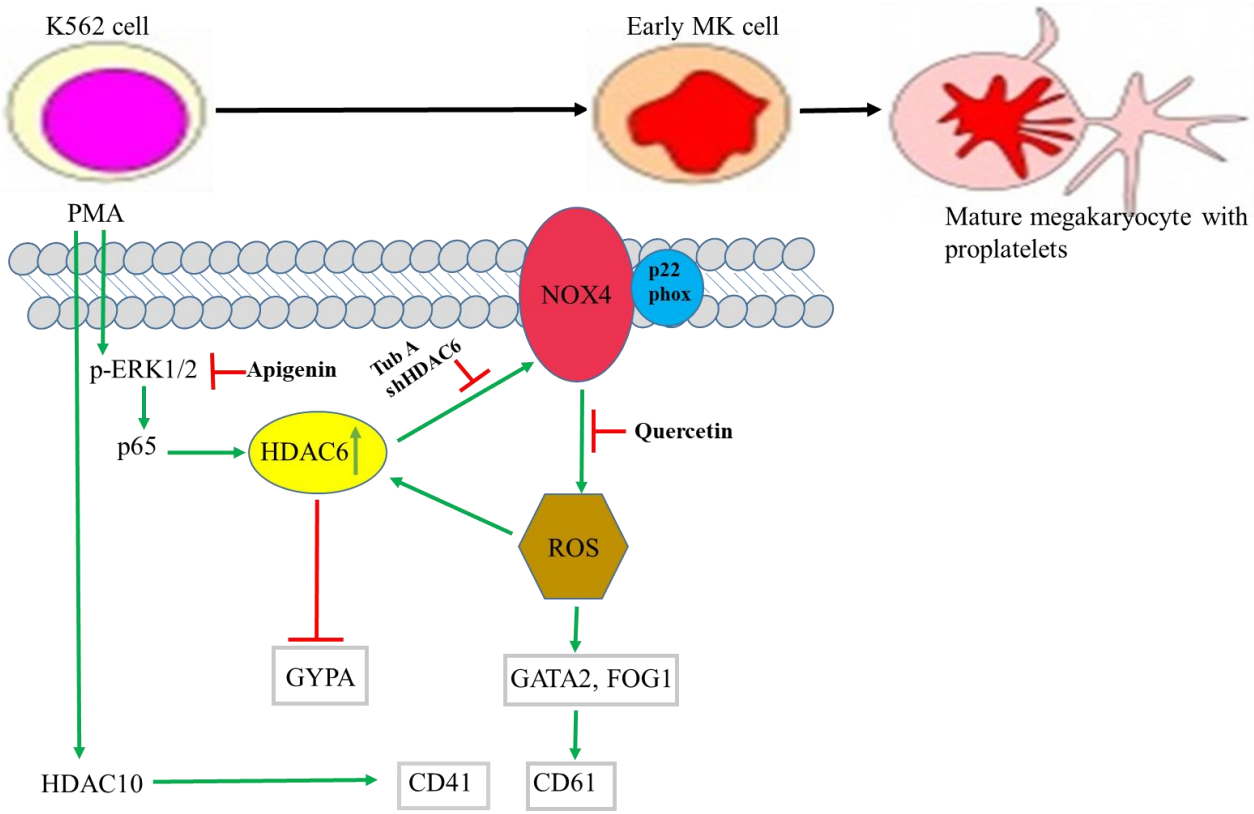

Fig 29: ***Overall summary of the results***

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