Proteomic Characterization of Hemogen in Erythropoiesis

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ABSTRACT

Hemogen (Hemgn) is reported as a tissue specific transcriptional regulator in testis as well as hematopoietic tissues. It is known that Hemgn positively regulates erythroid differentiation; however, the underlying molecular mechanism is not well understood. In the current study, using proteomic approach in combination with other molecular biology tools, we have attempted to decipher the role of Hemgn in differentiating Murine erythroblast leukemia (MEL) cells as a model system. Our study reveals that Hemgn predominantly interacts with transcriptional regulators, chromatin modifiers and histones. Furthermore, using Chromatin Immunoprecipitation and knockdown approach, we have demonstrated that Hemgn is recruited to the β-globin locus, which is known to be activated during erythroid differentiation. Based on the results, we speculate that Hemgn acts as a tissue specific histone chaperone that regulates transcription during erythroid differentiation.

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List of Abbreviations

Abbreviation	Expansion
aa	aminoacid
AML	Acute Myeloid Leukemia
BFU-E	Burst forming units -Erythroid
bp	basepair
CFU-E	Colony forming units -Erythroid
ChIP	Chromatin Immunoprecipitation
CLP	Common Lymphoid Progenitor
DMP	Dimethyl Pimelimidate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxycycline hyclate
EDTA	Ethylenediaminetetraacetic acid
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
GO	Gene Ontology
Hemgn	Hemogen
HOX	Homeobox
HRP	Horseshoe Radish Peroxidase
HS	Hypersensitive Region
HSCs	Hematopoietic stem cells
IgG	Immunoglobin
IP	Immunoprecipitation
IPTG	isopropyl β D thiogalactoside
LB	Luria-Bertani
LC-MS/MS	Liquid Chromatography - Tandem Mass Spectrometer
LCR	Locus Control Region
MEL	Murine erythroblast Leukemia
Mgn	Myogenin
mRNA	messenger RNA
NE	Nuclear Extract
NLS	Nuclear Localization Signal
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
PIC	Protease Inhibitors Cocktail
prom	promoter region

R	Regression Co-efficient
rpm	Rotations per minute
RT qPCR	Real time quantitative PCR
SDS	Sodium Doedecyl Sulfate
shRNA	short hairpin RNA
SN	Supernatant
TR	Tet Repressor
WB	Western Blot

Dedications and Acknowledgement

The last two years has been one long journey that I will always remember. The people I met, the friends I made and the lessons of life that I learnt will be cherished forever. Through the toughest of times, Mom, Dad and Vikram have always been there for me. Without the three of you, this thesis would have never been possible. The distance has only brought us closer. This thesis is dedicated to three of the most beautiful human beings I have ever known – Mom, Dad and Vik – this is for you!

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1. INTRODUCTION

Decades of persistent human effort on understanding the fundamental unit of life, the cell, has revealed the importance of genome in defining its state and fate. Faithful transmission of the genome is a key event that maintains the identity of species. Accumulation of alterations in the DNA sequence that constitutes the genome may result in either subtle changes leading to trait variation or in massive changes that may lead to speciation. These randomly occurring alterations in the genome are selected during evolution based on the advantage it confers to the organism. In some cases, these transmissible changes enhance the fitness of the organism during its struggle for existence. On the contrary, some of these traits acquired may decrease the ability of the organism to survive a challenging environment. This selection process has directed evolution towards building up the complexity of the organism by adopting different strategies that withstands time and the environmental insults encountered (Charles Robert Darwin, 2001). As unicellular organisms evolved to multicellular organisms, their cells have adopted the ability to exhibit different morphology and functionality in spite of having identical genome. This heterogeneity in the cells that constitute an organism is attributed to the heterogeneity in the structural organization of the genome. Therefore, understanding how the architecture of the genome i.e., the epigenome, plays its part in the determining the state and fate of the cell in higher organisms is important in understanding development (Rapp, R.A., and Wendel, F.J., 2005).

1.1. Chromatin structure:

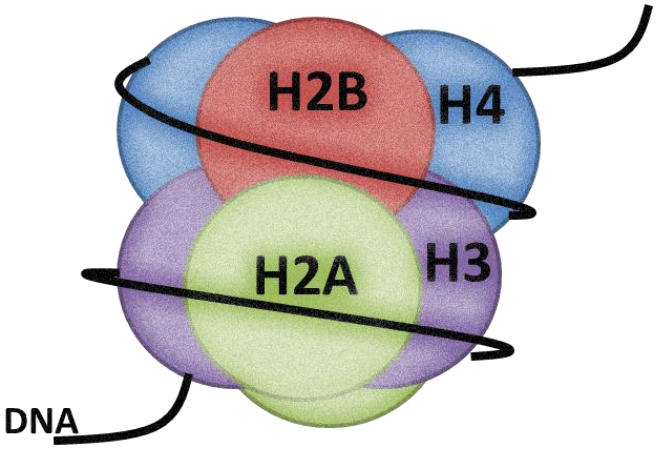


Figure 1. Schematic representation of a Nucleosome.

The genome in higher organisms is organized in the nucleus as a complex three dimensional structure called the chromatin. The chromatin is composed of DNA that is ordered in the form of protein-DNA complexes called the nucleosomes. The nucleosomes are made up of 147 bp of DNA wound as approximately two turns around a histone octamer. The histone octamer is made up of one H3-H4 histone tetramer and two histone H2A-H2B dimers (Fig. 1). The higher order structure of the chromatin is regulated by packing of these nucleosomes. Post-translation modification on the histones, the histone variants constituting the nucleosome, methylation status of the DNA and the linker Histone H1 play an important role in determining the packing of the nucleosomes into higher order structures (Guohong Li and Danny Reinberg, 2011). The chromatin is further condensed into complex structures with the help of proteins like cohesin, members of the mediator complex, etc., that facilitate looping (Kagey et al., 2010). These proteins mediate changes in the three-dimensional structure of the chromatin bringing distant regulatory elements containing enhancers in proximity to the gene and thereby

modifying the epigenetic landscape. As a result, there is usually either recruitment or increased localization of proteins that promote transcription.

Transcription is the process by which the information present in genome as genes is coded into messenger RNA (mRNA) molecules. These mRNA are in turn translated into protein molecules which are the building blocks of the cell. Transcription of genes is directly regulated by a class of proteins called transcription factors. These transcription factors bind to specific DNA sequences like the TATA box, etc. These specific DNA sequences are present usually in proximity to the 5' end of the gene called the promoters. When a transcription factor binds to DNA, there is a change in the tertiary conformation of the protein (Phillips, T. and Hoopes, L., 2008). These transcription factors then interact with basal transcription machinery as a consequence of which RNA polymerase is either recruited at specific loci or the active centers in the genome called the transcription factories (Cook, P.R., 1999 and Meng Xu and Peter R. Cook, 2008). Higher order structure and the nucleosomal positioning can act as a barrier to the transcription factor preventing them from binding to DNA. Nucleosomes when positioned over specific DNA sequence decrease its accessibility to transcription factors for binding (Campos EI, and Reinberg D, 2010). Nucleosomes also act as a hurdle to RNA polymerase during transcription (Bondarenko et al., 2006). The nucleosomal barrier is usually removed through eviction or reposition of nucleosomes. Several classes of proteins such as the chromatin modifying complexes, chromatin remodeling complex and histone chaperones play an important role in regulating transcription by preventing the stalling of RNA polymerase by nucleosomes (Tony Kouzarides, 2007, Cedric R. Clapier and Bradley R. Cairns, 2009, and Young-Jun Park and Karolin Luger, 2008).

1.2. Hematopoietic system – a brief overview:

Hematopoiesis is a complex developmental process that replenishes the blood system with its cellular components. During development, the early embryonic cells that are totipotent undergo commitment at several stages where massive re-arrangements in chromatin structure take place leading cells to differentiate towards specific lineages. This commitment process leads the cells to form a wide range of organs made up of different tissues. These tissues in turn are composed of a spectrum of cell types. The development of hematopoietic system and the role of different classes of bio-molecules in its development is well studied.

In the developing mouse embryo, commitment of primitive cells towards the hematopoietic lineage is first observed in the mid-primitive streak stage E7.0. These primitive embryonic cells at E7.0 show transient expression of embryonic globin genes (Palis et al., 1999). In the developing murine fetus, the fetal liver and spleen are the prime sites of hematopoiesis. After birth, the adult bone marrow becomes the major hematopoietic site (Yang et al., 2001a).

Because of their therapeutic potential, Hematopoietic Stem Cells (HSCs) are one of the most widely studied stem cells (Kondo et al., 2003). HSCs are harbored in adult bone marrow in a highly regulated micro-environment made up of cells that constitute the niche. The niche provides an environment favorable for the maintenance of multipotent HSCs and regulation of their mobilization. Mobilization of HSCs takes place as a result of alterations in the protein signals secreted by the cells that constitute the niche. The HSCs are in physical contact with the $Nestin^{+}$ Mesenchymal Stem Cells in the niche

which maintain the HSCs in their pluripotent state through expression of certain HSC maintenance genes (Méndez-Ferrer et al., 2010b). During stress condition, several cytokines and erythropoietin alter the micro-environment in the niche resulting in differentiation and mobilization. One of the several models proposed to explain differentiation of HSCs in the bone marrow suggests that several factors produced in the niche promote asymmetric division of stem cells where the more differentiated daughter cell exits the niche to propagate and further differentiate to form cells of different types that replenish the blood (Anne Wilson and Andreas Trumpp, 2006. Fig 2). The release of HSCs from the bone marrow follows a Circadian rhythm (Méndez-Ferrer et al., 2010b). This trafficking of HSCs from bone marrow to the blood stream is regulated by several signaling molecules like the chemokine CXCL12 in bone marrow (Sugiyama et al., 2006).

Hematopoietic stem cells are a pool of committed cells which can both re-populate themselves and differentiate into cells of the diverse hematopoietic lineages (Moore and Lemischka, 2006 and Wilson and Trump, 2006). There lies an intricate balance between the self-renewal and differentiation ability of the stem cells that is responsible for homeostasis and normal and healthy functioning of the circulatory system. Any perturbation to this balance could lead to leukemogenesis and may eventually result in death of the organism. Understanding the bio-molecules that regulate and maintain this homeostasis is important to design therapeutics.

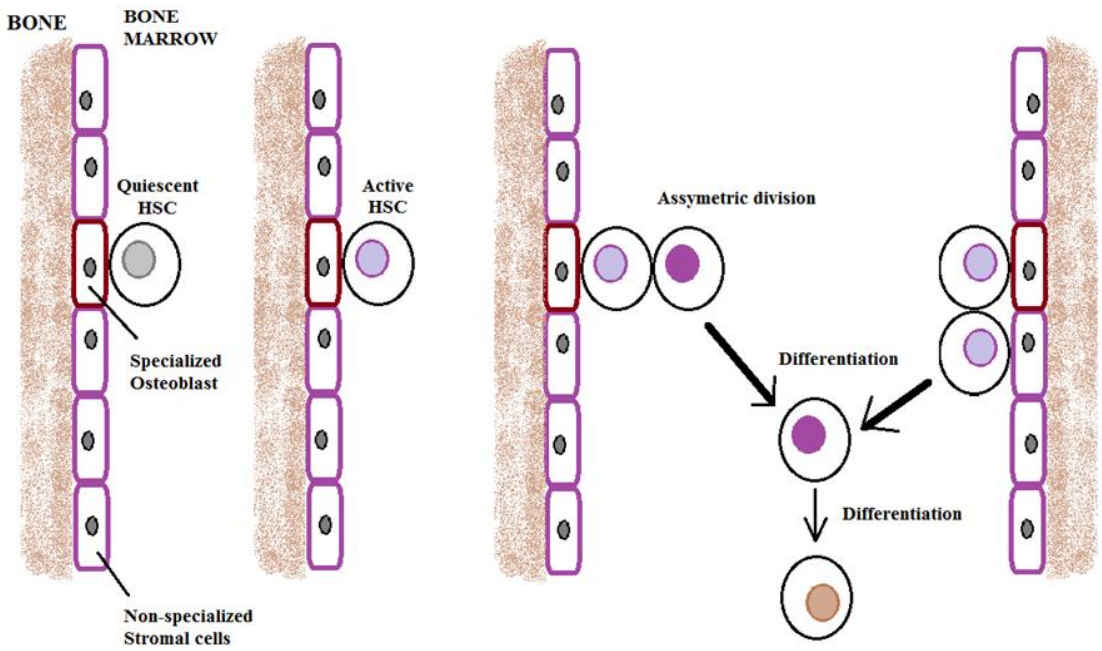


Figure 2. Maintenance, Self-renewal and Differentiation of Stem cells in the bone marrow niche. (Adapted from Anne Wilson and Andreas Trumpp, 2006).

1.3. Erythropoiesis – from HSCs to erythrocytes

The process of differentiation is highly regulated by several pathways involving many factors. The erythrocytes population in the body is regulated through a glycoprotein hormone called erythropoietin (Epo) (S E Graber, and S B Krantz, 1978, S E Graber, and S B Krantz, 1989). Once the HSC exits the niche, Epo in the bone marrow stimulates the growth and commitment of the early progenitor cells towards the erythroid lineage (S E Graber, and S B Krantz, 1989). Epo is expressed in fetal liver and adult kidney in response to hypoxia (Lin et al., 1985, Goldberg et al., 1988). In early progenitor cells, Epo binds to Epo Receptors (EpoR). This stimulates growth of erythroid progenitors like Burst Forming Units – Erythroid (BFU-E) and Colony Forming Units – Erythroid (CFU-E) and further promotes differentiation of early progenitors to pro-erythroblasts (Erslev,

A., 1987). EpoR exists as a dimer in the absence of Epo (Livnah*et al*., 1999). When Epo binds to the EpoR, several changes take place in the structural orientation of the dimer. This structural reorganization results in bringing the intracellular domains in close proximity. This allows the EpoR associated JAK2 to associate with each other and auto-phosphorylate, thus leading to activation of the internal signaling cascade. This signaling cascade brings about changes in the epigenetic landscape of the cell leading to differentiation towards the erythroid lineage (Remy et al., 1999).

1.4. Transcriptional regulation of erythropoiesis:

The interplay between extracellular and intracellular signals is transduced into a change in the epigenetic program of the hematopoietic stem cells. When an intracellular signal is translated into epigenetic alterations, transcription of several factors are either repressed or activated. The intracellular signals, generated as a response to the extracellular factors or signals, are amplified into a cascade of downstream signals. These signals are further processed into post translational modification of several proteins downstream. These post-translation modifications usually alter the interactome, the localization or the function of the protein. These post-translational modifications affect the transcriptional regulatory activity of nuclear proteins thereby bringing about a change in the transcriptional regulatory activity of Transcription factors. As a result, there is a gross change in the expression profile of various proteins in the cell (Nelly Khidekel and Linda C. Hsieh-Wilson, 2004). These events lead to changes in the phenotype of cell towards proliferation, commitment or differentiation.

1.4.1. Hemogen (Hemgn) – a novel transcription regulator:

Hemgn is a transcriptional regulator which is expressed specifically in hematopoietic tissue and testis (Yang et al., 2001a, Yang et al., 2003 and Li et al., 2007). Hemgn expression is first detected in the primitive blood islands of developing murine embryo at E8.5. At E11.5, Hemgn expression is detected in fetal liver and there is lower expression of Hemgn in circulating blood cells. In the adult mouse, Hemgn is expressed highly in bone marrow and spleen, primary sites of hematopoiesis. Hemgn expression pattern during mouse development corresponds to the ontogeny of hematopoiesis (Yang et al., 2001a, Yang et al., 2001b).

Hemgn is expressed as a tissue specific splice variants which may be regulated by alternative promoters. Hemgn exists as a 2.4 kb long splice variant in adult hematopoietic tissues. In round spermatids of pre-pubertal mice, Hemgn is detected as a 1.9kb splice variant. Both the splice variants have distinct untranslated regions but an identical coding region (Yang et al., 2003). In adult hematopoietic tissues, high expression of Hemgn is observed in $Lineage^-$ blast cells and $CD34^+$ stem/progenitor cells. However, no or low expression is seen in mature blood cells (Yang et al., 2001a).

Exogenous expression of EDAG, human homolog of Hemgn, in a transgenic mouse model under the human CD11a promoter results in myeloid hyperplasia and inhibited lymphopoiesis. CD11a is a lymphocyte specific gene and hence EDAG expression in cells of lymphocyte lineage resulted in a decrease in the ability of Common Lymphoid Progenitors (CLPs) to differentiate into CFU-pre-Bs (Colony Forming Units-pre-B-lymphoid) (Li et al., 2007). Over-expression of EDAG in myeloid cell line 32D results in

up-regulation of expression of erythroid and megakaryocytic differentiation related genes (Ding et al., 2010). GAL4 DNA binding domain tethered EDAG when co-transfected with GAL4 responsive luciferase coding plasmid into 293 T cells resulted in the activation of luciferase expression (Li et al., 2007). Therefore, the transcriptional regulatory activity of EDAG or Hemgn may play an important role in regulating erythroid/ megakaryocytic differentiation.

1.4.1.1.GATA1 – master regulatory factor of erythropoiesis:

The flanking region of the Hemgn gene consists of several conserved transcription factor binding sites that may regulate its expression. The 5' region of the Hemgn flanking region consists of a TATA box that is conserved across mouse and human. Two conserved GATA boxes and a GATA box containing a single mismatch between mouse and human are also present in the 5' untranslated region of Hemgn. Chromatin Immunoprecipitation experiments have shown that GATA1 directly binds to Hemgn promoter. Luciferase trans-activation assays show that the GATA boxes in the Hemgn promoter are critical for regulation of expression of the downstream gene (Yang et al., 2006). In addition to the GATA boxes, the promoter also harbors 4 putative HOXB4 binding sites.

GATA1 is a transcriptional factor expressed primarily in erythroid cells in addition to cells of few other hematopoietic lineages (Martin et al., 1990, Romeo et al., 1990 and Yamamoto et al., 1990). It is also expressed in the sertoli cells in testis (Ito et al., 1993 and Yomogida et al., 1994). GATA1 is expressed during both primitive and definitive erythropoiesis (Onodera et al., 1997). The mediator complex acts as a bridge between

RNA polymerase II and GATA1 and mediates trans-activation of the downstream genes (Stumpf et al., 2006). GATA1 expression is auto-regulatory (Tsai et al., 1991 and Hannon et al., 1991), and its expression is also regulated by other members of GATA family (Onodera et al., 1997). Transcription factor PU.1 can associate with GATA1 in hematopoietic cells. When PU.1 interacts with GATA-1, it decreases the DNA binding ability of GATA-1 repressing the commitment of cells towards the erythroid lineage (Zhang et al., 2000). In addition, GATA1 is also regulated by contact between mature and immature cells. When pro-erythroblasts are exposed to differentiated erythroblasts or death receptor ligands, GATA1 is subjected to caspase mediated degradation resulting in impaired erythropoiesis. This mechanism serves as a means to maintain the population of mature erythrocytes and prevent pro-erythroblasts from proliferating and further maintaining homeostasis (De Maria et al., 1999).

1.4.1.2. HOXB4– maintaining stemness of HSCs:

In myeloid progenitors that are expanded *ex vivo*, HOXB4 directly binds to Hemgn promoter. Hoxb4 is a member of the family of homeobox (HOX) genes that encode transcription factors. HOX cluster of genes play an important role in the determination of cell fate during embryogenesis (Krumlauf R, 1994). Hox A, B and C cluster genes are expressed in several hematopoietic cell types and leukemia (Helgason et al., 1996). Among the genes of HOXB cluster, HOXB4 expression is differentially regulated during hematopoiesis. The highest expression of HOXB4 is seen in the stem cell and the progenitor cell population. Very low expression of HOXB4 is observed in mature hematopoietic cells (Sauvageau et al., 1994). Higher expression of HOXB4 in early progenitor and stem cells suggest its importance in the early stages of hematopoietic

differentiation. HOXB4 positively regulates proliferation and self-renewal ability of hematopoietic stem cells as a result of which it plays a major role in maintaining the population of stem cells (Holland and Hogan, 1988). But HOXB4 does not affect the commitment of the progenitor or stem cells towards a specific hematopoietic lineage (Helgason et al., 1996). Over-expression of Hemgn partially recapitulates HOXB4 over-expression in murine bone marrow cells. Over-expression of Hemgn resulted in enhanced expansion of myeloid progenitors in culture and increased resistance to apoptosis (Jiang et al., 2010).

At the protein level, Hemgn shows only 43% identity between the mouse and human variant with the nuclear localization signal and a coiled coil domain being highly conserved. Hemgn is post-translationally modified at many aminoacid residues – 17 phosphorylation sites have been identified in Hemgn (Huttlin et al., 2010) and 24 phosphorylation sites (Bergström Lind S et al., 2011) and 5 acetylation sites have been reported for EDAG in the PTM database, Phosphosite Plus (Hornbeck et al., 2012). But the biological significance of these PTMs is not yet well understood.

1.4.1.3. NFE2 – a trans-activating complex in erythropoiesis:

Hemgn was identified to show increasing association with MafK during erythroid differentiation in murine erythroleukemia (MEL) cells (Brand et al., 2004). Factor p45 hetero-dimerizes with small Maf proteins to form NFE2 activation complex. NFE2 complex is important for the transcriptional regulation of erythropoiesis (Andrews et al., 1993a, Andrews et al., 1993b and Igarashi et al., 1994). Transcriptional Activity of NF-E2 complex, that contains the MafK protein, is important for the regulation of globin

genes expression during erythropoiesis (Kotkow, K., and Orkin, S.H.,1995). MafK is a member of the maf proto-oncogene family (Nishizawa et al., 1989). MafK has a highly conserved basic leucine zipper domain that binds to DNA (Kataoka et al., 1994). MafK localizes over the maf recognition elements (MARE) found within HS2 in the β-globin locus during terminal erythroid differentiation (Brand et al., 2004). During erythroid differentiation in MEL cells (Levenson, R. and Housman, D, 1979, Conscience, J. F., et al., 1977), MafK exchanges its interacting partner, Bach1 for p45 resulting in the formation of the transcriptional activator complex NFE2 on the β-globin locus. In addition to NFE2 complex formation, several other proteins also interact with MafK during differentiation (Brand et al., 2004).

Any abnormalities in the regulation of the transcription factors, which control cell fate decisions of the HSC (quiescence, self-renewal or differentiation), may lead to severe disease conditions like cancer. Acute Myeloid Leukemia (AML), a common cancer in adults, manifests from uncontrolled proliferation of the hematopoietic cells belonging to the myeloid lineage. Relapse of AML in the majority of the patients with complete remission exposes the severity that leukemic transformation of HSCs can impose (Cui et al., 2003). Transformation of stem cells into cancerous stem cell can increase the severity of the condition because of their ability to expand indefinitely. Moreover, stem cells in the bone marrow are refractory to most of the existing therapeutic approaches. It is well known that many hematopoietic transcription regulators are deregulated (i.e. mutated or aberrantly expressed) in leukemic patients (Tenen et al., 1997). This deregulation of transcription regulators may play an important role in leukemic transformation and may

contribute indirectly to decreased susceptibility of cancer cells to the existing treatment strategies.

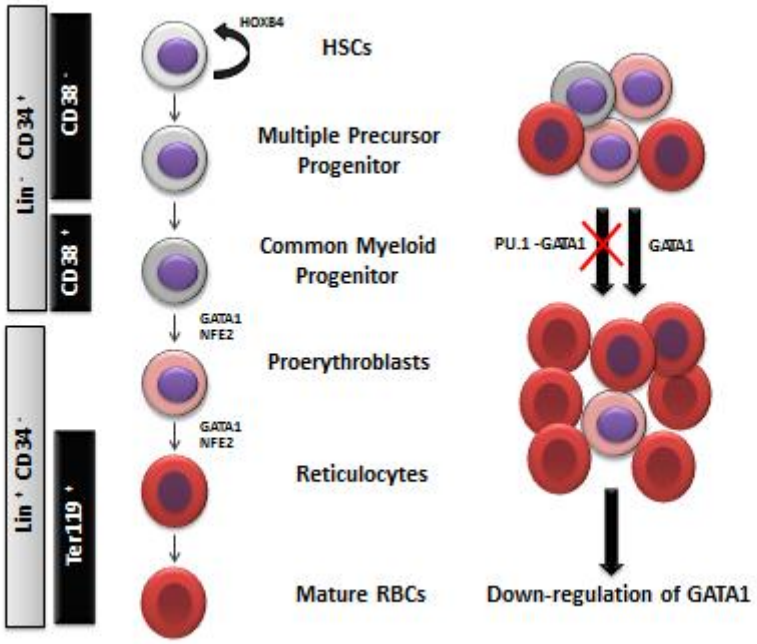


Figure 3.Transcriptional factors in erythroid differentiation.

1.5.Clinical importance of Hemgn or EDAG:

EDAG, the human homolog of Hemgn, is encoded in Chromosome 9q22 which is a well-known hotspot for leukemic chromosomal breakpoints (Yang et al., 2001a). Cell transformation and tumor formation is induced when EDAG over-expressing NIH3T3 cells are transplanted in nude mice (Li et al., 2004). High expression of EDAG transcript is found in several leukemic cell lines (Zhang et al., 2002). Moreover, EDAG expression in Acute Myeloid Leukemia (AML) patients having complete remission after therapy is significantly lower compared to patients with no remission. EDAG expression can be correlated to the refractory effects of tumor to therapy (An et al., 2005). EDAG down-

regulation inhibits growth and colony formation in K562, a myeloid leukemia cell line and over-expression of EDAG in IL-3 dependent cell line Ba/F3 cells results in decreased apoptosis and cell growth arrest on Il-3 starvation in a NF-κB dependent manner. Over-expression of EDAG increases the DNA binding activity and transcriptional activation activity of NF-κB (Li et al., 2004).

EDAG expression is hematopoietic specific. EDAG may play an important role in leukemogenesis since high expression of EDAG is found in several leukemia cell lines. High expression of EDAG increases tumorigenicity and therapeutic resistance in xenografts and leukemia patients respectively. Therefore, EDAG may play a key role in tumor development by regulating the expression of proteins that are involved in leukemic transformation. Though the transcriptional regulatory activity of Hemgn and EDAG has been established during hematopoiesis, **the molecular mechanism by which it regulates hematopoiesis specific gene expression remains poorly understood. Understanding the molecular function and the role of EDAG or Hemgn activity in hematopoiesis may potentially enable development of more efficient and targeted cancer therapy.**

1.6. Hypothesis:

Hemogen plays an important role in the transcriptional regulation of erythropoiesis.

1.7. Objective:

To characterize Hemgn and elucidate the molecular mechanism by which Hemgn acts as transcriptional regulator during erythropoiesis.

Aim 1: To study the role of Hemgn in differentiation of MEL cells

- By using shRNA mediated knock down of Hemgn in MEL cells and study the effect on cell growth and differentiation by using trypan blue assay, benzidine staining.

Aim 2: To identify Hemgn interacting proteins in MEL cells

- By using immunoprecipitation (IP) coupled with mass spectrometry to identify Hemgn interacting proteins in differentiated and undifferentiated MEL cells.
- Validate mass spectrometry results using western blot, reciprocal IP and size exclusion chromatography

Aim 3: To elucidate the molecular mechanism of Hemgn activity in differentiation of MEL cells

- By studying the transcriptional regulation of Hemgn on the β-globin locus using ChIP-qPCR, mRNA isolation and real time qPCR in MEL cells that can be induced to knockdown of Hemgn expression.

2. Materials and Methods

2.1. Cell culture:

1 to 2 x 10^6 cells/ml culture of MEL cells were used to inoculate fresh Growth media to a final cell concentration of 0.2 x 10^6 cells/ml. When a cell density of approximately 2 x 10^6 cells/ml was achieved, the media was changed by centrifugation at 1000 rpm, 5 min, room temperature and resuspending the cells in fresh growth media to a final cell density of 0.2 x 10^6 cells/ml. Trypan blue dye was used to count live cells with the help of a hemocytometer. Differentiation of MEL cells were induced by addition of DMSO (Sigma-Aldrich) to a final concentration of 2% in growth media containing 0.2 x 10^6 cells. MEL TR cells and MEL TR Hemgn knockdown cell lines were similarly cultured by using growth media containing appropriate antibiotic selection markers. Knockdown of Hemgn was induced by addition of Doxycycline hyclate (Sigma-Aldrich) to a final concentration of 5µg/ml to the media.

MEL growth media	RPMI-1640 (Hyclone, Thermo scientific) Pencillin Strepromycin (Multicell, Wisent Inc.) Standard Fetal Bovine Serum (Hyclone, Thermo scientific)
MEL TR growth media	RPMI-1640 (Hyclone, Thermo scientific) Pencillin Strepromycin (Multicell, Wisent Inc.) Tetracycline free Fetal Bovine Serum (Multicell, Wisent

	Inc.)
	Blasticidine (Multicell, Wisent Inc.)
MEL TR clone growth media	RPMI-1640 (Hyclone, Thermo scientific)
	Pencillin Strepromycin (Multicell, Wisent Inc.)
	Tetracycline free Fetal Bovine Serum (Multicell, Wisent Inc.)
	Blasticidine (Multicell, Wisent Inc.)
	G418 Sulfate (Multicell, Wisent Inc.)

2.2. Nuclear Extraction:

The cells were harvested by centrifuging at 2000 rpm for 10 min, 4°C. The packed cell volume was measured and the cells were resuspended in 5x volume of cold 1 X PBS (8g of NaCl, 0.2g KCl, 1.44g of $Na_2HPO_4.7H_2O$, 0.24g of KH_2PO_4, made up to 1L with water, pH adjusted to 7.4). The cells were then centrifuged again at 2000 rpm, 10min, 4°C and resuspended in 5x volume of Buffer A (10mM Potassium Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, Protease Inhibitor cocktail (PIC)). The cells in Buffer A were left on the ice undisturbed for 10 min. The cells were pelleted again as mentioned before and resuspended in twice the volume of Buffer A. The cells were lysed by douncing 15 strokes with Type B 15ml Kontes glass pestle. The lysate were centrifuged in a 30ml Nalgene centrifuge tubes at 25000 g for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in one volume of Buffer C (20mM Potassium Hepes pH7.9, 1.5mM $MgCl_2$, 0.6M KCl, 25% Glycerol, 0.5mM DTT, PIC). The extract

was dounced using 10 to 20 strokes and 25U of Benzonase was added to the extract for every 10^8 cells used for extraction. The mixture was incubated on the rotator for 30 min at 4°C. To the mixture, an equal volume of Buffer D (20mM K+ Hepes pH7.9, 5mM $MgCl_2$, 20% Glycerol, 0.5mM DTT, PIC) was added. The extract was centrifuged at 25,000 g for 30 min at 4°C. The supernatant was centrifuged at 14000 rpm, 30 min, 4°C, flash frozen and stored at -80°C. The protein concentration of the nuclear extract was measured using Bradford reagent (Bio-Rad).

2.3. Western Blotting:

The protein samples boiled for 5 min in 2X Protein loading dye (100mM Tris pH6.8, 20% Glycerol, 4% SDS, 0.4% Bromophenol blue, 25mM DTT) were loaded onto wells in SDS Poly-acrylamide gel and electrophoresis were performed using BIORAD SDS PAGE apparatus at 15mA per gel for 60 min at room temperature. The proteins were then transferred to a Nitrocellulose membrane by using BioRad Western Blot apparatus at 150 V for 75 min on ice. The Western blot membrane was blocked using 5% Milk in 1X PBS as blocking buffer at 4°C for 30 min on a rocker. The membrane was incubated with the primary antibody (thousand folds diluted antibody in 5% Milk in 1X PBS solution) and incubated overnight on a rotator at 4°C. The membrane was then washed thrice in PBST (1X PBS containing 0.05% Tween) and once in 1X PBS. The membrane was then incubated for an hour at 4° C in HRP-conjugated Secondary Antibody solution (5000 fold diluted Anti-Rabbit Goat Polyclonal Antibody in 1X PBS). The western membrane was again washed thrice in PBST (1X PBS containing 0.05% Tween) and once in 1X PBS. The membranes were treated with Luminol reagent (Thermo Scientific) and the specific signals were visualized using MR film (Kodak).

2.4. Transformation:

50 ng of DNA was added to Chemically Competent Bacteria prepared using Zymoresearch Kit as specified by their user manual. The mixture was then incubated on ice for 30 min. 1 ml of LB media (10g of Bacto-tryptone, 5 g of Bactoyeast extract and 10g of NaCl in 1l of water, pH7.0) was added to the mixture and incubated at 37°C with 225 rpm rotation for 40 min. Then the mixture was plated on to a pre-warmed LB Agar plate (15g of agar added for every 1l of LB broth). The plates were then incubated at 37°C overnight to obtain transformed colonies.

2.5. Protein expression and Purification:

BL21-pLysS (Stratagene) strain of *Escherischia coli* was transformed with sequence coding for 164 a.a. long N-terminal region of Hemogen cloned into PET28b+ vector. The transformed *E.coli* was selected on a LB Kanamycin^{+} plate. LB media containing 25µg/ml Kanamycin were inoculated with single colonies that were picked from the LB Kanamycin^{+} plate. The primary culture was incubated overnight at 37°C with 180 rpm rotation. Secondary culture was inoculated with 1% primary culture and incubated at 37°C with 180 rpm rotation. When the Optical density of the secondary culture reaches 0.6, the protein expression is induced by adding IPTG (Sigma Aldrich) to 1mM final concentration and incubated at 37°C with 180 rpm rotation for 4 hrs. The cells were then harvested by spinning down at 4000x g, 20 min at Room temperature. The cells were suspended in Lysis Buffer I (50mM NaH_2PO_4 (pH 8.0), 300mM KCl, 10mM Imidazole, PIC 1X) and subjected to lysis by Sonication (PULSE ON 5 sec, PULSE OFF 5 sec, 20% amplitude, 30 cycles). The Lysate was then centrifuged at 4,000rpm for 10 min at 4°C.

The supernatant was then added onto Lysis Buffer I prewashed TALON Metal Affinity beads. The beads were incubated for 2 hrs at 4°C on rotator. The beads were centrifuged at 1,000 rpm, 1 min at 4°C. The supernatant (SN) was discarded and the beads were washed thrice with five times the bead volume of Wash Buffer (50mM NaH_2PO_4 pH 8.0, 10% Glycerol, 8mM β-Mercaptoethanol, 600mM KCl, PIC) and incubated with elution buffer (50mM NaH_2PO_4 pH 8.0, 500 mM Imidazole, 10% Glycerol, 8mM Beta-Mercaptoethanol, 0.6M KCl, PIC) for 30 mins. The beads were then centrifuged again at 1000rpm, 4°C, 1 min and the Supernatant was used for the Western Blot Competition assay.

2.6. Western Blot Competition Assay:

MEL Nuclear extract was used as sample in SDS PAGE and transferred to a Western blot. The Western blot membrane was cut into small strips and the strips were blocked using 5% Milk in 1X PBS as blocking buffer. Meanwhile, the antiserum diluted to thousand folds in 5% Milk in 1X PBS solution were incubated overnight on a rotator at 4°C either with elution buffer or with increasing concentration of immunopeptide. Pre-immune sera for both the antibodies were also incubated in parallel with and without immunopeptide as a negative control. The pre-incubated antiserum and pre-immune sera were used to perform the western blot and visualize the protein of interest.

2.7. Construction of MEL TR Hemgn knockdown clone:

PGJ10 plasmid was digested using NEB enzymes BglII and NotI at 37°C for 2 hrs. Oligonucleotides coding for Hemgn targeting short hairpin RNA (shRNA, Invitrogen, siRNA sequence: GCAGTTGAACCTGAATTCA, Jiang et al., 2010) desalted and

phosphorylated at the 5' end were annealed. The annealed Oligonucleotides (0.09 pmoles) were then ligated to the double digested PGJ10 plasmids (75ng) using T4 DNA ligase (1.5 Units, Invitrogen) at 16°C overnight. 5µl of the ligation mixture was added to Z-competent DH5α cells and selected on LB Amp^+ agar plates. Single colonies were picked and 5ml of LB broth containing Ampicillin (100µg/ml final concentration) were inoculated. The inoculated LB was incubated overnight at 37°C with 225rpm. The plasmids were isolated from the clone using Miniprep kit (Qiagen) as per the manual. The positive clones were screened by subjecting plasmid to digestion with BglII (BglII and NotI restriction sites are destroyed on insertion of the oligonucleotide). Plasmid that was tested positive for insertion was prepared in large scale using Maxiprep kit (Qiagen). The insertion was confirmed by sequencing (performed by the StemCore Sequencing facility, OHRI). 16µg of plasmids were added to 15 x 10^6 MEL TR cells in MELTR growth medium and electroporation was performed in a 0.4mm cuvette at 260V and 950 µF (Gene Pulser Xcell, BioRad). After two days, the cells were transfered to MEL TR clone growth medium for G418 selection. Two weeks later the electroporated cells were serially diluted in a 96 well plate, and the clones obtained from single cells were isolated and further screened by Western blotting. Clones showing high degree of knockdown at protein level on addition of Dox to the media were selected.

2.8. Benzidine Staining:

1ml of Solution A (14.6 ml of acetic acid, 485.4ml of H_2O, 1g of Benzidine dihydrochloride) was mixed with 10µl of Solution B (29-32% H_2O_2 w/w aqueous solution). 10 µl of the mixed solution was added to 10µl of media containing cells and the mixture was incubated on ice for 2 min. The solution was then introduced into a

hemocytometer and the total number of cells and the number of blue cells (Benzidine positive) were enumerated in every chamber. The percentage of Benzidine positive cells was thus calculated.

2.9. Extraction of Genomic DNA:

The cells were harvested by centrifugation at 2000 rpm, 10 min, 4°C. The cells were washed twice with 5 times the packed cell volume of ice cold 1X PBS. The cells were then resuspended in 1 ml of Digestion Buffer (100mM NaCl, 10mM TrisHCl pH 8.0, 25mM EDTA, 0.5% SDS) for every 10^8 cells used for extraction. The mixture was incubated at 50°C for 12 to 18 hrs with 1400 rpm agitation. To the mixture, an equal volume of Phenol, Chloroform, Isoamyl alcohol mixture was added, mixed vigorously and centrifuged at 8000 rpm, 10min, room temperature. The aqueous phase was decanted into a fresh eppendorf and 0.1 volume of 3M Sodium Acetate pH5.2 was added. To the above, twice the volume of ice cold anhydrous ethanol was added, mixed well and incubated overnight at -20° C overnight. The mixture was then centrifuged at 14000 rpm, 30 min at 4°C. The pellet was washed with 70% ethanol, air-dried and resuspended in TE buffer.

2.10. Immunoprecipitation:

Protein A Dynabeads were resuspended by vortexing rigorously and required volume was pipetted onto a siliconized eppendorf tube. The tube was placed on a magnetic bar and the supernatant was discarded. The beads were then washed twice with 5 volume of 0.1M Potassium Phosphate Buffer pH8.2. The antibody solution was then added to the magnetic beads and incubated on a rotator for 1hr at 4°C. The supernatant was then

discarded and washed once with 5 volume of 0.1M Potassium Phosphate buffer. The beads were then washed twice with 0.2M Triethanolamine pH8.2. The bound antibody was then crosslinked to the beads by suspending them in freshly prepared 15mg/ml DMP (Thomson Pierce) solution (DMP in 0.2M Triethanolamine pH8.2) and incubated on a rotator at room temperature for 30 minutes. The cross-linking was performed thrice and the crosslinking was stopped by washing the beads with 50mM Tris HCl pH7.4. The beads were then washed twice with IP1M, 0.1M glycine pH 3.0 and IP100 solution (The IP wash buffers are denoted by IP followed by a number that denotes the concentration of KCl in mM or M, Composition of the IP wash buffer excluding KCl is as follows: 25mM Tris pH7.9, 5mM $MgCl_2$, 10% Glycerol, 0.1% NP-40, 0.3mM DTT, PIC). 10 µl of 10% NP-40 was added to 1ml of Benzonase treated MEL NE, mixed well and centrifuged at 14000rpm, 4°C, 30min. The Supernatant was filtered using a 0.2µm filter and the filtrate was incubated with Prot A Dynabeads (Invitrogen) on a rotator at 4°C for at least 4 hrs. for pre-clearing. The pre-cleared MEL NE was then added to the antibody cross linked beads and incubated on a rotator at 4°C overnight. The Supernatant was then removed and washed twice with IP150 and IP100 wash buffers by incubating the beads with the buffer on a rotator at room temperature for 5 min. The immunoprecipitate was then eluted by incubating the beads with Urea elution buffer (0.05% SDS, 50mM Tris pH8.3, 5mM EDTA, 6M urea) at 37°C for 30 min with agitation (700 to 1400 rpm).

2.11. Silver staining (Mass Spectometry Compatible):

The protein samples in 1X Protein loading dye were loaded onto wells in SDS Poly-acrylamide gel and the electrophoresis were performed using BIORAD SDS PAGE apparatus at 15mA per gel for 60 min at room temperature. The stacking gel was

removed and the separating gel was incubated in a fixing solution (50% ethanol, 5% acetic acid, in water) for 30 min on a shaker at room temperature. The gel was incubated for 10 min in wash solution (50% ethanol in water) on shaker at room temperature. The gel was then washed twice in water by incubating it on shaker at room temperature for 10 min each. The gel was then incubated in the sensitizer solution (0.02% Sodium thiosulfate) for 2 min followed by washing in water twice by incubating in water for 3 min each. The gel was then incubated in staining solution (0.1% Silver nitrate in water) for 30 min. The gel was then washed in water for a minute and in developing solution (0.04% formalin in 2% Sodium carbonate) for 30 sec. The gel was then incubated in developing solution with gentle swirling. When the bands appear, the gel is transferred to 5% acetic acid and the gel was incubated in it for 5 min. The gel was then transferred to 1% acetic acid and stored at 4° C.

2.12. Mass Spectrometry Sample Preparation:

The protein bands in the gel were visualized by using silver staining protocol (mass spectrometry compatible). The bands were excised using a sterile lancet into approximately $1mm^2$ small pieces and stored in 1% acetic acid. In-gel digestion was performed using trypsin as previously described (Shevchenko et al., 2007). Overnight digestions were performed using proteomic grade trypsin (Promega). Peptides from gel bands were extracted with 5% formic acid in acetonitrile, dried using the Vacufuge (Eppendorf) and rehydrated in 20 µL of 0.1% Trifluoroacetic acid in water. For analysis by LC-MS/MS, peptides were loaded at a rate of 20 µL/min onto a Michrom Capillary Peptide Trap using a Finnigan Surveyor HPLC (Thermo). Following the desalting step, peptides were eluted over 30-60mins using a 5-40% gradient of acetronitrile with 0.1%

formic acid at approximately 300nL/min. Eluted peptides bound to a second column (75µm x 100mm) packed with Zorbax SB-C18 5µ (Agilent) and were subsequently electrosprayed into an LTQ Orbitrap mass spectrometer (Thermo, USA). Mass spectrometry data was acquired in a data-dependant mode which selected the four most intense peaks from each MS spectrum for further fragmentation. All the Mass spectrometry raw files were processed using Sequest (Eng et al., 1994 and Qian et al., 2005) and Trans Proteomic Pipeline (Deustch et al., 2010, Seattle Proteome Center, Institute for Systems Biology).

2.13. Gel filtration Chromatography:

Differentiated MEL Nuclear extract was centrifuged at 14,000 rpm, 30 min, 4 C. The supernatant was filtered by passing it through a 0.2µm filter. The filtrate was loaded onto a Superose 6 column on AKTA FPLC system. The void volume as approximated by elution volume was 7.0 ml. Hence samples were collected as a fraction of 500 µl each from elution volume of 7.0ml when there is a sudden increase in the absorption of 280 nm light. The fractions were concentrated overnight using 10% trichloric acetic acid. Protein precipitate was pelleted by centrifugation and the pellets were washed with acetone subsequently to remove residual trichloric acid. Precipitated protein pellets were dissolved in loading buffer (143mM Tris pH6.8, 100mM KCl, 28.6% Glycerol, 5.7% SDS, 286mM DTT). 15µl of alternate fractions collected were loaded on to SDS Poly Acrylamide gel and Western blot was performed to visualize the migration pattern of the protein of interest.

2.14. Hemgn-Histone Interaction Studies:

Hemgn and Mock Rabbit IgG antibody (Santacruz) were crosslinked to the beads as mentioned in the immunoprecipitation protocol. Histones were extracted from MEL Nuclear Extract using the acid extraction protocol. Histones in 0.2N HCl were pH neutralized by the addition 1.5M Tris pH 8.6 buffer. The pH of the histone extract is then checked using pH strips. The pH neutralized acid extract were then centrifuged and the supernatant was filtered. The filtrate was used as the histone extract.

To study the interaction between DNA free histones and Hemgn complex, pH neutralized histone extract was added to MEL Nuclear extract (0.2mg) prepared in the absence of Benzonase activity. MEL Nuclear Extract with and without histone pre-incubation were then pre-cleared for at least 4 hrs. The precleared sample was then added to both Mock and Hemgn crosslinked beads. Further immunoprecipitation was performed as described in the immunoprecipitation protocol using IP150 and IP100 as the wash buffers. The proteins were eluted by boiling for 5 minutes in 2X SDS protein loading dye and the Hemgn histone interaction was studied by performing SDS PGE and Western Blot as mentioned in the previous sections.

To study the interaction between histone and the core Hemgn complex, histones were extracted, centrifuged and filtered as previously mentioned. The Hemgn and Mock Rabbit IgG IP were performed as mentioned in the immunoprecipitation protocol. The beads with immunoprecipitated protein were then washed with IP500 and IP100 wash buffer twice. The beads were then washed further with cold PBS containing 0.1% Ovalbumine (OVA). The OVA treated beads were further incubated with Histone extract

for two hours at 4°C on rotator. The beads were then washed twice with IP150 and IP100. The proteins were then eluted in 2X SDS protein loading dye by incubating it at 37°C for 30 min with 1400 rpm. Hemgn histone interaction was visualized using SDS PAGE and Western Blot.

2.15. Chromatin Immunoprecipitation:

Extraction of chromatin: MEL cells were grown to a cell density of ~$1X10^6$ cells/ml (up to 2 x 10^6 cells/ml). A total of $1X10^8$ cells were taken in each tube and centrifuged for 10 min. at 1500 rpm, room temperature. The supernatant was discarded. The pellet was resuspended in 39ml at 37°C preheated Crosslinking media (RPMI-1640 (Hyclone), 10% Standard Fetal Bovine serum) and transferred into a 50 ml falcon tube. 1.081 ml of Formaldehyde 37% (1% final) was added, mixed 4 times and rotated for 30 min. at Room Temperature (EXACTLY 30 min.). 2ml of Glycine 2.5M (0.125M final) was added. The tubes were mixed by inverting 4 times and then centrifuged for 10 min. at 1500 rpm, 4°C. The supernatant was discarded and the cross linked cells were washed with 30 ml of cold 1X PBS. The pellet was then transferred separately into 1ml eppendorf tubes using 1ml ice cold 1X PBS and resuspended by pipetting up and down. They were then centrifuged for 3 min. in a table-top centrifuge. The supernatant was removed carefully with pipet, flash frozen with Liquid Nitrogen and stored at -80°C.

The frozen cell pellets were thawed on ice. Each pellet was resuspended in 10X Packing Cell Volume (PCV) of Swelling Buffer (25mM Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.1% NP-40, 1mM DTT, PIC) and vortexed well. They were then incubated on ice for 15 min. and vortexed a few more times during the incubation. They were then lysed

by douncing 40X with pestle B (2 ml dounce). The lysate was then centrifuged at 2000 rpm for 5 min at 4°C and the supernatant was discarded. The pellet was resuspended in 400 µl of Sonication Buffer 1% SDS (50mM Hepes pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 1% SDS, PIC) in eppendorf tubes (~0.8% SDS final; ~500µl volume final) and sonicated using Bioruptor (Diagenode, water at 10^0C; High Power Setting; 30 cycles (30" ON and 1'' OFF)) for 75min. The sonicated sample was then transferred to a Falcon tube and Sonication Buffer No SDS (50mM Hepes pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, PIC) was added so that the final concentration of SDS reaches 0.1%. The sample was then centrifuged for 15 min. at 14 000 rpm at 4°C. 50µl of the supernatant is then used as Input for RT qPCR. To 8 ml of the chromatin added 8µl of (0.5µg/µl) sonicated λ DNA (= 2µg final) and 40 µl (1/100 vol.) of ovalbumin (OVA, 100 mg/ml) (= 1mg/ml final) which is then pre-cleared and used for immunoprecipitation.

Pre-clearing of Chromatin: 10 µl of Dynabeads M280 protein A or G in each of them were taken in a small Falcon tube for pre-clearing. Each tube was washed twice with 1ml of Sonication Buffer 0.1% SDS (50mM Hepes pH7.9, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, PIC). 1ml of Sonication Buffer 0.1% SDS + 2µl (=1µg) of sonicated λ DNA + 10µl (1/100) of OVA (100mg/ml) was added to them and they were incubated on the rotator for 1hr (minimum) at 4°C. The supernatant was discarded and the extracted chromatin was added and incubated on the rotator for 1 hr (minimum) at 4^0C.

Chromatin Immunoprecipitation: 20 µl of dynabeads M280 were taken in each tube. The beads were washed twice with 1ml of IP100 Buffer. The Supernatant was removed

by pipetting and the appropriate antibody solution was added to the tubes. The tubes were incubated on a rotator for 2h at room temperature (minimum). The supernatant was discarded and the beads were then washed with 1ml of Sonication Buffer 0.1% SDS each. The beads were then incubated on the rotator for 1h (minimum) at 4°C. The supernatant was then discarded and the pre-cleared chromatin was added to the antibody bound beads. The beads were incubated overnight at 4°C. The supernatant was discarded and the beads were washed 2X with 1ml of cold Sonication Buffer 0.1% SDS. They were then washed twice with cold Wash Buffer A (50mM Hepes pH7.9, 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, PIC), cold Wash Buffer B (20mM Tris pH8.0, 250mM LiCl, 1mM EDTA, 0.5% NP40, 0.5% Sodium deoxycholate, PIC) and cold TE Buffer. Then 200µl of Elution Buffer (50mM Tris pH8.0, 1mM EDTA, 1% SDS) at room temperature was added and mixed by vortexing. The beads in elution buffer were incubated at 65°C in heated vortex at 1400 rpm for 10 min. The first eluate was collected in a fresh eppendorf. The elution was repeated and the two eluates were pooled together. To each elution and PCR input tube made up to 400µl with elution buffer, 16µl of NaCl (5M) (=0.2M final) and 4µl of RNAse A (DNAse free) (1mg/ml) (=10µg/ml final) were added and incubated at 65°C for 4h to reverse crosslink the chromatin. The DNA was then purified from this mixture by centrifuging each tube for ~ 1 min. (table top centri.). 4 µl of EDTA (0.5M) and 1 µl of proteinase K (10µg/µl) were then added to each tube and incubated at 42°C for 2h. The sample can now be stored at -20 C if required. The DNA was extracted by adding equal volume of Phenol, Chloroform and Isoamyl alcohol mixture or Chloroform and Isoamyl alcohol mixture. The samples were agitated vigorously by vortexing. The sample was then centrifuged for

10 min at 8000 rpm at room temperature. The SN (aqueous phase) was taken and further extracted with equal volume of chloroform. After chloroform extraction, the aqueous phase was decanted into a fresh eppendorf and 5 µl of Glycogen (20ug/ul), 40 µl (1/10 vol.) of Na Acetate (3M) pH5.5 and 0.8 ml of Et-OH cold (-20°C) (=2 vol.) were added. The samples were mixed vigorously by vortexing and kept overnight at -20°C. The samples were centrifuged at 14,000 rpm for 30 min. at 4^0C. The pellets were washed with Et-OH 70% (0.5ml) and centrifuged at 14,000 rpm for 30 min. at 4°C. The supernatant was discarded and the pellets were air dried. The pellets were resuspended in 10mM Tris pH7.4 by vortexing and allowing the DNA to dissolve at room temperature for 30 min. The samples were then subjected to real time quantitative PCR (RTqPCR) to find enrichment of specific protein over a specific gene locus using appropriate primers. The samples can be stored at -20°C if required.

2.16. mRNA isolation and RTqPCR:

Cells were harvested by centrifugation at 2000 rpm, 10 min, 4°C. The supernatant was discarded and 1 ml of RNA STAT 60^{TM} (TelTest Inc) was added to the pellet and mixed vigorously by vortexing. The rest of the mRNA isolation was performed according to the RNA STAT60^{TM} manufacturer's protocol. The concentration of the obtained RNA was measured by reading the absorption at 260nm using Spectrophotometry (Nanodrop, Thermo Scientific). The expression level of genes of interest were assessed using RTqPCR. Runs were performed using the Corbett Rotor-Gene 6000 (Lifesciences). PCR was performed using gene-specific primers under the following PCR conditions: First hold at 48°C for 30 min, second hold at 95°C for 10 min, 45 cycles of denaturation at 95°C for 20 sec, annealing at 55°C for 20 sec, extension at 72°C for 34 sec. The first hold

at 48°C was performed for reverse transcription of RNA and hence was not used for quantification of enrichment of ChIP samples.

Contents	Volume (µl)
10X PCR Buffer (Applied Biosystems)	2.5
25mM $MgCl_2$	5.5
10mM dNTP	2
10mM Labelled Probe	0.25
10mM Forward Primer	0.5
10mM Reverse Primer	0.5
5 Units/µl AmpliTaq Gold™ (Applied Biosystems)	0.2
40 units/µl RNAse Inhibitor (Promega)	0.25
50 Units/µl Multiscribe Reverse Transcriptase (Applied Biosystems)	0.625
5ng/µl RNA	5
	made up to 25µl with water

For RTqPCR of ChIP samples, the composition of the reaction mixture is as follows:

Contents	Volume (µl)
10X PCR Buffer (Applied Biosystems)	2.5
25mM $MgCl_2$	5.5
10mM dNTP	2
10mM Labelled Probe	0.25
10mM Forward Primer	0.5
10mM Reverse Primer	0.5
5 Units/µl AmpliTaq Gold™ (Applied Biosystems)	0.125
DNA sample	5

	made up to 25µl with water

MEL genomic extract diluted serially to different concentrations was used as a standard for all the qPCR reactions. A standard curve was plotted for all the primer probe sets using the known standards that were used during every PCR run in parallel to the samples. The samples were quantified using the linear equation that fits the standards. The square of the regression co-efficient (R^2), that determines the fitness of the data to the equation, for standards of all the primer probe sets used in the experiment were greater than 0.99.

3. RESULTS

3.1. Expression and Purification of N-Terminal region of Hemgn in BL21 pLysS:

Two antibodies 9474p and 9520p were previously made in the lab injecting the N-Terminal region of Hemgn (1-164 a.a.) as immunopeptide in two individual rabbits. The antisera collected from the rabbits were tested for their specificity for the endogenous Hemgn in Western Blot competition assay using purified N-terminal region of Hemgn.

The N-terminal region of Hemgn containing a His tag was expressed in BL21 pLysS and purified using Metal Affinity Resin as described in the methods section 2.5. The protein was run on a SDS PAGE in parallel with standard BSA protein (different concentrations) as a standard on the SDS PAGE and the amount of protein was quantified visually based on the band intensity and the size of the protein. From Fig. 4., the protein expressed and purified from BL21 pLysS was observed to be of good purity. The theoretical size of the purified protein is 17 kDa, but using Coomassie Blue Staining three bands of protein were observed in the purified protein sample. The three bands were all identified to be the N-Terminal region of Hemgn using mass spectrometry during previous studies in the lab. This anomalous migration of the protein in PAGE may be contributed either to post-translational modifications or the amino-acid composition of the peptide. Charged amino-acid residues are present in stretches in the N-Terminal region of Hemgn which may lead to poor binding of SDS, in the loading dye, to the protein. Hence the migration of protein may be altered due to poor SDS binding, resulting in a shift in their electrophoretic

mobility. This protein sample thus purified was further used in the Western blot competition assay.

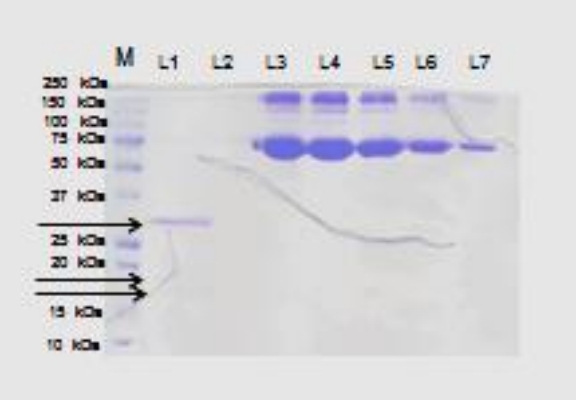


Figure 4. Coomassie Blue Staining - Purified Amino-terminal region of Hemogen (1-164 aa) used as immunopeptide for competition assay. M - BioRad Precision Plus All Blue Marker, L1 – Purified N-terminal region of Hemogen, L3-7 - Bovine Serum Albumin of concentrations 1, 0.75, 0.5, 0.25, 0.1mg/ml respectively. The arrows point to bands that correspond to N-Terminal Hemgn.

3.2.Western Blot Competition assay:

Western Blot competition assay works on the principle that when a specific immunopeptide is added to the antibody during western blot, the immunopeptide competes with the membrane immobilized substrate for the antibody. This results either in the disappearance or a decrease in the intensity of the specific signal. 9474p and 9520p antiserum western blot were performed with purified N-terminal region of Hemgn used in competition to the substrate (MEL Nuclear Extract containing Hemgn protein) immobilized on the membrane. The antisera diluted in blocking buffer were pre-incubated with either plain buffer or buffer containing different concentrations of

immunopeptide. The pre-incubated primary antibody solutions were added to membranes containing equivalent amount of substrate and incubated overnight. The theoretical size of Hemgn is 55kDa but the antibodies used in other literature suggest that the observed electrophoretic mobility of Hemgn on SDS PAGE is close to the 100kDa standard marker (Li et al., 2007). Performing Western blot, a major band was observed at a range close to 100kDa in addition to few other bands with both the homemade antibodies (Fig. 5). On testing both the antibodies, the signal migrating close to 100 kDa standard marker protein disappeared when revealed with immunopeptide pre-incubated antiserum by Western blotting. Hence the signal migrating closer to the 100kDa standard marker may correspond to Hemgn.

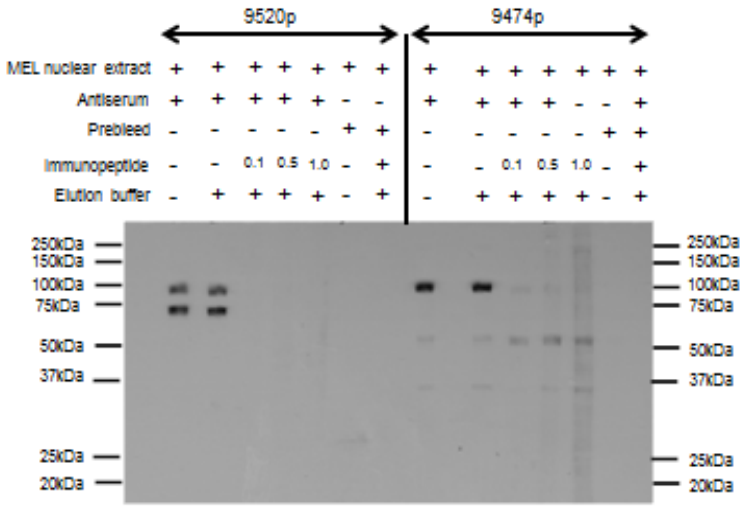


Figure 5. Immunopeptide Competition Assay. The Western Blot membranes have equivalent amount of MEL Nuclear Extract and the contents of the solution used to reveal the western blot is indicated above the western blot. (+) indicates presence and (-) indicates absence. The numbers indicate the ratio of immunopeptide used to the total amount of antiserum protein used (in terms of total protein).

When the competition assay was performed with 9520p, a band migrating close to 75kDa standard marker was observed to disappear on competition in addition to the 100 kDa band. The 75kDa band was not observed during Hemgn western blots with 9474p antiserum or a commercial antibody M180. This additional band may be an uncharacterized isoform of Hemgn that is identified only by the antiserum or may be a non-specific band arising from cross-reaction between the Hemgn antibody and another protein in MEL Nuclear Extract containing similar aminoacid sequence.

3.3. Immunoprecipitation:

The antibodies were further validated for immunoprecipitation (IP). Hemgn pull-down assays were performed with MEL cell nuclear extract using the two antisera 9474p and 9520p and a commercial antibody M180, raised against the intermediate region of Hemgn (Fig. 6F). Medium stringency salt (300mM KCl containing IP buffer) wash condition was used to remove non-specific proteins bound to the antibody. The immuno-precipitated proteins were eluted by boiling for 5 min in 2X SDS PAGE dye containing Dithiothreitol. They were then visualized using Western blotting. The western blot containing elute from IP performed with N- terminal region specific antibodies were revealed with the M-180 antibody, which is specific to the intermediate sequence of Hemgn, and the reciprocal experiment was also performed using M180 antibody for IP and the 9474p/9520p antibodies for Western blot. We detected Hemgn in eluates from IPs that were performed with all three Hemgn specific antibodies and we did not observe any detectable amount of Hemgn in the corresponding mock IPs that were performed in parallel (Fig. 6 A-C). Therefore, all the antibodies can immunoprecipitate Hemgn from MEL nuclear extract.

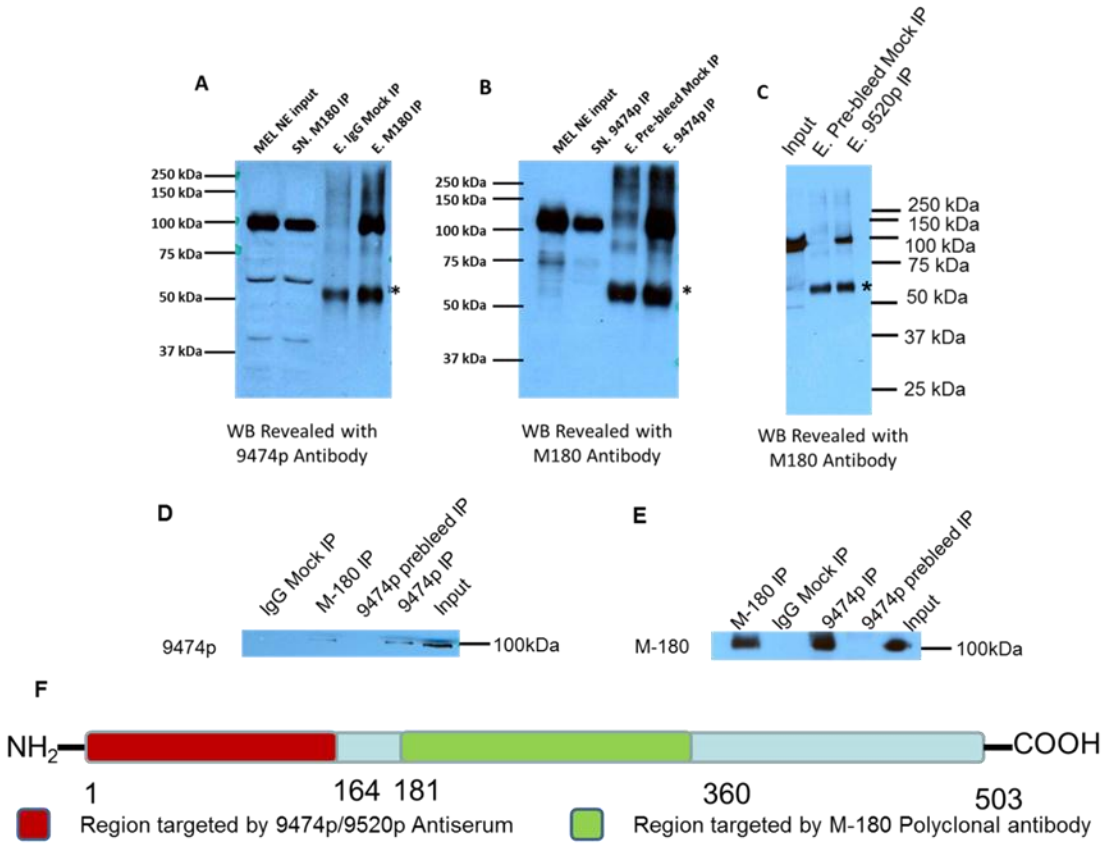


Figure 6. Immunoprecipitation of Hemogen. The contents of each lane in the western blots have been described above each lane. Immunoprecipitation was performed with M-180 and revealed with 9474p antibody(A), 9474p IP was revealed with M-180 antibody (B) and 9520p IP was revealed with M-180 antibody (C). Western Blot containing eluate from IP revealed with D) 9474p antibody and E) M-180. * denotes IgG Heavy chain. (F) Schematic representation of the region of Hemgn which were used for making the homemade antisera and the commercial antibody.

Moreover, the immunoprecipitated Hemgn migrated close to the 100kDa standard marker protein in western blots, as observed earlier with the competition assay (Fig. 5). We noticed that the amount of Hemgn immunoprecipitated is higher in the 9474p IP eluate in comparison to an equivalent amount eluate from 9520p IP when both fractions are

revealed with the M-180 antibody. This suggests that 9474p is more efficient in immunoprecipitation. Furthermore, we noticed previously that the 9520p antibody detected an anomalous protein signal migrating close to 75kDa (Fig 5.) that disappeared during competition assay and the band was not observed in the Hemgn IPs performed with both the antiserum 9474p and the commercial antibody M180. Since the nature of the anomalous 75 kDa protein was not known, 9520p was not further utilized. Instead we used the 9474p and commercial M180 antibodies for further experiments. To compare the efficiency of M-180 and 9474p antibodies for immunoprecipitation, further western blot assays were performed on membranes containing equivalent amount of elution from 9474p, M-180 (Hemgn) and corresponding mock IP. All the IPs were performed in parallel in similar conditions. The elutions from both the IPs along with their corresponding mock IP elutions were loaded onto the same gel and transferred into a single membrane. Western blots were performed with these membranes using both M-180 and 9474p separately (Fig. 6D,E). From both 9474p and M180 western blots, stronger signal or higher enrichment of Hemgn was observed for Hemgn Immunoprecipitation with 9474p antiserum when compared with M180 antibody. Hence, 9474p was used for all further Hemgn IPs performed during the course of the study.

3.4. Knockdown of Hemgn expression:

The antibodies were further validated for their specificity using a gene expression knockdown strategy. A Hemgn specific short hairpin RNA coding sequence was cloned into PGJ10 vector (Fig.7). The construct expresses the inserted shRNA in the presence of tetracycline analog, Doxycycline hyclate (DOX). The PGJ10 shRNA construct was introduced into MEL cells expressing the TET Repressor (MEL TR) by electroporation.

After electroporation, the cells were allowed to grow and recover in MELTR growth medium and G418 sulfate was added to the media 48 hrs. after electroporation for positive clone selection. Two weeks later the electroporated cells were serially diluted in a 96 well plate, and the clones obtained from single cells were isolated and tested further for Dox-dependent knockdown of Hemgn.

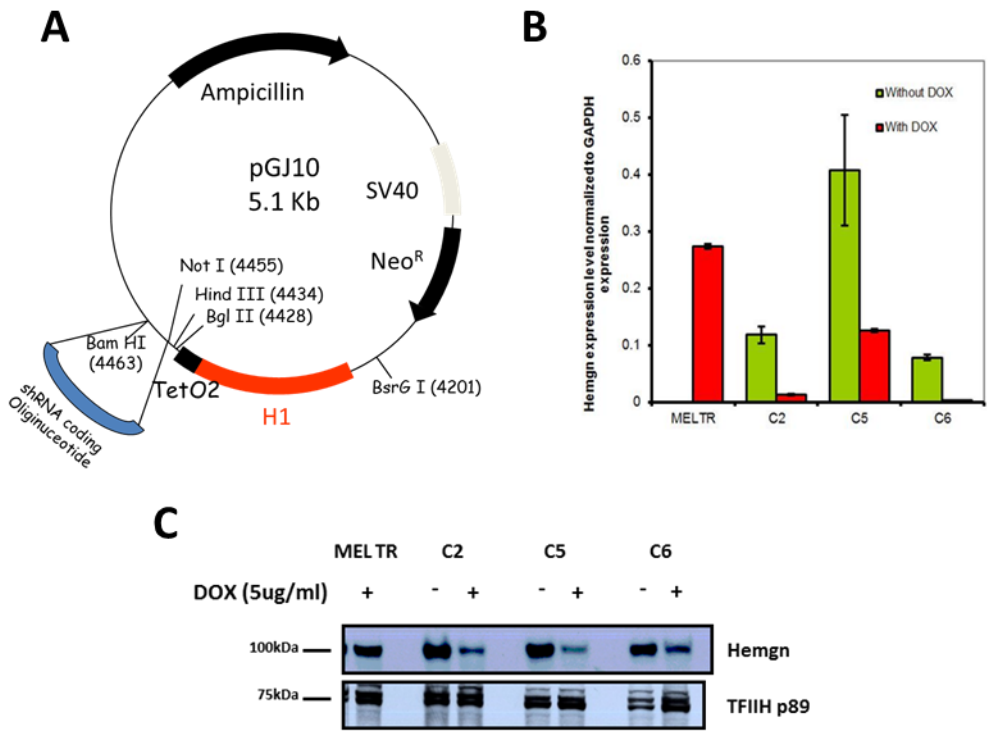


Figure 7. Knockdown of Hemgn expression in MEL TR cells (A) pGJ10 map. (B) The relative expression of Hemgn in comparison to GAPDH was studied before and after DOX induction of Hemgn knockdown in Hemgn shRNA expressing MELTR clones C2, C5,C6. MELTR parental cell line treated with DOX was taken as a control for Hemgn expression. (C)Western Blot shows Hemgn knockdown at protein level in the clones C2, C5 and C6 upon DOX treatment. DOX treated parental MELTR was used as a control. TFIIH p89 protein levels serve as a loading control.

Six clones were obtained after the single clonal selection. Positive clones for Hemgn knockdown were screened by using western blot to study the Hemgn expression at the protein level in the presence and absence of DOX induction (Fig.7C). Using 9474p antiserum for western blotting, four positive clones were obtained. Clone 2 had the maximum knockdown of Hemgn expression and clone 1 had the least knockdown (Supplementary Fig. 1). Clone 3 and clone 4 had no visible changes in Hemgn expression at the protein level. There was a decrease in the intensity of the band migrating below the 100kDa standard marker protein on Hemgn knock down (Fig. 7C). This further validates the result of competition assay and immunoprecipitation where Hemgn was observed to be migrating close to the 100kDa standard protein marker. The knock down of Hemgn expression at the transcript level was confirmed by isolating mRNA from the positive clones with and without DOX induction and on measuring the Hemgn mRNA expression level using RT-qPCR. There was a significant decrease in the Hemgn expression at the mRNA level in the positive clones C2, C5 and C6 whereas no significant decrease in Hemgn expression level in parental MEL cell (Negative control) with DOX induction (Fig 7B).

3.5. Expression Profile of Hemgn during differentiation of MEL:

MEL cells are erythroid lineage committed transformed cells that are highly proliferative and can be induced to differentiate with DMSO (Levenson, R. and Housman, D, 1979, Conscience**,** J. F., et al., 1977). Our previous studies in MEL cells identified Hemgn as a MafK interacting protein that exhibits increased association with MafK after differentiation (Brand et al., 2004). Therefore, we were interested to study the expression profile of Hemgn during differentiation. Therefore using Western blot, we studied

Hemgn expression at the protein level during differentiation in MEL (Fig. 8A). The nuclear extracts from MEL cells, that were induced to differentiate with DMSO, were quantified using Bradford Assay and equivalent amount of protein was loaded onto the SDS PAGE gel based on the quantification. TFIIH p89 was used as the internal loading control. Other nuclear proteins such as G9a and GLP were also studied in parallel as a control. ImageJ analysis was performed on the western blots to quantify the protein bands based on densitometry. Increase in Hemgn expression was observed in comparison to TFIIH during differentiation. About three fold increase in Hemgn expression was observed at the protein level in MEL cells after four days of differentiation with DMSO (Fig. 8B). The protein expression level of G9a and GLP remained fairly constant in comparison to TFIIH p89 during the course of MEL differentiation with DMSO. Hemgn expression is therefore upregulated during DMSO induced differentiation of MEL cells.

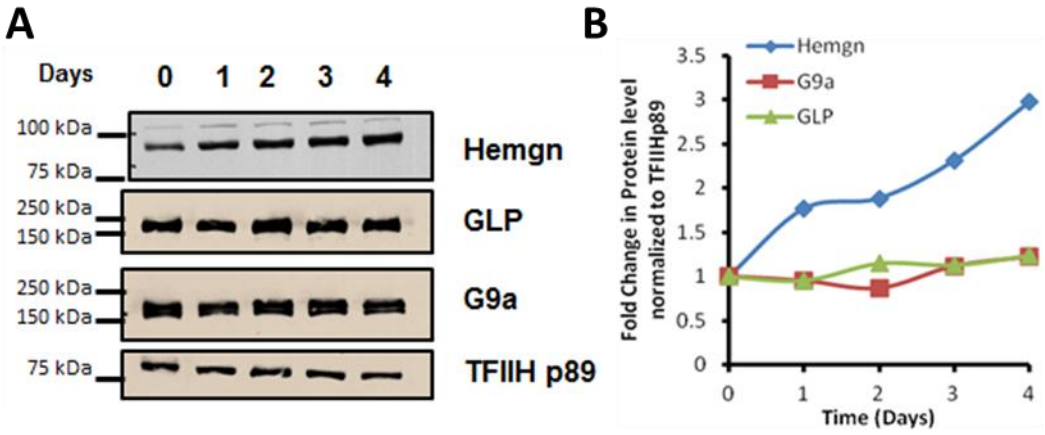


Figure 8. Expression profile Hemgn in differentiating MEL cells. (A) Using Western blot, the protein expression level of Hemgn and other nuclear proteins were studied every 24 hrs when MEL cells were induced to differentiate with DMSO up to 4 days. Equal amount of protein samples were loaded based on the total protein amount (estimated

using Bradford assay) and TFIIH p89 was used as the loading control. (B) The graph represents the fold change in the protein expression in comparison to undifferentiated MEL (0 day). The protein expression level as visualized by western blot was quantified using densitometry based software ImageJ. The protein expression level was normalized based on the TFIIH p89 densitometry values.

3.6. Effect of Hemgn knockdown in MEL on cell growth and differentiation:

There is an upregulation of Hemgn expression during differentiation in MEL. So, we were further interested in studying the effect of Hemgn on cell growth in both proliferation and differentiation conditions. We used Hemgn expression knockdown approach to study its effect on cell growth in MEL cells. The live cell density of parental MEL cells and Hemgn knock down clones C5 and C6 grown in the presence and absence of DOX were counted every day using Trypan blue assay. In normal growth conditions, Hemgn knockdown did not have a significant effect on cell growth in comparison to the parental MEL cells (Supplementary Fig 2 and 5). Whereas, in differentiation conditions (.i.e., in the presence of DMSO), there was a significant decrease in cell growth on Hemgn knockdown in the clones (Fig. 9A). There was no significant difference in the cell count in parental MEL cells in the presence and absence of DOX in differentiation conditions serving as a negative control. This result suggests that Hemgn is important for cell growth during MEL cell differentiation.

The effect of Hemgn knockdown on hemoglobin synthesis, a marker of erythroid differentiation, in MEL induced by 2% DMSO was studied by using Benzidine staining. During Benzidine staining, Oxidized Benzidine (blue in color) is formed as a result of the

peroxidase activity of Hemoglobin (Kapralov et al., 2009). Undifferentiated MEL cells are not positive for Benzidine staining because of their low globin expression. When induced to differentiate with DMSO, there is a hundred fold increase in their globin expression after 2-4 days of induction (Fig. 9C). Hence differentiated cells which highly express globin are stained blue and can be enumerated with Benzidine staining. On inducing clones C2, C5 and C6 and parental MELTR to differentiate with DMSO, the percentage of Benzidine positive cells were recorded every 24 hrs. The clones C2, C5 and C6 in the absence of DOX and the DOX treated parental MELTR cells express globins and showed comparable percentage of Benzidine positive staining throughout the course of differentiation. When clones were treated with DOX, they showed significantly lower percentage of Benzidine positive cells in comparison with the parental MELTR and clones without DOX induction between days 4-7 (Fig. 9B-D). The percentage of Benzidine positive cells profiled over a time course suggests that knockdown of Hemgn expression inhibits differentiation in MEL. The experiment was performed twice and a similar trend was observed in both the experiments (Supplementary Fig. 6). Therefore Hemgn may play an important role in hemoglobin synthesis directly by regulating α and/or β globin transcription or may affect hemoglobin synthesis indirectly by inhibiting terminal differentiation.

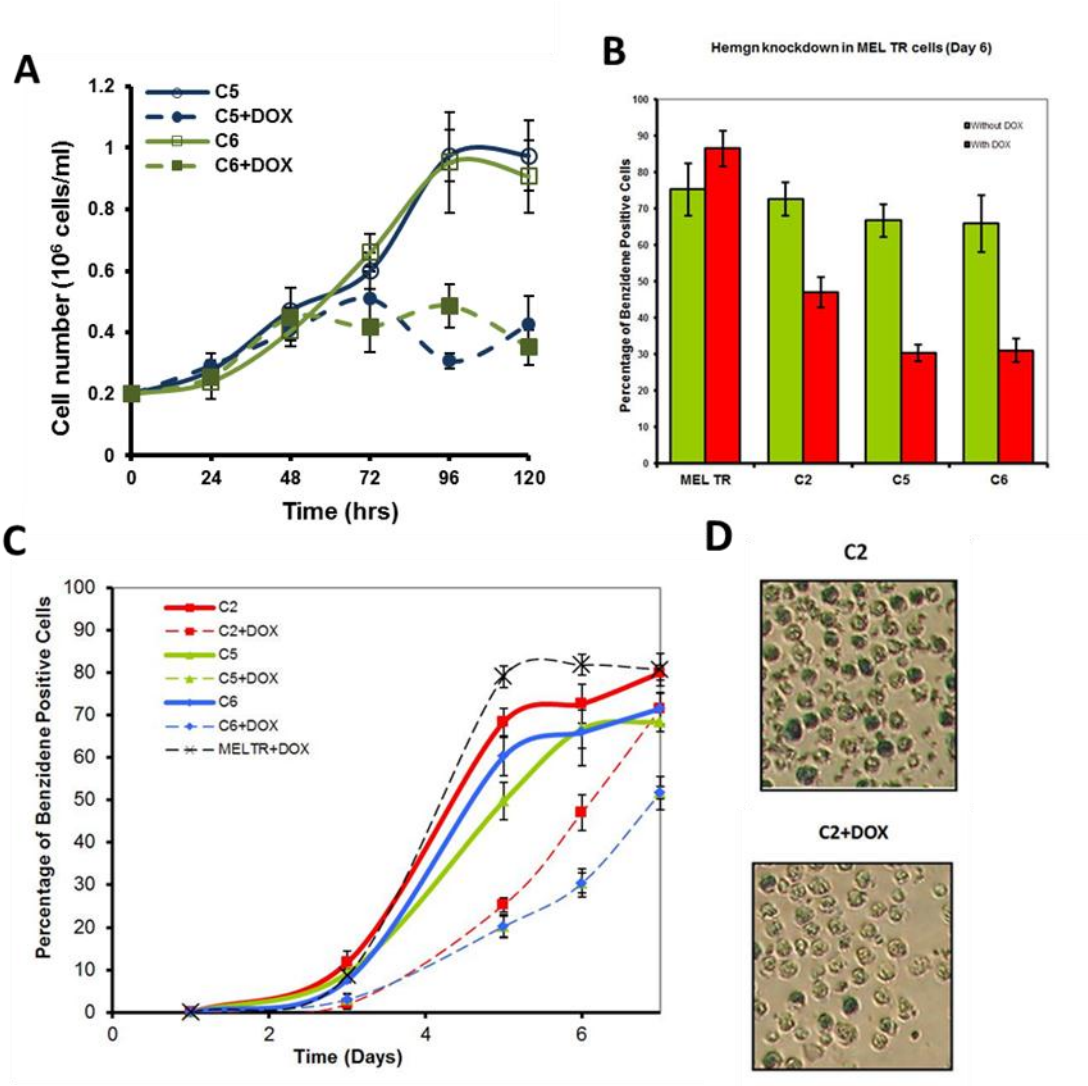


Figure 9. Effect of Hemgn knockdown on cell growth and differentiation in MEL. (A) Hemgn expression was knocked down in clones 5 and 6 using DOX induction. A significant decrease in the cell number was observed with knockdown during differentiation by using Trypan Blue assay. Each data point in the graph represents an average of 4 readings. (B, C and D) The effect of Hemgn knockdown on differentiation was studied by using Benzidine staining (stains the erythroid differentiation marker, hemoglobin) in the clones 2, 5 and 6 that knocks down Hemgn expression with DOX induction. (C) There is a significant decrease in the percentage of Benzidine positive cells during the course of differentiation with DMSO upto 7 days when Hemgn expression was

knocked down using DOX induction in the clones. Parental MELTR treated with DOX was used as the negative control. The growth curve of DOX treated C5 clone follows similar trend as DOX treated C6 clone and hence not clearly visible in the graph. (B) represents the percentage of Benzidine positive cells during day 6 of differentiation in the clones and the parental control. (D) The pictures of Benzidine stained clone 2 with (bottom panel) and without DOX induction (top panel) on day 7 of differentiation show a larger number of cells being stained by Benzidine staining in cells without DOX induction.

3.7.Hemgn Immunoprecipitation and Mass Spectrometry:

MafK was first identified as a transcriptional regulator that associates with Hemgn more after differentiation in MEL cells (Brand et al., 2004). Benzidine staining assay with Hemgn knockdown cells suggests that Hemgn may play an important role during erythroid differentiation in MEL. To further understand the mechanism by which Hemgn may play an important role in erythroid differentiation, we screened for other proteins that interact with Hemgn during growth and differentiation conditions. We performed Immunoprecipitation (IP) with our Hemgn antibody (9474p) and used Rabbit IgG IP as our negative control. Undifferentiated MEL and Differentiated MEL (with DMSO for 4 days) nuclear extracts, that were Benzonase treated, were used for the IP. Benzonase is an enzyme that digests both DNA and RNA independent of the polynucleotide secondary structure. Benzonase was added during nuclear extraction to extract chromatin-associated proteins (Supplementary Figure 12, 13 and 14). The wash condition for the IP was standardized by using different salt stringency (100mM, 300mM, 500mM and 1M KCl concentration in IP wash buffer, Supplementary Fig. 16) in the wash buffer. The eluates

were loaded onto a 10% Polyacrylamide Gel and electrophoresis was performed (Fig. 9). The pull down was visualized by subjecting the gel to silver staining. Since the pull down was the best with 100mM KCl wash condition, 100mM salt wash condition was used for all the immunoprecipitation for studying the Hemgn interactome. On subjecting the IP elution samples to Polyacrylamide Gel Electrophoresis (Fig. 10), gel band excision, in-gel trypsinization and LC-MS/MS, we identified a total of 2768 and 2394 proteins pulled down with Hemgn in Undifferentiated and Differentiated conditions respectively by Sequest analysis (Eng et al., 1994, Qian et al., 2005). The list of proteins was obtained after subtracting proteins identified in the mock IP. The list was further filtered by removing common contaminants like keratin and other cytoskeleton proteins. The list was then consolidated by removing proteins having less than 1 probability and being represented by less than 2 unique peptides. A total of 268 and 195 unique proteins were identified from the mass spectrometry analysis as proteins that interact with Hemgn directly or indirectly in growth and differentiation condition respectively.

Entrez ID	Gene Symbol	Description	Protein Probability (0 day)	No. of Unique Peptides (0 day)	Protein Probability (4 day)	No. of Unique Peptides (4 day)
93966	Hemgn	Hemogen	1	7	1	12
		Trascription elongation factors				
21973	Top2a	DNA topoisomerase 2-alpha	1	56	1	58
20926	Supt6h	Transcription elongation factor SPT6	1	54	1	19
12995	Csnk2a1	Casein kinase II subunit alpha	1	9	1	5
21974	Top2b	DNA topoisomerase 2-beta	1	21	1	36

		Transcription Machinery				
20021	Polr2c	DNA-directed RNA polymerase II subunit RPB3	1	4	1	4
73826	Poldip3	Polymerase delta-interacting protein 3	1	10	1	7
270627	Taf1	TAF1 RNA polymerase II	1	3	1	3
		SUMOylation machinery				
107932	Chd4	Chromodomain-helicase-DNA-binding protein 4	1	52	1	64
19386	Ranbp2	E3 SUMO-protein ligase RanBP2	1	33	1	46
19387	Rangap1	Ran GTPase-activating protein 1	1	10	1	9
		Transcription factors				
207165	Bptf	Bromodomain PHD finger transcription factor	1	4	1	6
66660	Sltm	Isoform 1 of SAFB-like transcription modulator	1	8	1	4
		Chromatin modifying enzymes				
110147	Ehmt2	Isoform 1 of Histone-lysine N-methyltransferase EHMT2	1	10	1	6
433759	Hdac1	Histone deacetylase 1	1	6	1	5
77683	Ehmt1	histone-lysine N-methyltransferase EHMT1 isoform 2	1	9	1	6
19821	Rnf2	E3 ubiquitin-protein ligase RING2	1	6	1	3
		Chromatin associated proteins				
74355	Smchd1	Structural maintenance of chromosomes flexible hinge domain-containing protein 1	1	11	1	4
233532	Rsf1	remodeling and spacing factor 1	1	3	1	9
		Other proteins				

12412	Cbx1	Chromobox homolog 1 (Drosophila HP1 beta)	1	3	1	3
11335	Cbx3	Chromobox protein homolog 3	1	6	1	6
17220	Mcm7	DNA replication licensing factor MCM7	1	9	1	6
17217	Mcm4	DNA replication licensing factor MCM4	1	5	1	4
66867	Hmg20a	Isoform 1 of High mobility group protein 20A	1	5	1	4
17191	Mbd2	Isoform 1 of Methyl-CpG-binding domain protein 2	1	5	1	5
12237	Bub3	Budding uninhibited by benzimidazoles 3 homolog	1	6	1	3
26914	H2afy	Isoform 1 of Core histone macro-H2A.1	1	9	1	9
12648	Chd1	Chromodomain-helicase-DNA-binding protein 1	1	9	1	4
217578	Baz1a	Bromodomain adjacent to zinc finger domain protein 1A	1	17	1	16
17688	Msh6	DNA mismatch repair protein Msh6	1	6	1	3
94112	Med15	Mediator of RNA polymerase II transcription subunit 15	1	6	1	4

Table 1. Partial list of proteins identified to interact with Hemgn in both differentiated and undifferentiated MEL using Mass spectrometry. The protein spectra obtained from LTQ Orbitrap were processed using Sequest and Trans Proteomic Pipeline. Probabilities were obtained for the proteins by using protein prophet during Trans proteomic pipeline analysis.

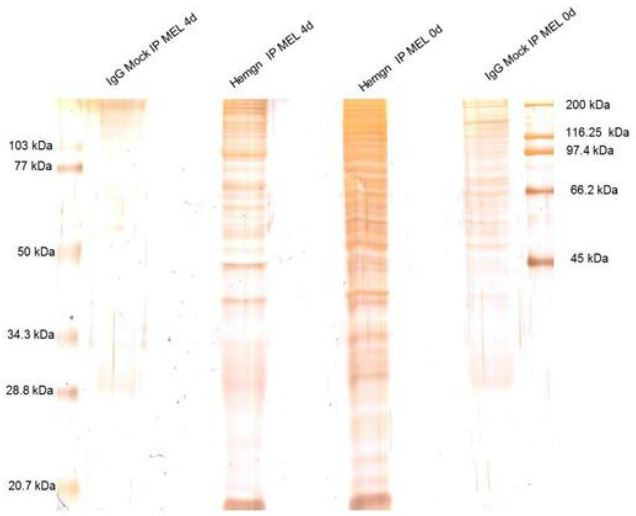


Figure 10. Silver stain of the Polyacrylamide gel subjected to mass spectrometry. Elutions from IgG Mock and Hemgn IP with differentiated MEL Nuclear extract and Hemgn IP and IgG Mock IP with undifferentiated MEL Nuclear Extract (Left to Right) were added loaded onto SDS PAGE and silver staining was performed. The bands on the gel were excised and subjected to protein extraction, trypsinization and Mass spectrometry analysis.

3.8. Validation of Hemgn Interactome Mass Spectrometry by Western Blot:

Proteins that were identified to interact with Hemgn using Mass Spectrometry were validated in both growth and differentiation conditions by using western blot. G9a and GLP were known to interact with p45 which forms NFE2 complex with MafK, previously identified to interact with Hemgn, during erythroid differentiation in MEL (Chaturvedi et al., 2009 and Brand et al., 2004). Since G9a, GLP, Histones H3 and H2B were all identified to interact with Hemgn using Mass Spectrometry in growth and differentiation conditions, their presence in Hemgn IP was studied by performing WB with the appropriate antibody. G9a, GLP, Histones H3 and H2B were found to be

enriched only in Hemgn IP in comparison to the Mock Rabbit IgG IP (Fig. 11). Therefore, these factors are enriched with Hemgn in MEL in both growth and differentiation conditions.

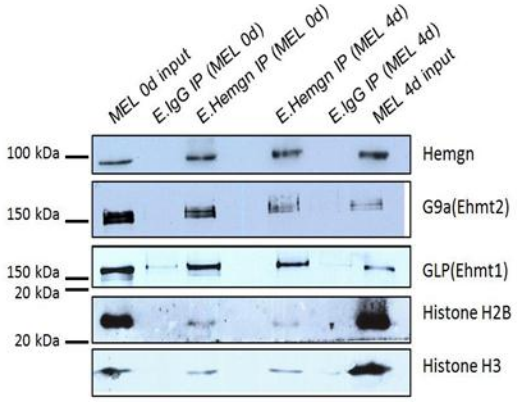


Figure 11. Validation of Hemgn Interactome by Western Blot. G9a (Ehmt2), GLP (Ehmt1), Histone H2B and Histone H3 were identified to interact with Hemgn during Mass spectrometry analysis. Hemgn IP was performed with undifferentiated (Mel 0d, Left) and differentiated MEL Nuclear extract (MEL 4d, Right) and the IP input and elutions from Mock IgG IP and Hemgn IP were loaded on the gel and Western Blot was performed for the interacting proteins to validate Mass Spectrometry analysis. (E. denotes eluate)

3.9. Reciprocal IP:

The interaction between Hemgn and the interacting proteins that were identified using Mass Spectrometry was validated by using IP – western blot. Some of the proteins were further validated using reciprocal IP and western blot. Immunoprecipitation was performed using antibodies for the interacting protein and the presence of Hemgn in the pull down was tested by using Western blot. IP was performed with G9a (Ehmt2), GLP

(Ehmt1), Med1, Med12 (Ref. Table 1 for Mass Spectrometry data) and their corresponding Mock IgG IP with Benzonase treated MEL Nuclear Extract. Hemgn was found to be enriched only in the IP of protein of interest and not in the corresponding mock IgG IP that were performed in parallel in similar conditions (Fig. 12). Therefore, interaction of Hemgn with members of the G9a-GLP complex and Mediator complex as identified by Mass Spectrometry is confirmed using Reciprocal IP.

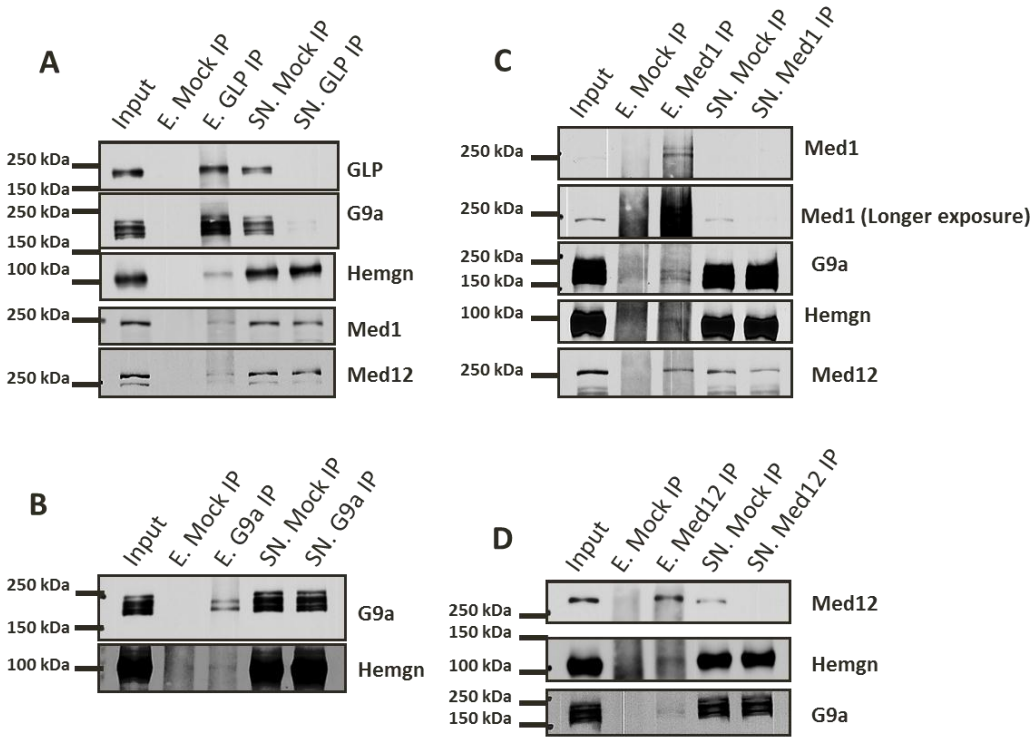


Figure 12. Validation of Hemgn Interactome using Reciprocal IP. (A-D) IP was performed with antibodies against the interacting proteins and probing for Hemgn using Western blot. On performing G9a, GLP, Med1 and Med 12 IP (A-D respectively), Hemgn was found to be enriched in all the IP in comparison to their corresponding Mock IgG IP using MEL Nuclear extract (growth condition, MEL 0 day) treated with Benzonase. Input, elutions from mock IgG IP and Portein of Interest IP and the Nuclear

extract supernatant after Mock IgG IP and Protein of Interest IP were loaded on the gel (Left to Right). (E. denotes eluate and SN. denotes supernatant.

3.10. Size Exclusion Chromatography:

Our mass spectrometry studies revealed several interesting proteins to interact with Hemgn. The complex that Hemgn forms with some of these proteins identified by IP – mass spectrometry studies and reciprocal IP was further characterized on the basis of their migration profile using size exclusion (or gel filtration) chromatography. Differentiated MEL nuclear extract was loaded onto a gel filtration chromatography column and western blot was used to visualize the elution profile of proteins of interest. The major fraction of G9a, GLP, Med1 and Med 12 migrated as bigger complex peaking between 2000kDa and 669kDa standard proteins (Fig. 12A). Though Hemgn co-migrates with the interacting proteins that were studied, the major peak of Hemgn migration was observed to occur between 669kDa and 75kDa Standard marker. Hence, a major fraction of Hemgn forms smaller complexes. Coomassie staining was performed on SDS Polyacrylamide gel (Data not shown) where all the fractions collected from Gel filtration column was loaded as described in Fig 13. Several other proteins were also found to co-migrate at fractions where majority of Hemgn was found to migrate but the identity of these proteins and their interaction with Hemgn are not known.

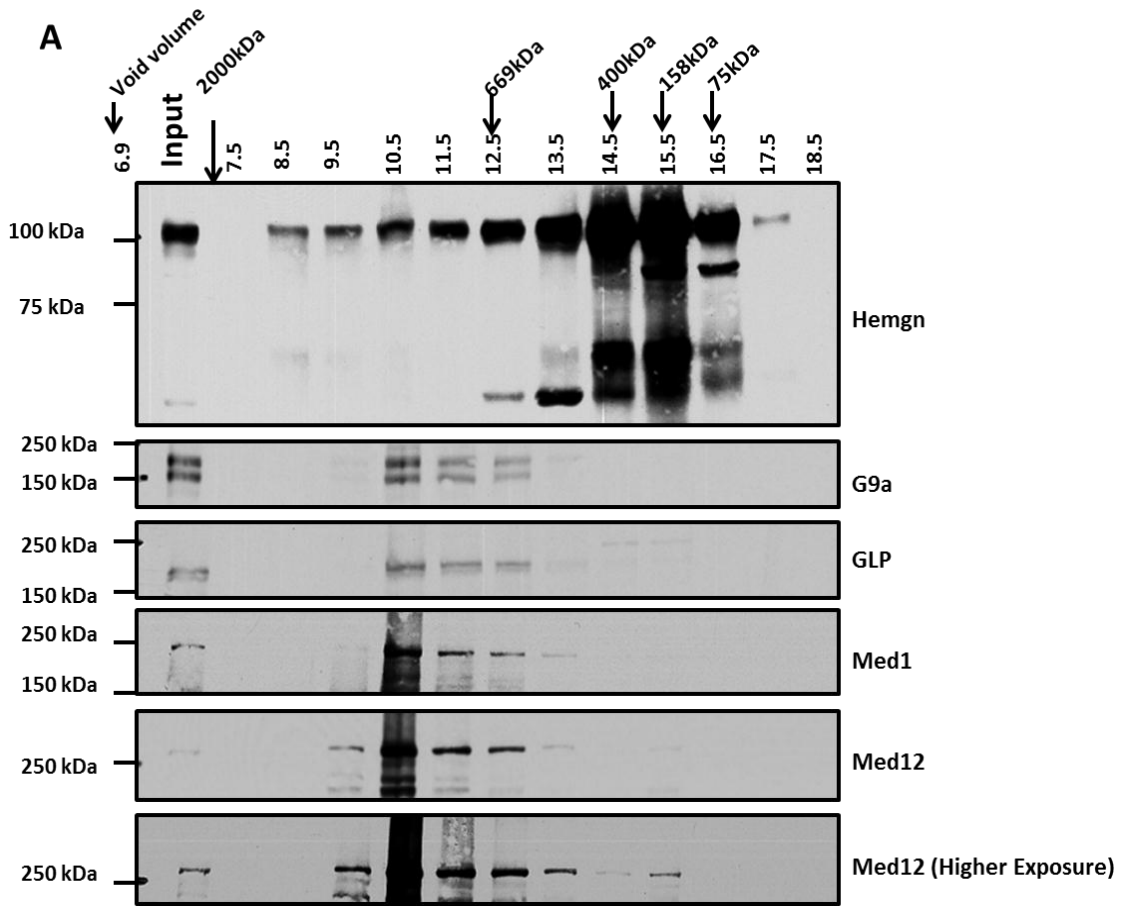


Figure 13. Migration of Hemgn and interacting proteins on Gel filtration Chromatography. Differentiated MEL Nuclear Extract was loaded on to the Gel filtration column. The arrows indicate the migration of standard proteins and the molecular weights of the known standard protein are mentioned above the arrow. The numbers above each lane indicate the volume at which the fraction was collected. The eluate was collected as 0.5 ml fractions each. The migration of pattern of various proteins was observed by performing western blot with their respective antibody.

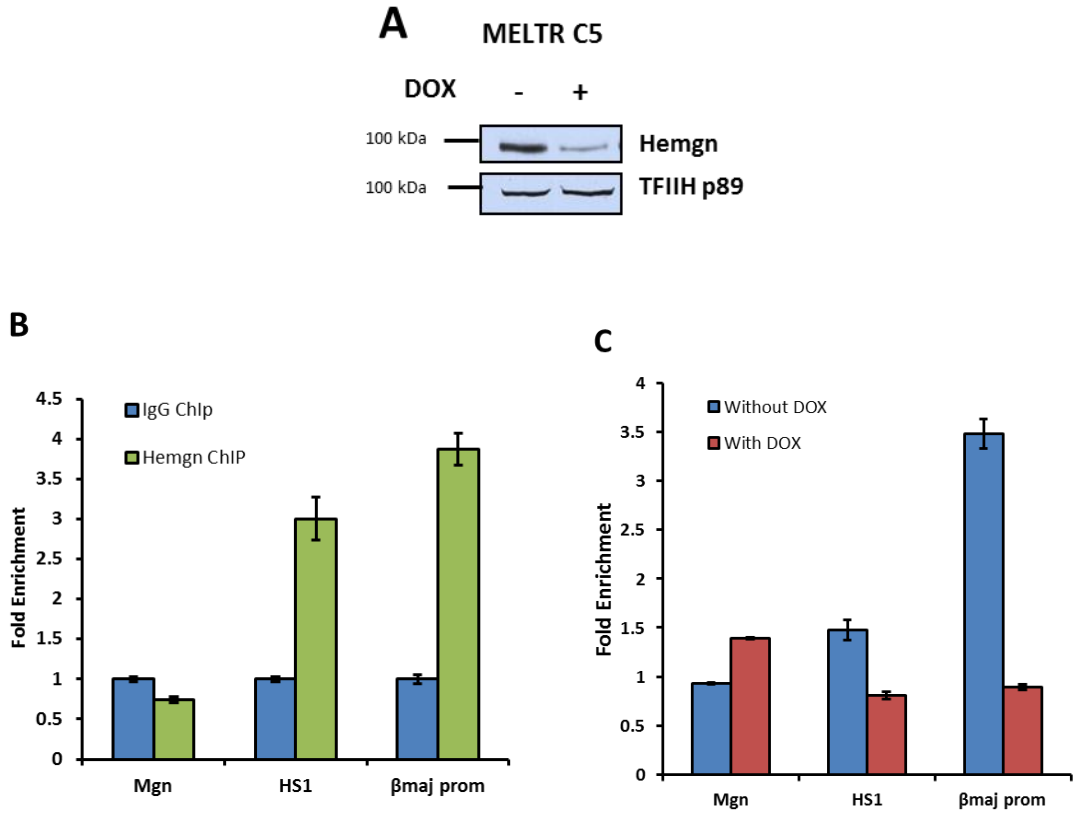
3.11. Hemgn Chromatin Immunoprecipitation:

Knockdown of Hemgn has a significant effect on the hemoglobin expression as inferred from previous Benzidine Staining experiment (Fig 9B-D). G9a and GLP that were found

to interact with Hemgn play an important role in Hemoglobin synthesis by regulating transcription at the β-globin locus by binding to the chromatin and modifying the histones (Chaturvedi et al., 2009). Therefore, to study the localization of Hemgn over chromatin, we used Chromatin immunoprecipitation and probed over the β-globin locus using Realtime qPCR for possible binding. Using myogenic specific gene Myogenin promoter region as a negative control region, we observed enrichment of Hemgn on different regions of the β-globin locus (Hypersensitive sites and gene promoter) in comparison to mock Rabbit IgG ChIP in cross linked MEL cells cultured in growth conditions (Fig. 14B). To test the specificity of our antibody for ChIP, we performed ChIP in the Hemgn knockdown clones with and without DOX induction (Fig 14C). The enrichment of Hemgn over the β-globin locus in comparison to Mock ChIP decreased when the clones were induced with DOX. Therefore, 9474p antiserum is efficient and specific for Hemgn chromatin Immunoprecipitation.

The murine β-globin locus is organized into a highly ordered three-dimensional structure that spans about 100kbp. It consists of a Locus Control region (LCR) that contains 6 hypersensitive regions and is present at about 40-60 Kbp upstream of globin genes (Fig 1E). The LCR plays a major role in the regulation of globin transcription and transcription factors are recruited to the promoters through the LCR (Tolhuis et al., 2002). Moreover, Hemgn knockdown in differentiating MEL cells results in a reduced percentage of Benzidine positive cells. Therefore, Hemgn chromatin Immunoprecipitation was performed in both undifferentiated and differentiated MEL cells to investigate differences in Hemgn binding over the β-globin locus during differentiation. The ChIP-qPCR experiments suggests that Hemgn is enriched in both the

LCR regions and the promoter region of genes of the β-globin locus during both differentiated and undifferentiated MEL cells, in comparison to the enrichment of Hemgn over Myogenin promoter. However, increased recruitment of Hemgn is observed at the promoter regions of genes present in the β-globin locus in MEL cells upon differentiation in comparison with the rest of the regions of the β-globin locus studied (Fig 14D). About two fold increase is observed in Hemgn recruitment at the embryonic gene e_y promoter and adult gene βmaj promoter during differentiation.



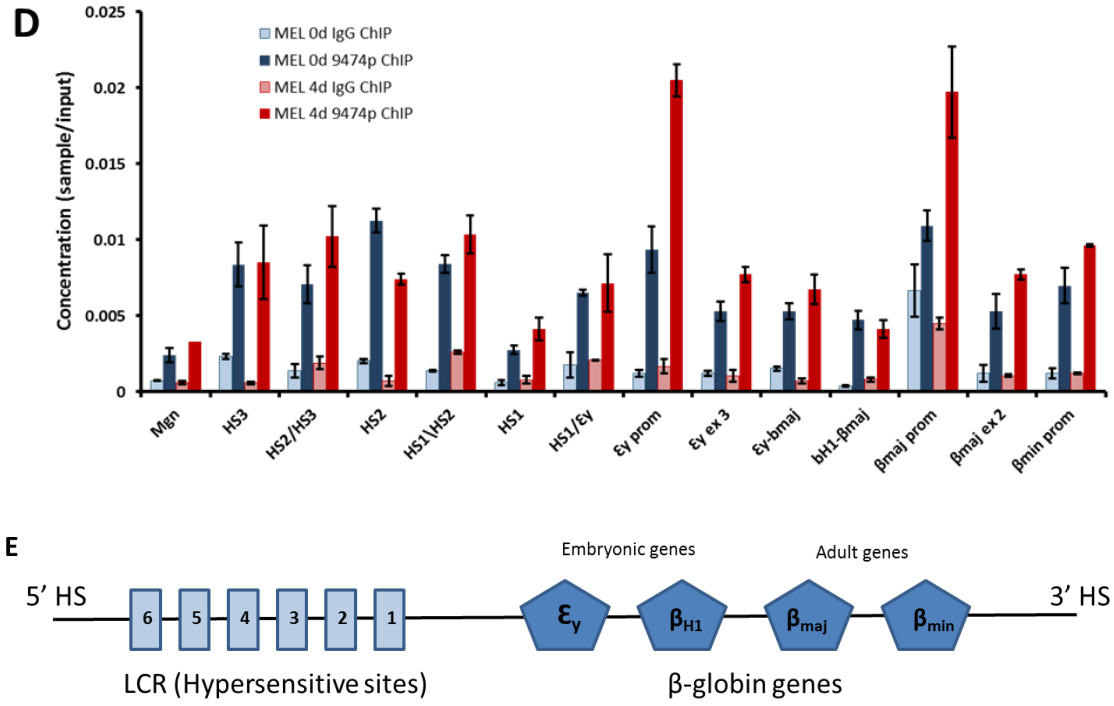


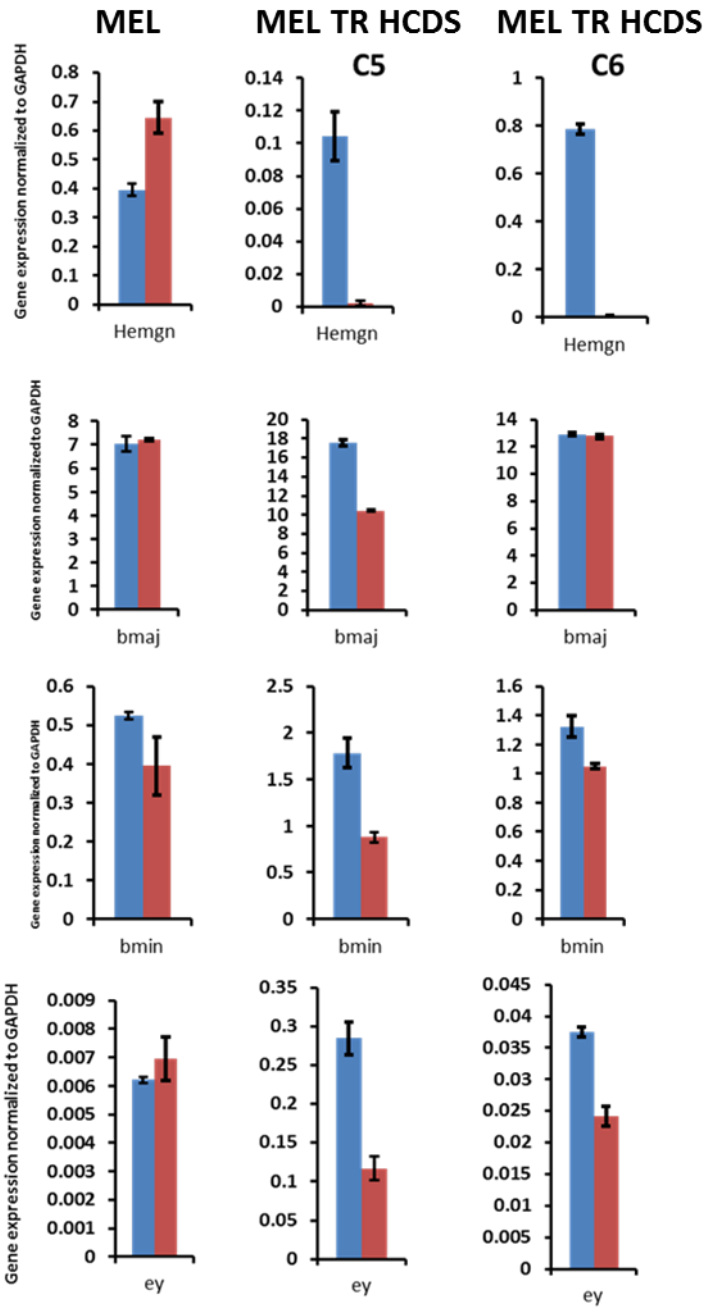
Figure 14. Hemgn is recruited on the β-globin locus. (A) Knockdown of Hemgn in MELTR clone 5 was induced by addition of DOX and the knockdown at the protein level was checked by using western blot. TFIIH p89 was used as a loading control. (B) ChIP was performed using 9474p Hemgn antisera using crosslinked MEL cells. IgG Mock ChIP was performed in parallel. The enrichment over Myogenin promoter (Mgn) and various regions of the β-globin locus (Hypersensitive region, HS1 and promoter region of βmaj gene, βmajprom) were studied using Realtime qPCR. (C) Hemgn Knockdown clone C5 was treated with and without DOX, crosslinked and ChIP was performed with 9474p and Mock IgG antibody. There was a decrease in the enrichment of Hemgn on β-globin loci and no difference in enrichment was found on the negative control, Mgn, validating antibody for Hemgn ChIP. (D) Hemgn (9474p) ChIP and Mock IgG ChIP were performed with crosslinked MEL (Blue) and Differentiated MEL cells (Red) and

enrichment was studied in different places of β-globin locus (HS represents Hypersensitive Sites in the β-globin locus, prom reperesents the promoter regions and ex. represents the exons, / and – represents the intermediate regions) taking Mgn as the negative control. (E) Schematic representation of the β globin locus.

3.12. Effect of Hemgn on β-globin transcription:

The Hemgn ChIP assay and Benzidine staining results revealed that Hemgn binds to the β-globin locus and may directly regulate its transcription. Since Benzidine staining is not quantitative, we performed mRNA isolation, cDNA preparation and real time quantitative PCR (RTqPCR) in Hemgn knockdown clones with and without DOX induction to probe into the effect of Hemgn on the transcriptional regulation of the β-globin genes. MEL cells with the presence and absence of DOX was used as a negative control to take the effect of DOX on the cells into consideration. Constitutively expressed gene, GAPDH, gene expression values were used to normalize any variation between the samples. Using Hemgn as a positive control, we observed more than 95% knockdown in the expression of Hemgn at the mRNA level only in the clones with DOX induction whereas no significant decrease was observed in the parental MEL cell line in the presence of DOX (Fig. 15). e_y gene is an embryonic globin gene that is poorly expressed in the MEL cells. A Significant decrease in the level of e_y gene expression was observed in both the clones whereas no significant decrease was observed in the parental MEL cells on DOX induction. A significant decrease was observed in both the adult globin gene, βmin and βmaj, transcription in clone 5 whereas there was no significant difference in the βmaj gene expression level in clone 6 and parental MEL control on DOX induction. Since the level of Hemgn knockdown is greater in clone 5 in comparison with the clone 6, the

decrease in βmaj transcription is greater in clone 5 or the decrease in βmaj transcription in clone 5 may also be non-specific (due to clonal effect). There was a decrease in the βmin gene expression in both MEL and clone 6 to similar extent. From gene expression analysis at the transcript level, Hemgn knockdown appears to affect the transcription of the β-globin genes.



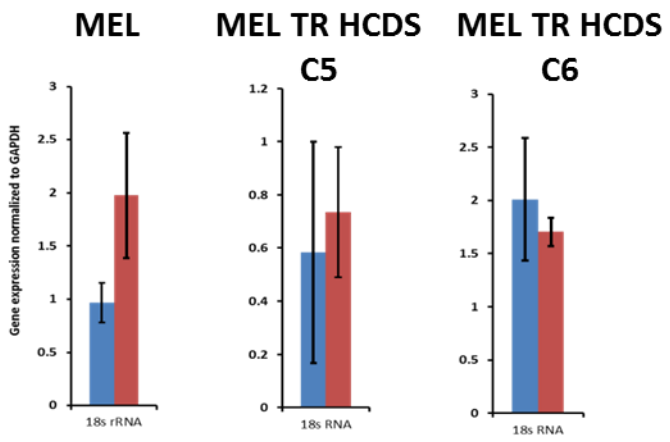


Fig 15. Effect of Hemgn knockdown on the transcriptional regulation of β-globin locus. The knockdown of Hemgn was induced in clones C5 and C6 using DOX. Parental MEL cells were used as a negative control. Expression of Hemgn and β-globin genes βmaj, βmin and e_y were studied at the transcript level. GAPDH, a constitutively expressed gene, transcript level was used to normalize the expression between different samples. Constitutive gene 18s RNA expression was used as a negative control.

3.13. Hemgn - Histone interaction:

Benzonase treatment was included in our standard MEL Nuclear extract protocol to extract chromatin-associated proteins. The protein extraction profile in nuclear extracts treated with different amount of Benzonase and with different incubation time periods for enzymatic activity were studied using Western blot (Supplementary Fig. 14). Various Histones such as H2A, H2B, H3 and H4 were observed to be enriched in the nuclear extracts with benzonase treatment whereas the amount of Hemgn extracted remained comparable in all the different samples. Hemgn IP with the nuclear extracts with and without benzonase treatment was performed, in parallel, to compare the amount of histones immunoprecipitated. There was a significant increase in the amount of histone

pulled down with Benzonase treatment (Fig. 16A). This increase in Histone pull-down may be due to higher amount of histones in nuclear extract on Benzonase treatment. In order to characterize the Hemgn- Histone complex further we performed Hemgn IP with Benzonase treated Nuclear Extract and washed them with buffers containing increasing salt concentration (Fig. 16D). The amount of histone pull down decreased significantly when washed with buffer containing salt concentration greater than 500mM. Hence, Hemgn and histones forms a weak complex that dissociates with higher salt concentrations.

In addition to histone enrichment in the nuclear extract, benzonase activity may also result in higher amount of DNA free histones as a result of its nuclease activity. Histones are organized as nucleosomes which are wound by DNA. Benzonase nuclease activity may result in digestion of DNA wound around the histone octamer exposing surface that may interact with Hemgn. In order to study the substrate specificity of Hemgn core complex (with respect to presence and absence of DNA interaction with histones), we further characterized histone-Hemgn interaction using MEL nuclear extract devoid of Benzonase treatment and pre-incubated it with Histone extract (mimics Nuclear extract containing DNA free histones) as an input for Hemgn IP. When normalized based on the Hemgn content of the IP input, the histones exogenously added to the nuclear extract did not result in any significant increase in the amount of histones present in the nuclear extract (endogenous histones) when visualized by western blot (Fig. 16B). On Hemgn IP, the amount of histones pulled down after addition of DNA free histones to MEL NE was several folds higher. When DNA was extracted from equal volume of nuclear extract and histone extract used in the previous experiment, the amount of DNA present was

relatively higher in the nuclear extract whereas no visible traces of DNA were found in the histone extract ensuring that Hemgn can interact with the histones even in the absence of DNA (Supplementary Fig. 19). We speculate that the endogenous histones, present in Nuclear extract devoid of Benzonase treatment, exist as nucleosomes since the DNA extracted from the nuclear extract when electrophoresed on an agarose gel shows characteristic nucleosome laddering pattern. Therefore, Hemgn complex can interact with histones in the absence of DNA.

To further investigate on the interaction of Hemgn with DNA free histones, we incubated the DNA free histones with immunoprecipitated Hemgn. During our preliminary studies, we observed that Hemgn loses interaction with most of the proteins when treated with high salt concentration buffers (500mM or more, Supplementary Fig. 16). We also observed that Histone – Hemgn complex dissociates at higher salt concentrations. Hence we washed Hemgn IP with 500mM KCl containing wash buffer and performed silver staining and western blot to ensure the dissociation of histones and other weakly interacting proteins (Supplementary Fig 18). Then the core complex of Hemgn which survives 500mM KCl salt wash was then incubated with histone extract. DNA free histones were pulled down with the Hemgn core complex specifically whereas no visible amount of histones was pulled down with the Mock IgG IP that was treated similarly in parallel (Fig 16C). The Hemgn core complex may interact with DNA free histones.

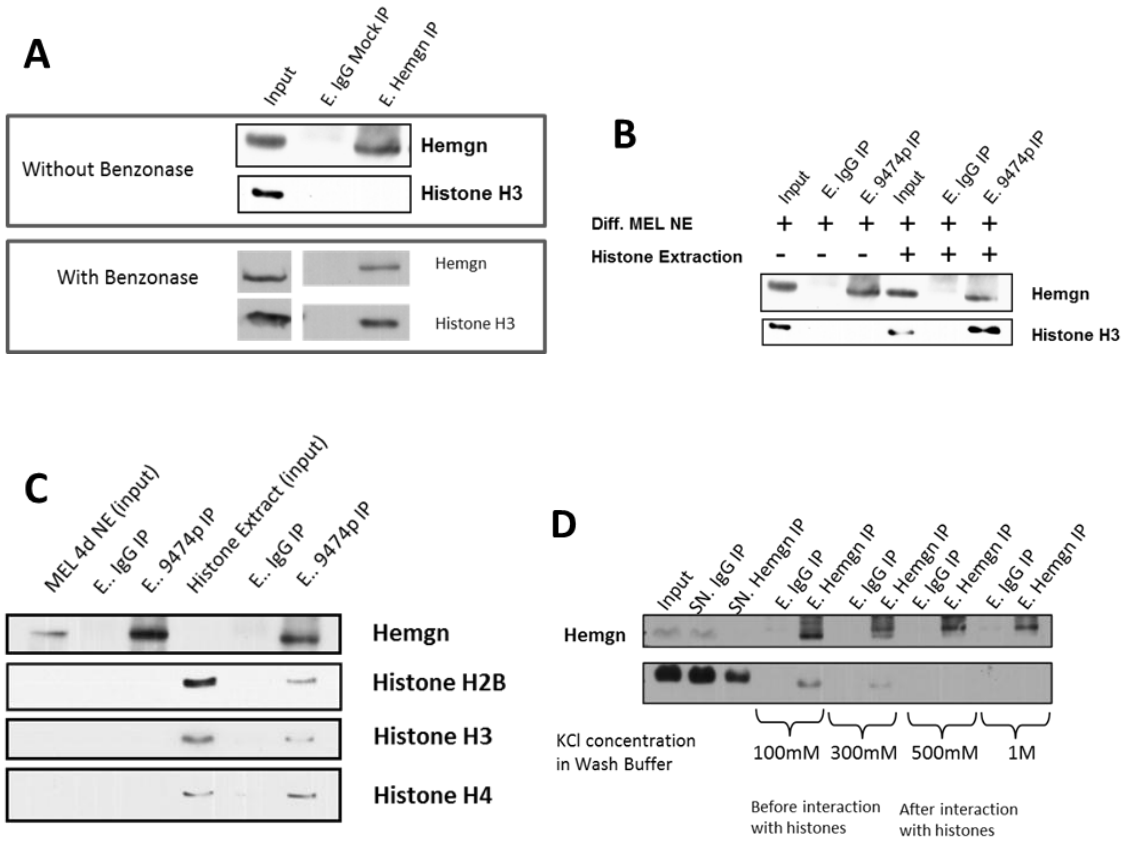


Figure 16. Hemgn interacts with DNA free histones. (A)Hemgn IP was performed with MEL Nuclear extract with (bottom) and without (top) Benzonase and enrichment of histones was specifically found in Hemgn IP with Nuclear extract with Benzonase treatment. (B) Histone enrichment was found in Hemgn IP when Acid extracted histones were added to the Nuclear extract in the input. (C) Histone extract (Lane 4 from left) was added to Mock IgG and Hemgn antibody pull down after washing the pull-down with high salt stringency wash buffer(IP500, Lanes 2 and 3 from left). Using Western blot, Histones were pulled down specifically with the Hemgn IP.(D) Hemgn IP was performed with Benzonase treated MEL Nuclear extract and washed with increasing salt stringency in wash buffer. Hemgn histone complex dissociated with higher salt (more than 500mM) strigency wash.

4. DISCUSSION

Hemgn is a 55kDa nuclear protein that is expressed in the hematopoietic tissue and testis (Yang et al., 2001a, Yang et al., 2001b, Lu et al., 2001, Yang et al., 2003). In the hematopoietic tissue, Hemgn is reported to be highly expressed in Hematopoietic stem cells and its' expression is down-regulated upon differentiation (Yang et al., 2006). Our previous studies in human cord blood suggest that EDAG, the human homolog of Hemgn, shows a biphasic pattern of expression during *ex vivo* erythropoiesis. During early hematopoiesis, Hemgn is highly expressed in $CD34^{+}$ $CD38^{-}$ cells (Multipotent precursor cells) and its expression is comparatively lower in $CD34^{+}CD38^{+}$ cells (Oligopotent precursors) at the transcript level. During further differentiation, there is an up-regulation of Hemgn expression in the erythroid lineage restricted precursor cells. Towards the terminal differentiation, by which enucleated mature erythrocytes are formed, there is a down-regulation of Hemgn expression (Palii CG., and Brand, M., unpublished data). The role of Hemgn in both the early progenitor cells and lineage restricted progenitor cells remains poorly characterized. In this report, we use a proteomic approach to characterize Hemgn and its role in the terminal differentiation of erythrocytes using Murine Erythroblast Leukemia cells (MEL cells).

4.1. Hemgn core complex interacts with free histones:

EDAG, the human homolog of Hemgn, is a positive regulator of erythroid and megakaryocytic differentiation (Ding et al., 2010). Our knockdown studies in MEL cells revealed that Hemgn can act as a positive regulator of erythroid differentiation. But the molecular function of Hemgn in regulating erythropoiesis is not well understood. The

amino acid sequence of Hemgn, when subjected to Motif analysis using SCANSITE or other motif analysis tools, does not reveal any conserved motif that allows prediction of molecular function. Interestingly, the mouse and human homolog of Hemgn show only 43% identity with the nuclear localization signal (NLS) domain and a coiled-coil domain responsible for dimerization being highly conserved. The Rat homolog RP59 shares 70% homology with murine Hemgn having almost identical NLS and coiled-coil domain (Yang et al., 2001). Previous studies in the lab revealed that Hemgn localizes in the nucleus in both differentiated and undifferentiated MEL cells. Though the NLS is highly conserved among the human, murine and rat homologs of Hemgn, no conserved histone or DNA binding domains were found. Hemgn is organized as a protein with a N-Terminal basic domain (34-78 residues, net charge +15) and a C-Terminal acidic domain (450-480, Net charge -11). The presence of an acidic domain in Hemgn is a feature highly characteristic of Histone chaperones, a family of proteins that interact with Histones. They are characterized by stretches of acidic amino acids as a common theme in spite of having no sequence similarity among each other (Das et al., 2010).

During our nuclear extraction procedure, we observed higher enrichment of histones in the nuclear extract when treated with Benzonase, an enzyme with secondary structure independent DNase and RNAse activity, whereas the amount of Hemgn extracted remained comparable (Supplementary Fig. 14). Surprisingly, the histones pulled down with Hemgn during Hemgn IP increased significantly with Benzonase treatment (Fig. 16A). This drastic increase in the association of Histones with Hemgn may either be attributed to an increase in the amount of histones in the extract or an increase in the amount of DNA free histones in the extract due to the nuclease activity of benzonase.

When the nuclear extract, which was not treated with Benzonase, was pre-incubated with DNA free histones before performing Hemgn IP, a high amount of histones were pulled down in comparison with the pull down performed with nuclear extract alone eventhough the amount of histones in comparison to Hemgn is almost the same in both inputs (Fig 16B). The histones in the histone extract added were ensured to be in DNA free conformation by extracting DNA from it and visualizing it on an agarose gel (Supplementary Fig. 19). Hence, we speculate that the majority of the histones in the nuclear extract may be present in nucleosomal conformation whereas the histone extract contains primarily DNA free histones. Hemgn complex may interact more efficiently with DNA free histones. We also speculate that the addition of Benzonase, during Nuclear extraction, digests the majority of RNA and DNA associated with proteins thereby resulting in release of histone octamers from the nucleosomes. Since Hemgn interacts with DNA free histones, the increase in histone pulled down during Hemgn IP with Benzonase treated nuclear extract can be explained to be a result of Hemgn binding to DNA free histones.

Many histone chaperones interact with their substrates with weak affinity (Das et al., 2010). When the Hemgn complex immunoprecipitated was treated with increasing concentration of salt, Histone-Hemgn complex dissociated at higher salt concentrations (500mM and more, Fig. 16D). Therefore, DNA free histones can interact with the Hemgn core complex but with weak affinity. When immunopurified Hemgn from MEL Nuclear extract was incubated with histone extract, histones were pulled down (Fig. 16C). Since the purity of the immunopurified Hemgn was not assessed, the nature of the interaction between Hemgn and histones cannot be termed definitely as direct or indirect interaction.

Using Recombinant Hemgn and histones, the direct interaction studies can be performed to study the nature of interaction and the substrate specificity of Hemgn. *In vitro* assembled nucleosomes can also be used as a negative control in similar studies to further confirm the specificity of Hemgn interaction with DNA free histones.

4.2. Hemgn interacts majorly with Chromatin modifying proteins:

MEL cells are erythroid lineage committed transformed cells that are highly proliferative and can be induced to differentiate with DMSO (Levenson et al., 1979, Conscience et al., 1977). Our previous studies in MEL cells identified Hemgn as a MafK interacting protein that exhibits increased association with MafK on differentiation (Brand et al., 2004). Ctbp1, a transcription corepressor (Jeffrey D. Hildebrand and Philippe Soriano, 2002), was also identified to directly interact with human homolog of Hemgn, EDAG (Wang et al., 2011). In our study, using a mass spectrometry based proteomic approach; we have identified several proteins that interact with Hemgn either directly or indirectly in both differentiated and undifferentiated MEL. Ctbp1, which was identified to interact with EDAG, was also found in our list of proteins identified by mass spectrometry to interact with Hemgn in both differentiated and undifferentiated conditions (Data not shown). Ctbp1 acts as a co-repressor by its interaction with three different chromatin modification mediating complexes – histone Deacetylases (HDACs), histone lysine methyl transferase (like G9a/GLP complex) and histone lysine demethylases (like LSD1) (Shi et al., 2004, Tachibana et al., 2005). CtBP1 can also act as a platform for SUMOylation of other factors (Kuppuswamy et al., 2008). From our mass spectrometry study, we identified several members of the SUMOylation machinery (Table 1) and histone lysine methyl transferases and demethylases. When the aminoacid sequence of Hemgn was subjected to

Eukaryotic Linear Motif software ELM (Dinkel et al., 2011), CtBP interacting motif PxDLS was found between 418-422 aminoacid residues. This suggests that CtBP1 may directly interact with murine Hemgn.

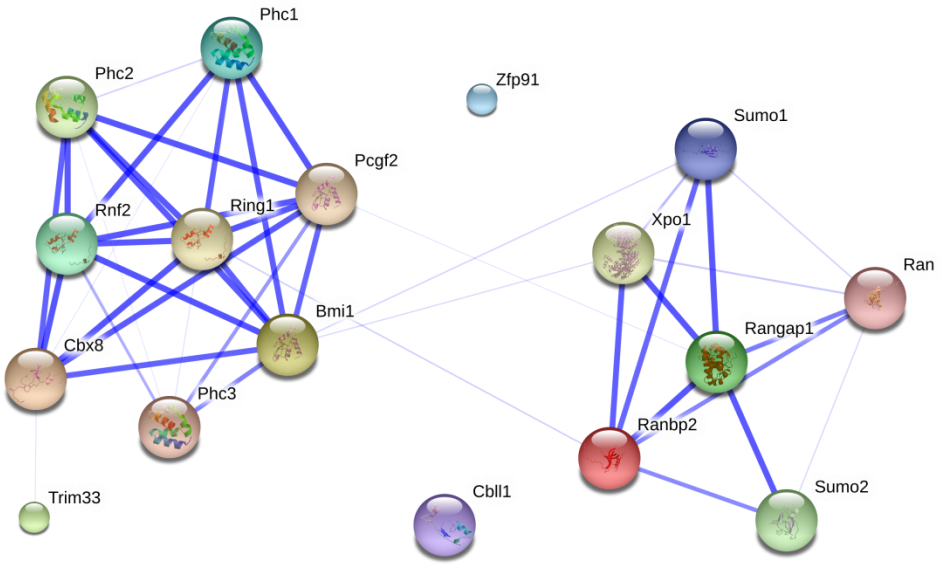


Figure 16. Proteins complex that acts as a SUMOylation machinery that were identified to interact with Hemgn with Mass Spectrometry (Data not shown).

From our study, we identified a large number of proteins to interact with Hemgn. When Hemgn immunoprecipitated proteins were treated with higher salt containing wash buffers (500mM or more), they dissociate from Hemgn core complex (may or may not contain proteins other than Hemgn). Therefore, the majority of the proteins in the Hemgn interactome interacts with Hemgn with weak affinity. When we classified the protein based on the function using GO based tool DAVID (Huang et al., 2009), we found a large fraction of the proteins to be associated with transcriptional regulation. To include as many proteins as possible we listed proteins that were identified to specifically interact

with Hemgn with a probability of greater than 0.8 and having at least 1 unique peptide. Common contaminants were removed from the list. When the protein lists identified in differentiated and undifferentiated condition were subjected to Gene Ontology (GO) based classification (Table 2), 151 (from a list of 571 proteins in total) and 131 proteins (from a list of 493 proteins in total) were identified to be associated with transcriptional regulation in undifferentiated and differentiated conditions respectively. Many other proteins were also found to be associated with Chromatin organization. 68 and 64 proteins were found to be associated with Chromatin organization in Undifferentiated and Differentiated conditions respectively. When proteins that were identified in both differentiated and undifferentiated conditions were subjected to GO based Gene function classification, about 24% of the proteins identified were associated with transcriptional regulation and 14% were identified to be associated with chromosome organization. Though many of the transcriptional regulators have DNA dependent transcriptional activity, a significant fraction of the proteins also have chromatin modifying activity. The mass spectrometry data was also analyzed using MASCOT algorithm. Most of the proteins identified by SEQUEST algorithm (Eng et al., 1994, Qian et al., 2005) were also identified using MASCOT algorithm. A Significant proportion of MASCOT analyzed data are associated with transcriptional regulation activity.

			common				4 day				0 day			
Term	Transcription activator activity	transcription repressor activity	covalent chromatin modification	chromosome organization	transcription regulation	\transcription activator activity	transcription repressor activity	covalent chromatin modification	chromosome organization	transcription regulation	transcription activator activity	transcription repressor activity	covalent chromatin modification	chromosome organization	transcription regulation
Count	8	10	13	33	66	16	20	23	64	114	17	17	20	68	127
%	3.49345	4.366812	5.676856	14.41048	28.82096	3.361345	4.201681	4.831933	13.44538	23.94958	3.10219	3.10219	3.649635	12.40876	23.17518
P Value	0.056958	0.001999	4.51E-09	1.64E-16	8.58E-20	0.005111	5.31E-06	6.10E-15	7.22E-31	3.17E-26	0.006681	8.55E-04	1.34E-10	6.43E-30	2.13E-27
List Total	177	177	180	180	218	364	364	365	365	450	411	411	432	432	522
Pop Hits	259	211	96	404	1546	259	211	96	404	1546	259	211	96	404	1546
Pop Total	13288	13288	13588	13588	17854	13288	13288	13588	13588	17854	13288	13288	13588	13588	17854
Fold Enrich	2.318871	3.557983	10.22245	6.166172	3.496339	2.255166	3.460236	8.919064	5.897409	2.925623	2.122105	2.604859	6.552855	5.294188	2.809696
Bonferroni	0.999998	0.361233	3.71E-06	9.15E-14	1.35E-17	0.838657	0.001888	7.36E-12	8.71E-28	7.62E-24	0.913436	0.268203	1.85E-07	8.88E-27	5.29E-25
Benjamini	0.336687	0.026021	3.09E-07	4.57E-14	2.69E-18	0.043518	1.18E-04	9.20E-13	8.71E-28	1.52E-24	0.050729	0.007975	6.85E-09	4.44E-27	8.81E-26
FDR	52.70037	2.522135	6.95E-06	1.67E-13	1.03E-16	6.784862	0.007279	9.88E-12	1.17E-27	4.10E-23	8.812991	1.170424	2.20E-07	1.06E-26	2.77E-24

Table 2. GO based Functional Classification of proteins that were identified using Mass Spectrometry to interact with Hemgn.

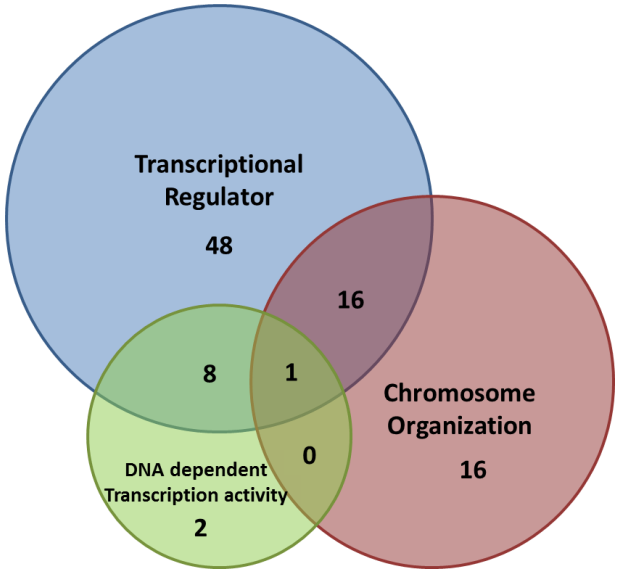


Figure 17. Venn diagram representing the GO based Functional Classification of transcriptional regulator proteins that were found to interact with Hemgn in both differentiated and undifferentiated MEL.

Our proteomic screen for Hemgn interacting protein identified G9a and several members of the G9a-GLP complex to associate with Hemgn in differentiated MEL cells (Table 1). G9a, otherwise known as Ehmt2, is a N-Lysine methyltransferase that methylates H3K9 and H3K27 in the euchromatic regions (Tachibana et al., 2002, Wu et al., 2011). G9a forms homodimers or heterodimerizes with G9a-like protein (GLP or Ehmt1) (Tachibana et al., 2005) and brings about epigenetic modifications to the chromatin locus that it binds to. Its methyltransferase activity is responsible for the establishment of repressive marks on the Histone H3. But our previous studies have established that the G9a complex can also have histone methyltransferase independent activity which enables it to act as a co-

activator of transcription. G9a is also known to interact with p45, which is a member of the NFE2 core complex, with which Hemgn was previously identified to interact with. Moreover, G9a-GLP complex plays an important role in terminal differentiation by regulating β-globin expression during MEL differentiation (Chaturvedi et al., 2009). Therefore, we performed Hemgn IP and confirmed its interaction with G9a and GLP by using Western Blot in both differentiated and undifferentiated MEL cells (Fig. 10). To further validate the interaction, we performed Reciprocal IP with G9a and GLP antibody in MEL cells and confirmed the presence of Hemgn in G9a and GLP IP using Western blot (Fig. 11A-B).

Our Mass Spectrometry studies also revealed that Hemgn can interact with members of the mediator complex (Data not shown). We confirmed the interaction between Mediator complex and Hemgn using reciprocal IP with Med1 and Med 12 and performing Hemgn WB (Fig. 11C-D). Mediator complex interacts with major fraction of G9a (Ding et al., 2008, Chaturvedi et al., 2009). G9a and mediator complex recruitment at the adult globin genes in the β-globin locus takes place during DMSO induced differentiation of MEL resulting in the activation of gene expression (Chaturvedi et al., 2009). Gel filtration chromatography of Differentiated MEL Nuclear Extract reveals that Hemgn co-migrates with the major fraction of G9a complex and mediator complex (Fig. 12A). Conversely, a major fraction of Hemgn does not co-elute with the major fraction of G9a and Mediator complex. Hemgn migrates primarily as a smaller complex. Though Hemgn and G9a do co-elute, their migration profile in Gel filtration are different i.e., their peak or the migration of major fraction of protein are at different elution volumes. In addition to the histone modifying proteins, we also found several variants of histones H2A and H3 to

interact with Hemgn in our mass spectrometry data and confirmed the interaction using western blot in both conditions (Fig. 10).

EDAG, the human homolog of Hemgn, regulates hematopoiesis by regulating proliferation, differentiation and resists apoptosis through NFkB activation (Li et al., 2004). Hemgn expression in MEL cells is up-regulated when induced to differentiate with DMSO (Fig. 7). When Hemgn expression is knocked down during differentiation there is a significant decrease in the cell number. Moreover, the erythroid differentiation marker Hemoglobin, as visualized by Benzidine staining, was observed to be poorly expressed when Hemgn expression is knocked down during differentiation (Fig. 8B-D). Therefore, Hemgn plays a very important role during erythroid differentiation in MEL.

In a previous report, EDAG, human homolog of Hemgn was found to play an important role in the terminal differentiation of the myeloid cell line 32D by using both over-expression and knockdown strategies (Ding et al., 2010). A similar knockdown strategy in MEL cells was used in our study to phenotypically characterize the role of Hemgn in the murine erythroblast cell line, MEL (Levenson, R. and Housman, D., 1979). Knockdown of Hemgn in MEL cells inhibited hemoglobin synthesis when induced to differentiate with DMSO. Knockdown of G9a expression in differentiating MEL cells also results in inhibited globin production (Chaturvedi et al., 2009).

4.3. **Role of Hemgn at the β-globin locus**

The Murine β-globin locus is organized into a highly ordered three-dimensional structure that spans about 100kbp. It consists of a Locus Control region (LCR) that contains 6 hypersensitive regions and is present at about 40-60 Kbp upstream of globin genes. The

LCR plays a major role in the regulation of globin transcription and transcription factors are recruited to the promoters through the LCR (Tolhuis et al., 2002). Our previous studies on the β-globin locus showed that G9a is recruited to the β-globin locus in a p45 NF-E2 dependent manner and then G9a spreads over the β-globin locus (Chaturvedi et al., 2009). p45 along with MafK is an important part of the transcriptional activation complex NFE2 that transcriptionally regulates the globin expression in differentiated MEL cells. Moreover, Hemgn was first identified in our previous studies as a factor that shows increasing association with MafK after differentiation in MEL cells (Brand et al., 2004). Our study has revealed that Hemgn is also recruited to the β-globin locus (Fig 13). Knockdown of Hemgn results in depletion of Hemgn localization at the β-globin locus (Fig. 13C). On Hemgn knockdown, we observed a decrease in the expression of the adult β globin genes at the transcript level (Fig. 14). This explains at least partially the decrease in the percentage of Benzidine positive cells observed, since Benzidine marker stains Hemoglobin. Our observation is also in accordance with previous studies in the human erythroid cell line 32D, where over-expression of EDAG resulted in up-regulation of various adult as well as fetal globin genes. In 32D cell line, in addition to upregulation of β-globin genes, there is also an up-regulation in the expression of both adult and fetal alpha globin genes (Ding et al., 2010). Disruption of transcriptional activity at the α-globin locus may also contribute to the decrease in the percentage of Benzidine positive cells during Hemgn knockdown. This can be confirmed by studying the effect of Hemgn knockdown on the transcription of α-globin genes and the recruitment of Hemgn at α-globin locus using ChIP-qPCR.

Our study reveals that Hemgn is recruited to both the embryonic and adult β-globin gene promoters in MEL (Fig. 13D). Previous studies from our lab has shown that knockdown of G9a, that interacts with Hemgn and localizes over both the embryonic and adult genes, results in down regulation of adult β globin genes and re-activation of embryonic β globin genes (Chaturvedi et al., 2009). Knockdown of Hemgn expression in MEL negatively regulates expression of both the adult and embryonic genes. Therefore, Hemgn activity is independent of G9a histone methyltransferase activity at the embryonic genes of the β-globin locus. Embryonic genes are poorly expressed in MEL and possess repressive histone marks by virtue of activity of several chromatin modifying enzymes. But the effect of Hemgn on the histone marks at the β-globin locus is not known. A Native Chromatin Immunoprecipitation assay can be used to study the changes in the histone mark in the β-globin loci on Hemgn knockdown. We speculate that basal expression of embryonic genes exists in differentiated MEL cells due to the open chromatin structure of the β-globin locus. This basal level transcription at the embryonic globin gene could be regulated by Hemgn recruitment.

Though Hemgn interactome reveals that Hemgn interacts with transcriptional repressors and activators, our study suggests that Hemgn may positively regulate transcription at the β-globin locus. Nucleosomes, consisting of the histone octamer, act as a major hurdle to RNA polymerase during elongation (Bondarenko et al., 2006). Histones often regulate the expression of the genes that they are present on by - 1. recruitment of transcriptional regulators, 2. Altering the structure of the genomic loci, 3. Impairing cryptic transcription by preventing anomalous RNA polII recruitment 4. Acting as a barrier to RNA pol II passage. The eviction of histones and its re-deposition during transcription is required for

structural stability of the loci and maintenance of histone code which in turn regulate subsequent transcription (Ng et al., 2003, Carrozza et al., 2005). Chromatin remodeling factors and histone chaperone regulate transcription enabling histone eviction and deposition thus allowing the passage of RNA polymerase through the gene. Embryonic globin gene expression is repressed during differentiation of MEL. Surprisingly, Knockdown of Hemgn in MEL impairs the transcription of embryonic globin genes since embryonic genes are expressed poorly in MEL cells. But our Chromatin Immunoprecipitation assay however shows that Hemgn can bind to the embryonic gene, e_y promoter. Therefore we speculate that Hemgn may be associated with the basal transcriptional machinery. The ability of Hemgn core complex to interact with Histone further suggests that Hemgn may be involved in the chromatin remodeling or histone dynamics. From our study and based on the signature of the amino acid sequence, we propose a model for Hemgn activity (Fig .18). Hemgn may acts as a tissue specific histone chaperone and alter the histone dynamics at transcribed genes allowing the passage of the basal transcription machinery thus activating transcription. This transcriptional activation may directly or indirectly play an important role in erythropoiesis.

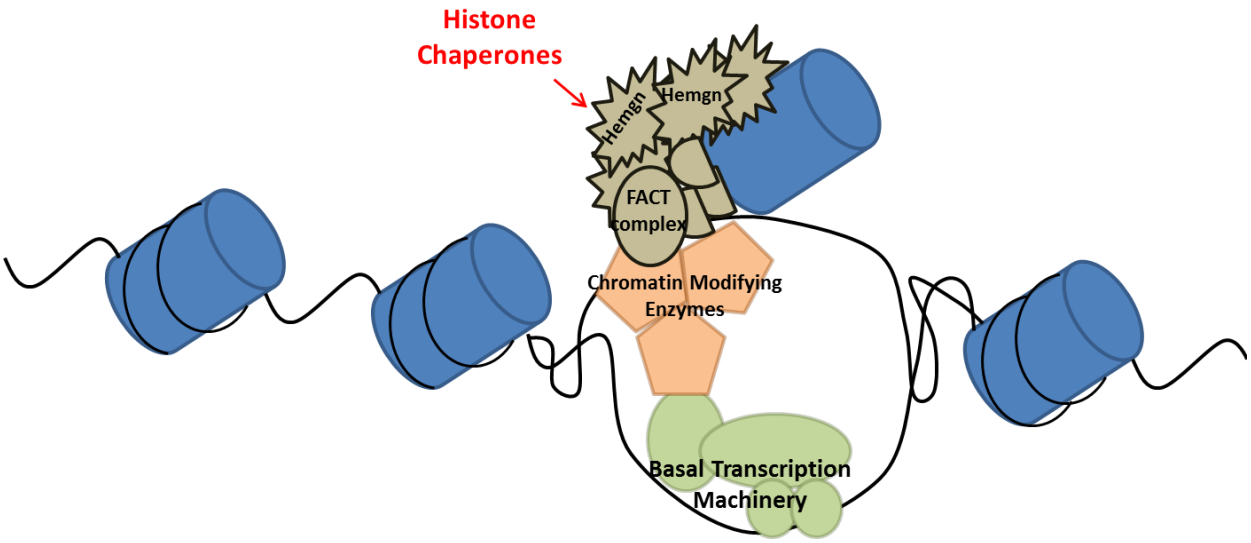


Figure 18. Proposed Model for Hemgn activity. Hemgn interacts with the basal transcription machinery directly or indirectly. It is recruited to the chromatin during transcription. Hemgn positively regulates transcription by interacting with DNA free histones and removing the nucleosomal barrier during transcription allowing the passage of RNA polymerase across the gene.

4.4. Conclusion:

Erythroid differentiation is a complex process that is regulated by several factors. From our study, we have observed that Hemgn may play an important role in the transcriptional regulation during erythropoiesis. Our proteomic screen has revealed several transcription factors, chromatin remodeling factors, chromatin modifying factors, transcriptional basal machinery and the transcriptional elongation machinery that are important during erythroid differentiation to interact with Hemgn. Moreover, we have observed that Hemgn can be recruited directly or indirectly to the chromatin at the β -globin locus. Knockdown of Hemgn resulted in perturbation of the normal β-globin genes

transcription. Moreover, the basal level transcription of the embryonic genes which were normally repressed in MEL cells was further reduced. Therefore, Hemgn may enable transcriptional activation at the β-globin locus. This transcriptional activity of Hemgn can be a result to the direct or indirect interaction of Hemgn with Histones. This transcriptional regulatory activity of Hemgn may play a key role in MEL cells differentiation. Further investigation is necessary to understand the role of the molecular mechanism of Hemgn in transcriptional regulation in detail.

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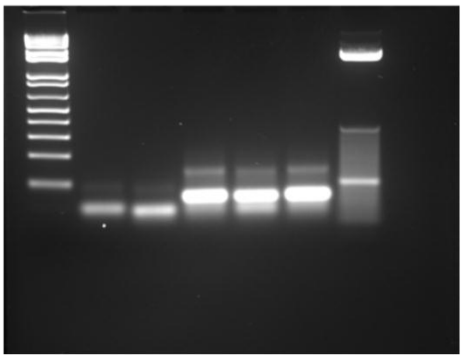
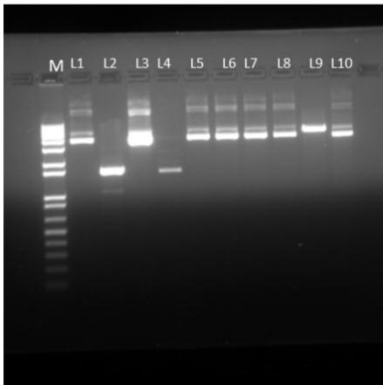
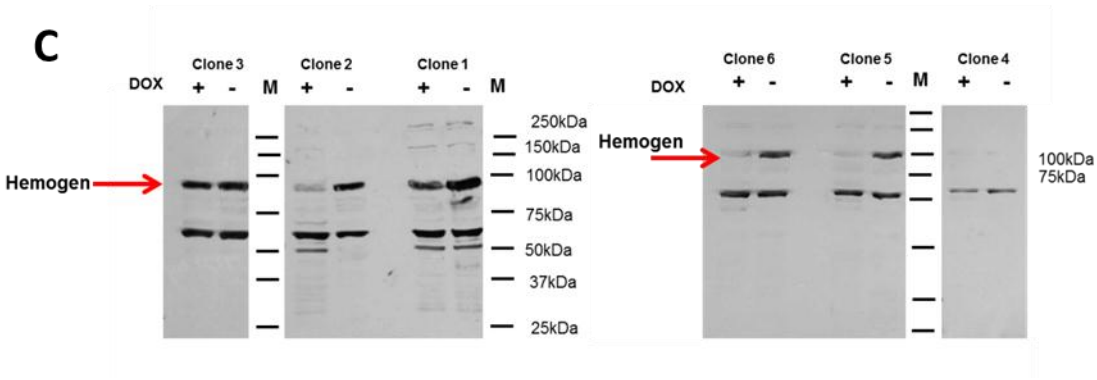
APPENDIX

A

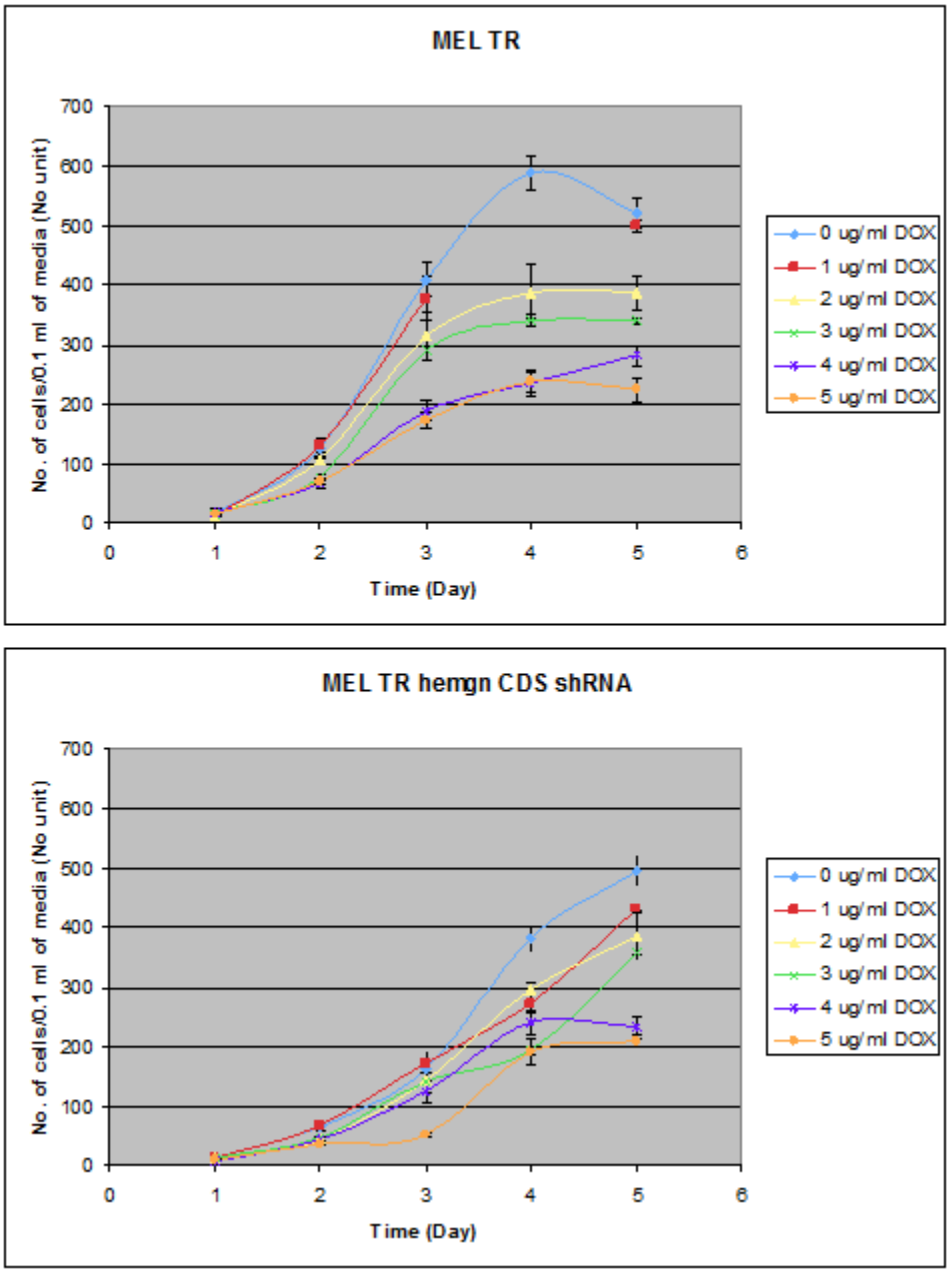


B

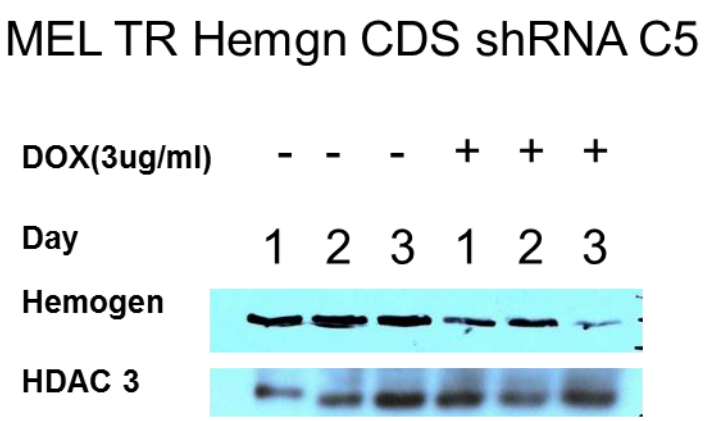




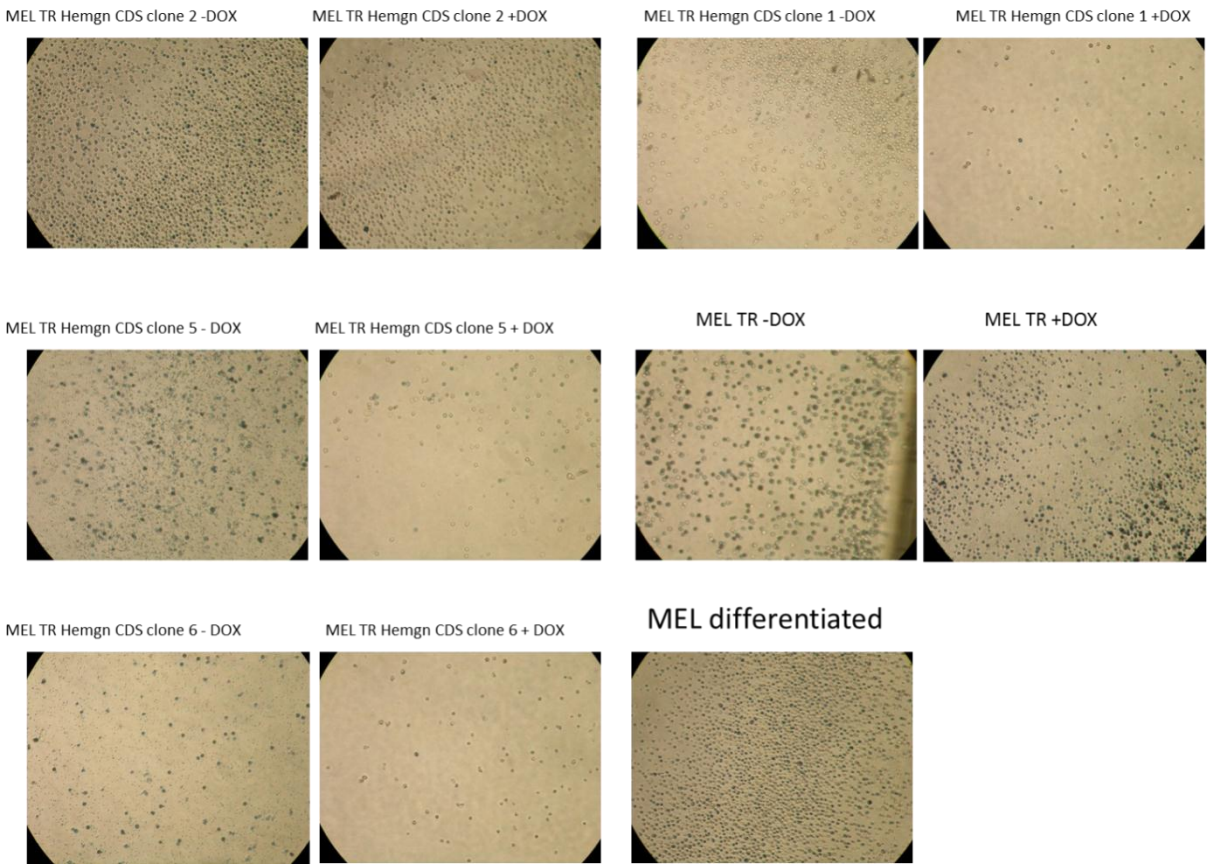
Supplementary Figure 1. (A) The efficiency of annealing of the complementary oligonucleotides was tested by running them on a 2% Agarose gel along with single stranded oligonucleotides. The description over each lanes represent the following, M - 1kbp ladder, M2 - 10bp ladder, L1 – shRNA sense, L2 – shRNA antisense, L3 – MuHemgnCDS shRNA, L4 - MuHemgnUTR shRNA and L5 - HuHemgnCDS shRNA. (B) Plasmid was extracted from transformed single colonies and screened for oligonucleotide insertion by subjecting them BglII digestion. BglII sites are destroyed on oligonucleotide insertion. The description over the lanes indicate the following, M – 1kb Ladder, L1-4 – PGJ10 –shRNA Hemgn CDS (Human, clone 1-4), L5-8 – PGJ10 – shRNA Hemgn CDS (Mouse, clone 1-4), L9 – PGJ10 (Bgl II restricted) and L10 – Unrestricted PGJ10.(C) Screening of MELTR cells for Hemgn knockdown clones. MEL TR cells that were electroporated with PGJ10 shRNA Hemgn CDS construct, selected and serially diluted using 96 well plate to obtain clonal population. Nuclear extract was performed from these clones with (+) and without (-) the presence of 5µg/ml DOX. Hemgn Western blot was performed using the nuclear extract and the knockdown was studied at the protein level.



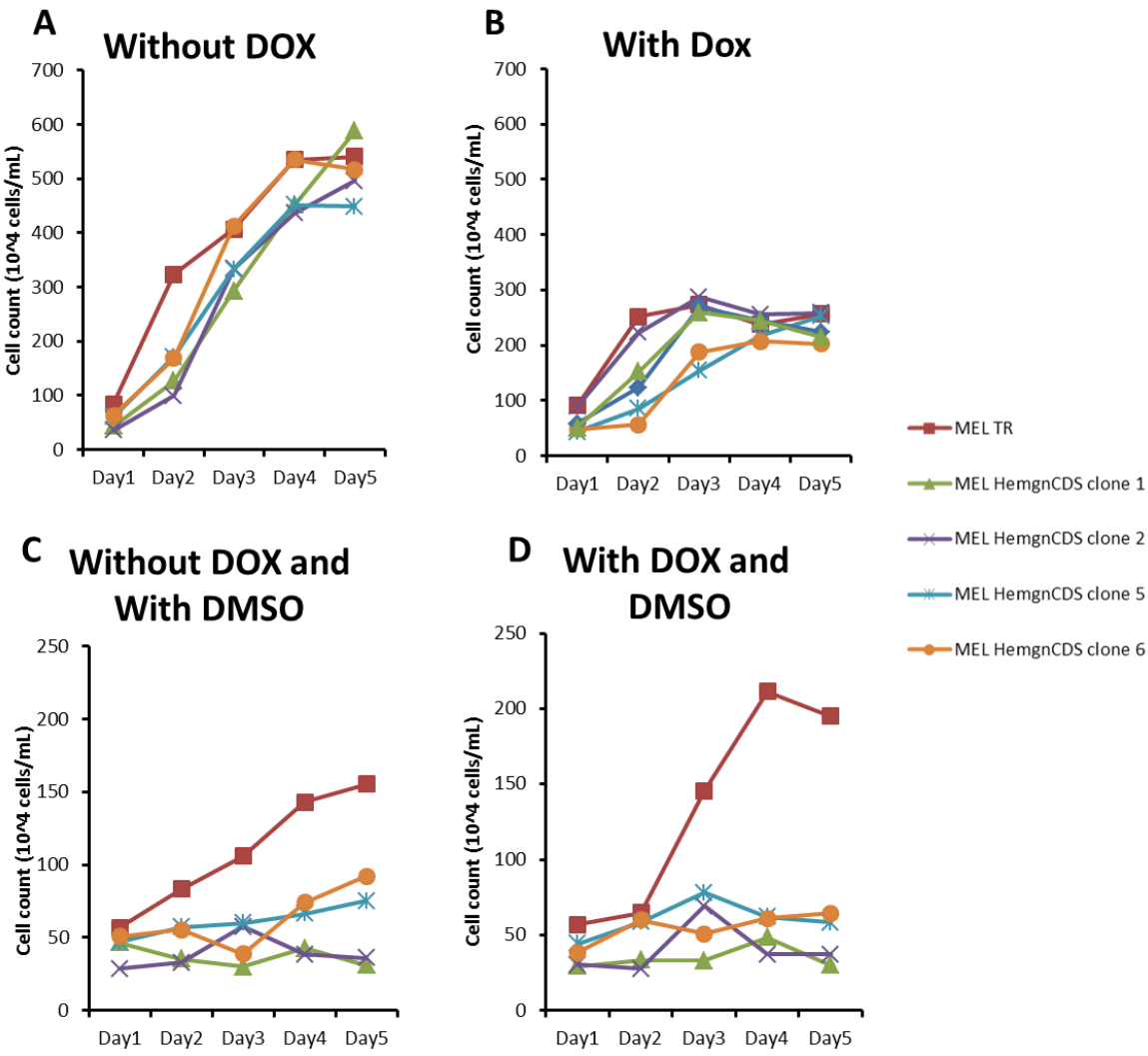
Supplementary Figure 2. Effect of DOX induction on the growth of parental MEL TR and the Hemgn knock down clone C5. Different concentration of DOX as indicated in the graph legend was used in the experiment. The data points are an average of four readings from the same experiment ± SD.



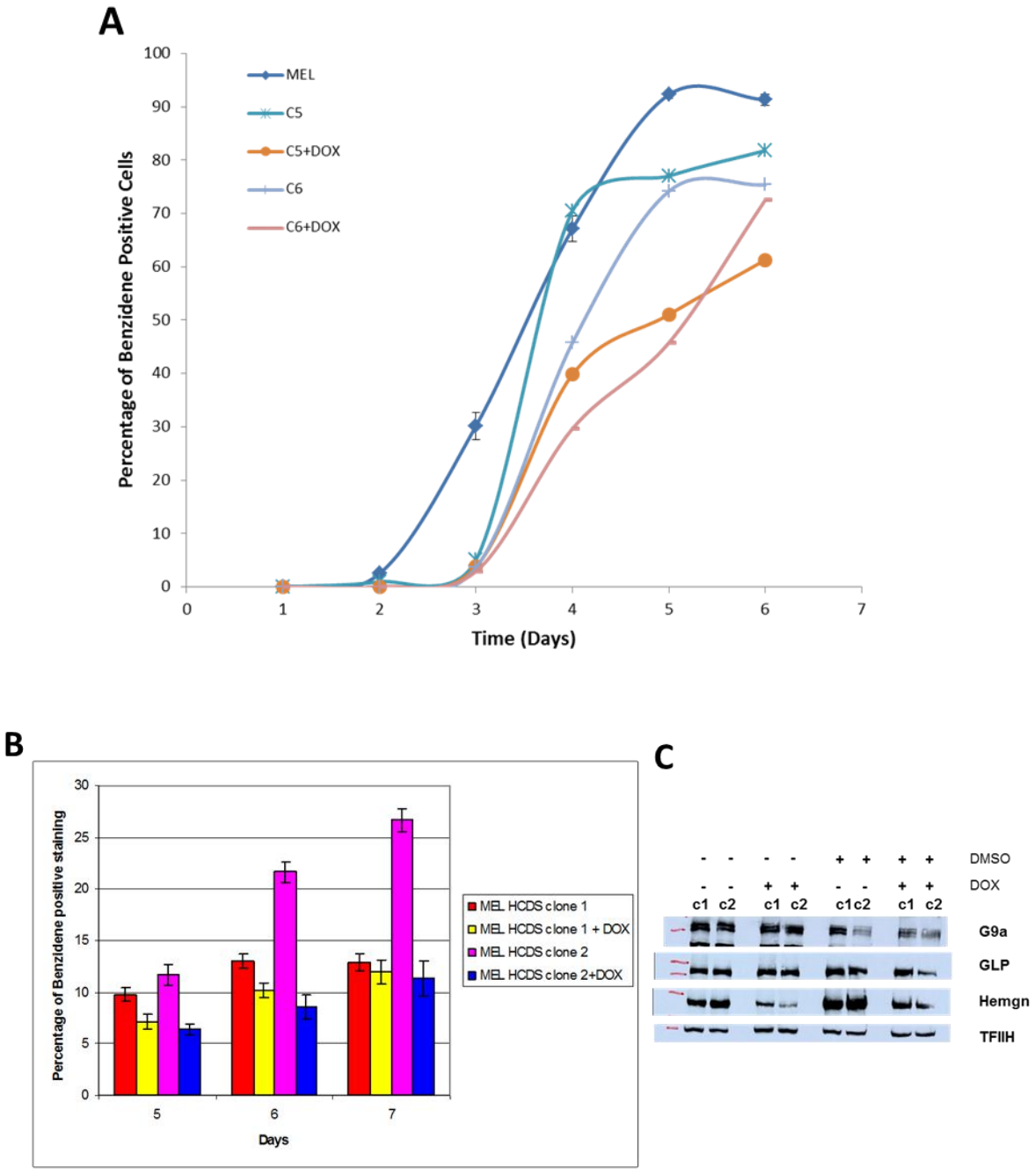
Supplementary Figure 3. Standardization of condition for Hemgn knockdown studies. Nuclear extraction was performed at a time profile (every 24 hrs) for Hemgn knockdown clone 5 in the presence and absence of DOX. HDAC3 was used as a loading control. Best knockdown at the protein level was observed at day 3 of DOX induction. Hence, 3 or more days of DOX induction was used as a standard condition to study the effect of Hemgn knockdown in all the studies.



Supplementary Figure 4. Benzidene staining picture of MEL, MEL TR and Hemgn knock down clones after 6 days of 2% DMSO and 5µg/ml DOX induction. The description of the sample and the presence (+) and absence (-) of DOX is indicated above each field. MEL and parental MEL TR were used as a positive control for Benzidene staining and DOX treated MEL TR was used as a negative control for the effect of DOX on Benzidene staining. Clones 2,5 and 6 show appreciable decrease in the percentage of cells showing Benzidene positive staining on DOX induction whereas clone 1 shows poor or no appreciable change in the percentage of Benzidene positive staining on DOX induction.

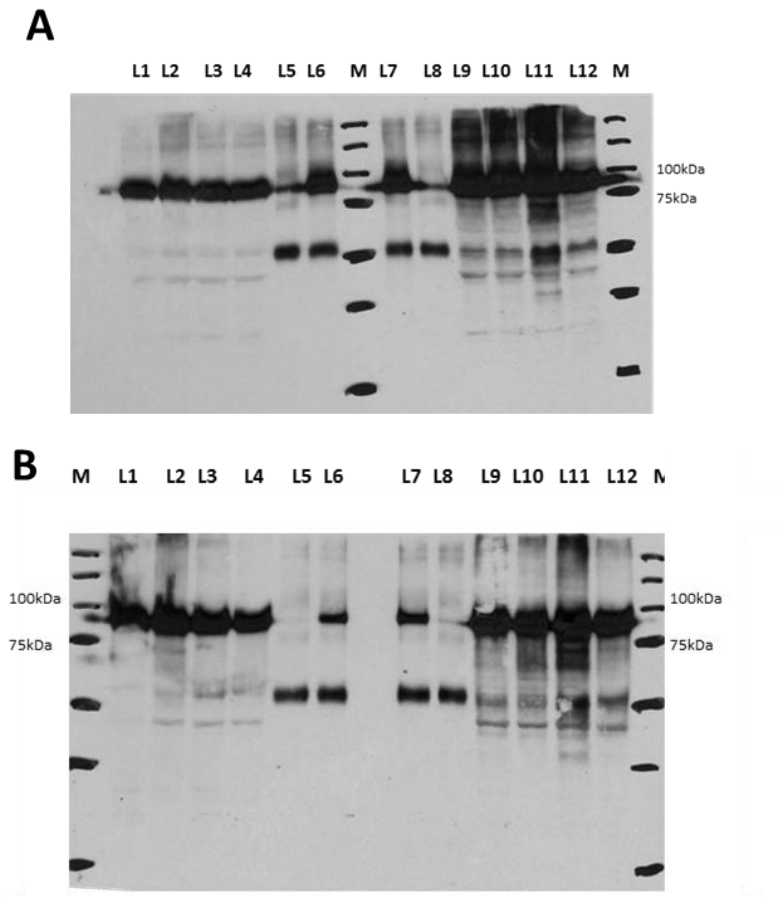


Supplementary Figure 5. Growth kinetics of MEL TR Hemgn knockdown clones in Growth and Differentiation condition. The growth kinetics was studied every 24 hrs with and without DOX and DMSO induction using trypan blue assay. The condition in which the experiment was performed is indicated in each graph title. MEL TR parental cells were used as negative control. Each data point is represented by an average of 4 readings from the same experiment.



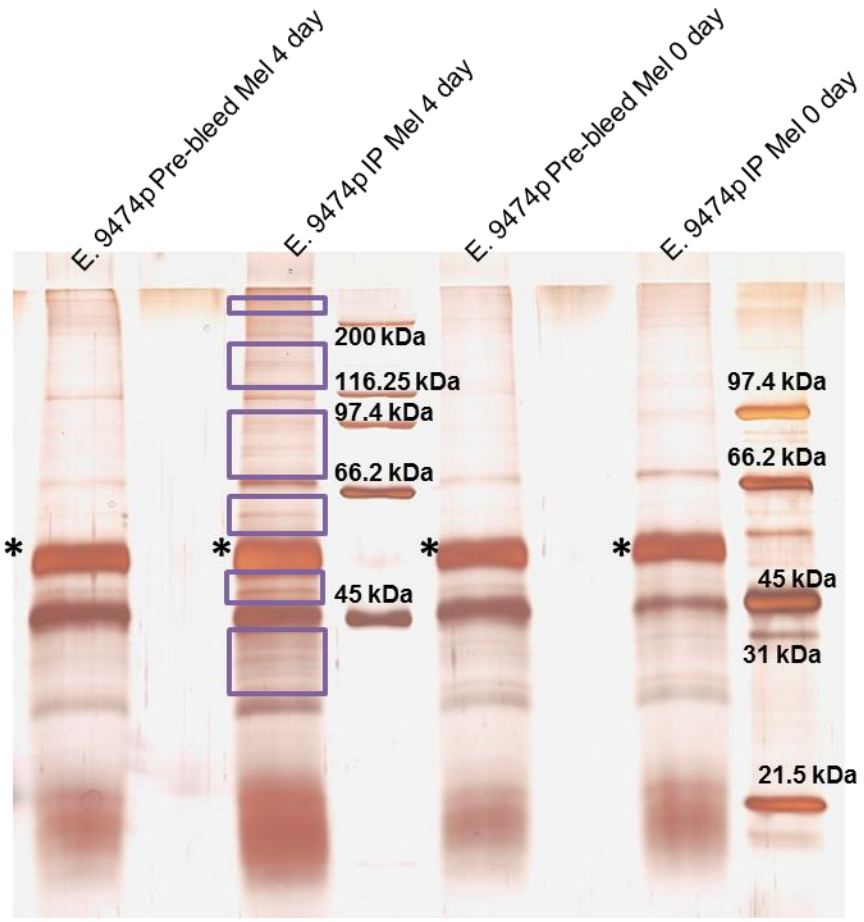
Supplementary Figure 6. (A) The effect of Hemgn on differentiation was studied by using Benzidene staining in the Hemgn knockdown clones 5 and 6. Knockdown of Hemgn expression was induced by addition of 5µg/ml of DOX. There is a significant decrease in the percentage of Benzidene Positive cells during the course of differentiation with 2%

DMSO upto 6 days when Hemgn expression was knocked down using DOX induction in the clones. Parental MELTR treated with DOX was used as the negative control. (B) Benzidene staining was performed on clone 1 and clone 2 with and without DOX induction in the presence of DMSO on day 5,6 and 7 of differentiation. The reading represent an averafe of 4 reading ± SD. (C) Nuclear extract was performed on clone 1 and clone 2 on day4 of DMSO and DOX induction and the knockdown of Hemgn and its effect on G9a and GLP expression at the protein level was studied using Western blot. TFIIH p89 western blot was used as a negative control.



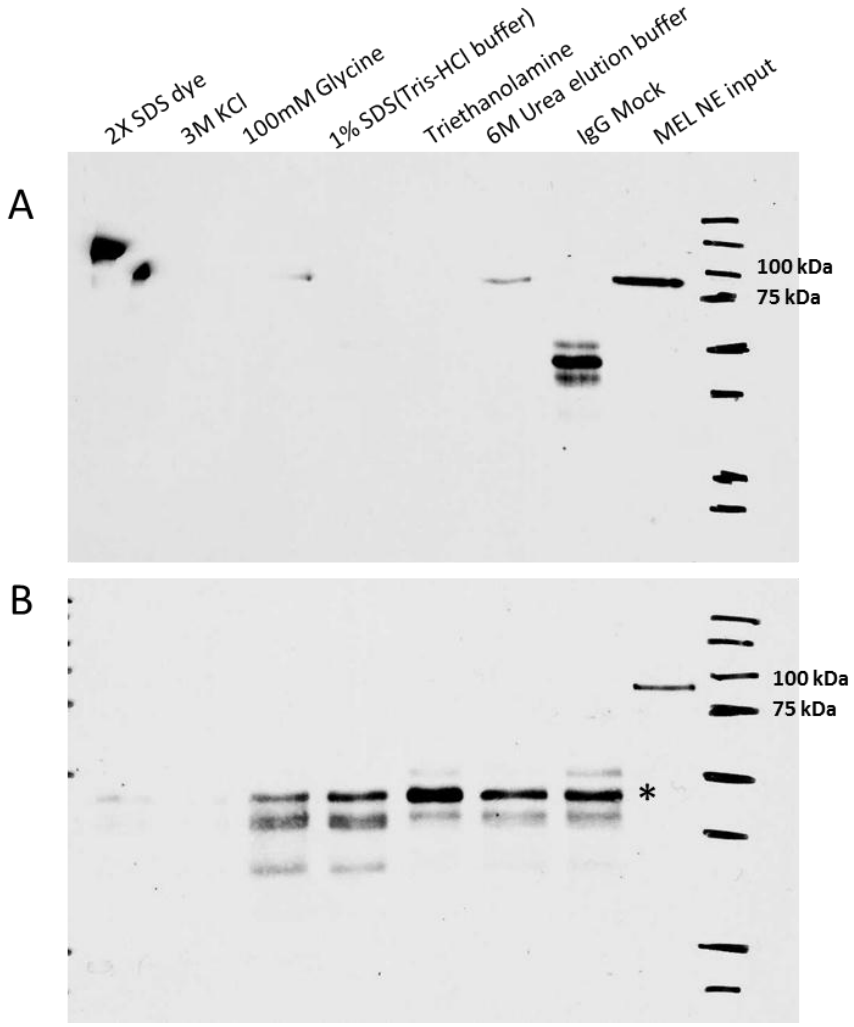
Supplementary Figure 7. Standardization of Hemgn Immunoprecipitation Condition (A) 9474p IP and (B) 9520p IP was initially performed and Hemgn profile at each step of the

experiment was observed using M180 (commercial Hemgn Antibody) western blot. The description above each lane indicates the following, M – marker, L1 – differentiated MEL Nuclear Extract, L2 – differentiated MEL NE preclearing SN, L3 – differentiated MEL NE prebleed SN, L4 – differentiated MEL NE primary antibody SN, L5 – differentiated MEL NE prebleed Elution, L6 – differentiated MEL NE primary antibody Elution, L7 – MEL 0 d NE primary antibody Elution. L8 – MEL 0 d NE prebleed Elution, L9 – MEL 0 d NE primary antibody Elution, L10 – MEL 0 d NE primary antibody SN, L11 – MEL 0d NE preclearing SN and L12 – MEL 0d NE.



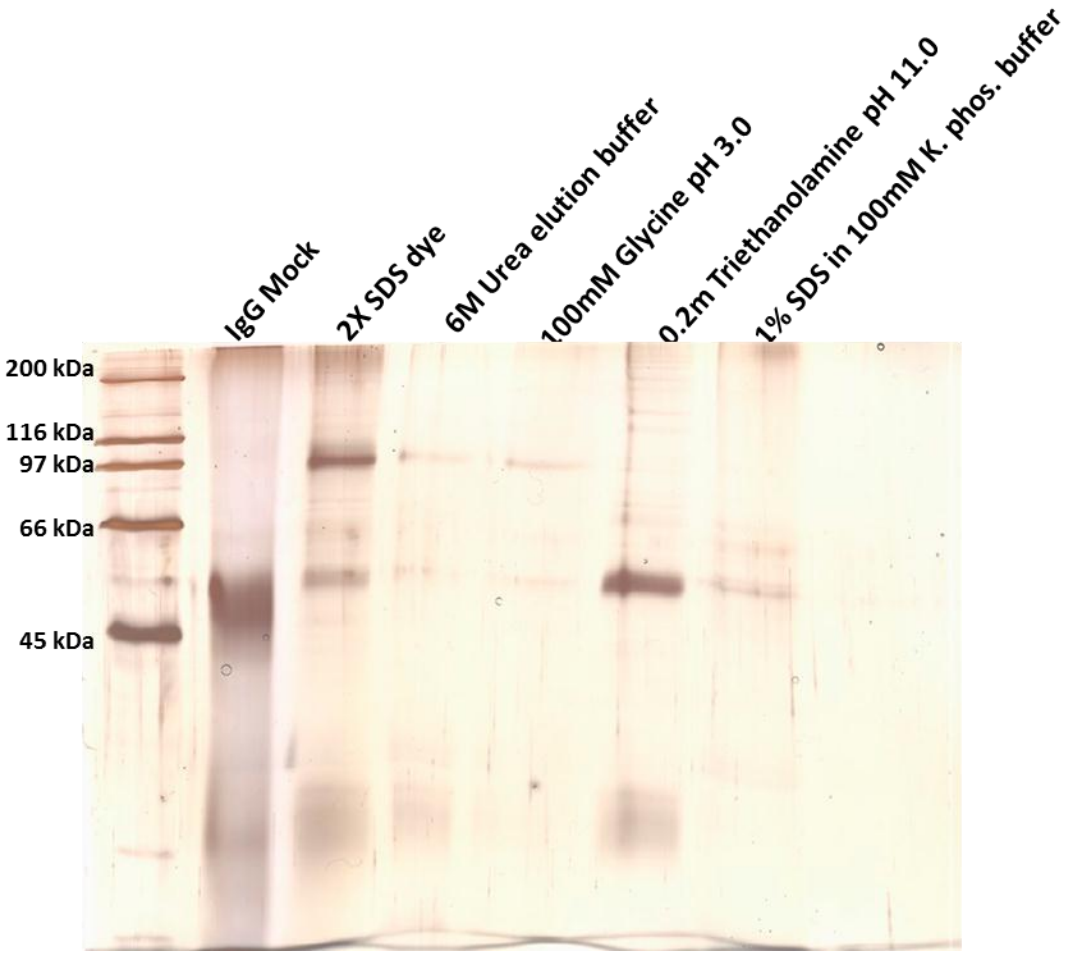
Supplementary Figure 8. Hemgn IP was performed with both differentiated and Undifferentiated MEL Nuclear extract using 9474p antiserum and its corresponding

prebleed in parallel. The elutions were loaded onto a 10% SDS polyacrylamide gel and electrophoresis was performed. Silver staining was performed on the gel. The boxes indicate the portion of gel that were excised and subjected to Mass Spectrometry analysis. * indicates the heavy chain. The numbers indicate the size of the standard marker proteins. “E.” represents elution from Immunoprecipitation that was obtained by boiling the IP beads in 2 X SDS dye.

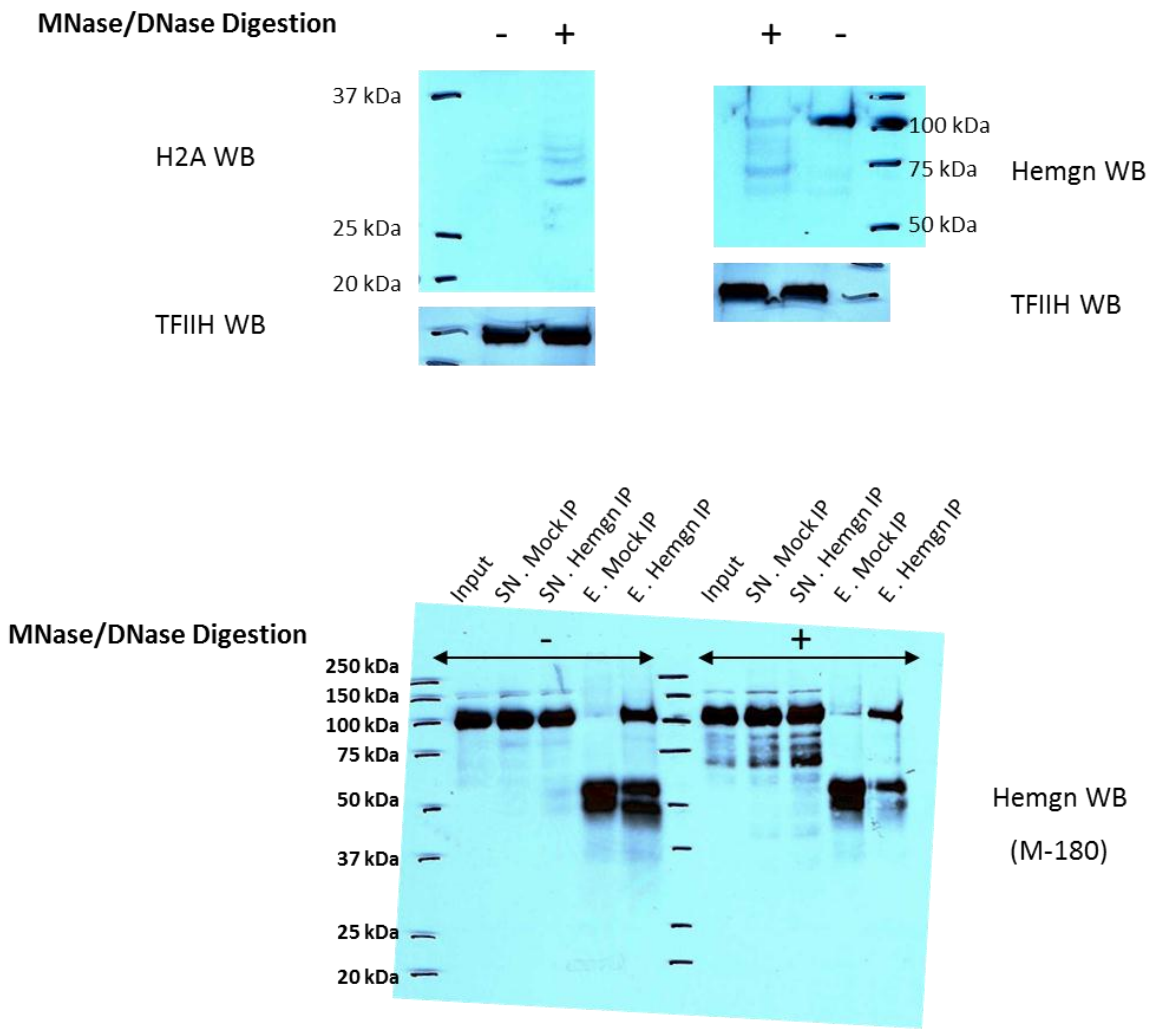


Supplementary Figure 9. Standardization of elution conditions for Hemgn IP. Hemgn IP was performed with 9474p antibody and Mock IP was performed with IgG antibody. (A)

Equivalent amount of beads were eluted with the elution conditions as specified above each lane whereas the negative control, mock IgG IP, was eluted by boiling with 2X SDS dye. Hemgn Western blot was performed with M180 antibody to compare the efficiency of elution of Hemgn in each condition. Hemgn was observed to be eluted by incubating the IP beads in 2 X SDS dye, 6 M urea or 100mM glycine solution at room temperature for 30 min. (B) Beads were boiled in 2 X SDS dye after elution and reminiscent Hemgn in beads after elution were tested using Hemgn Western blot and no Hemgn was found to be present on the beads after treatment. * indicates IgG Heavy chain.



Supplementary Figure 10. Standardization of elution conditions for Hemgn IP by silver staining profile. Hemgn IP was performed with 9474p antibody and Mock IP was performed in parallel with IgG antibody. Equivalent amount of beads were eluted with the elution conditions as specified above each lane whereas the negative control, mock IgG IP, was eluted by boiling with 2X SDS dye. Silver stain was performed on gel containing the samples from different elution conditions and compared. No or very light signals of IgG Heavy chain was detected to be eluted when elution was performed by incubating the IP beads in 2 X SDS dye, 6 M urea or 100mM glycine solution at room temperature for 30 min.

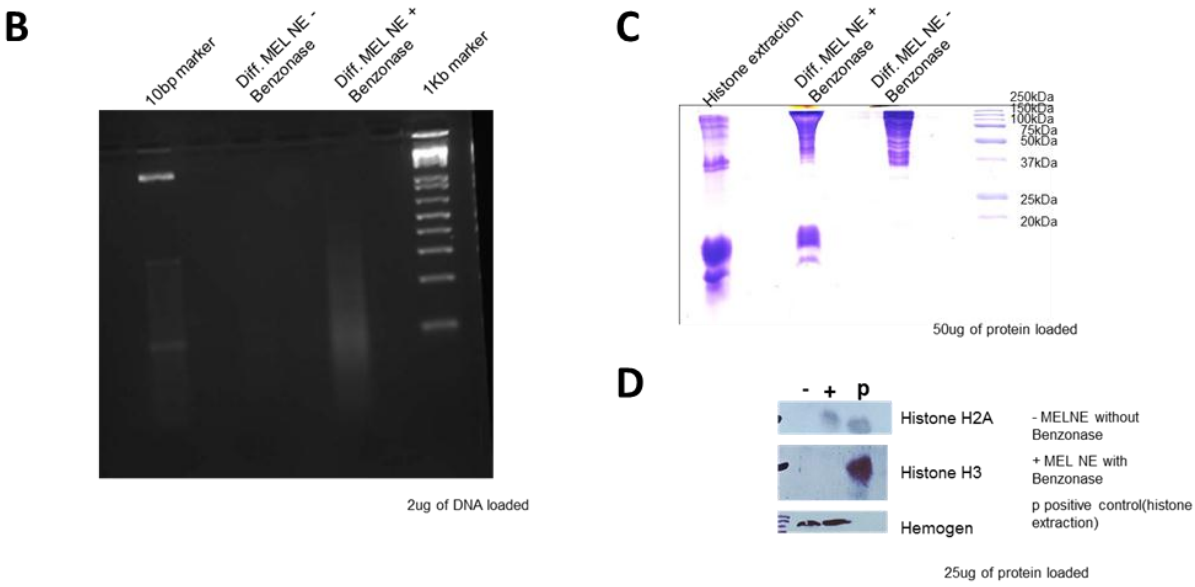


Supplementary Figure 11. Nuclear extraction was prepared by including MNase and DNAse in the standard Nuclear extract protocol to enrich histones. H2A WB and Hemgn WB were performed on equivalent amount of NE with TFIIH as the loading control. IP (below) was then performed with Nuclear extract treated (+) and not treated (-) with MNase and DNAse . There was no significant difference in Hemgn pulldown or extraction with MNase and DNAse mixture. Though Histones were enriched with MNase and DNAse treatment, the enrichment levels remained poor.

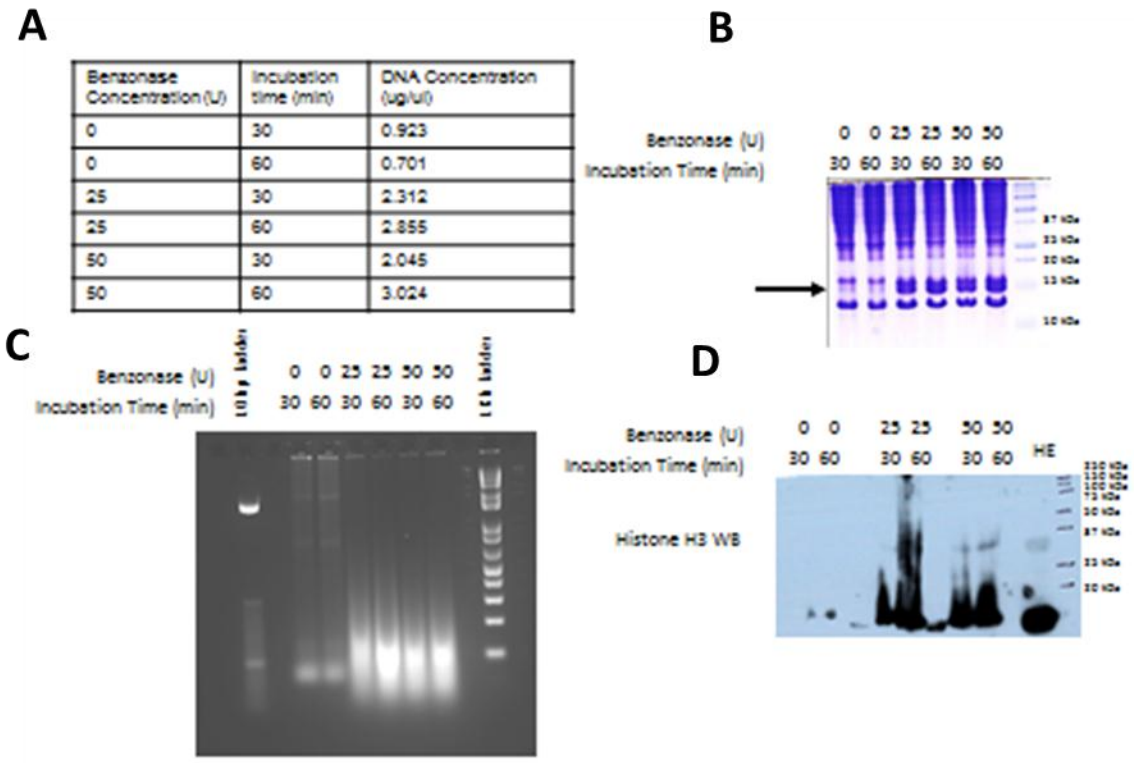
A

Sample	DNA Concentration
MEL 4d - Benzonase	465.5 ng/ul
MEL 4d + Benzonase	727.59 ng/ul

Sample	Protein Concentration
MEL 4d - Benzonase	1.053 ug/ul
MEL 4d + Benzonase	1.385 ug/ul

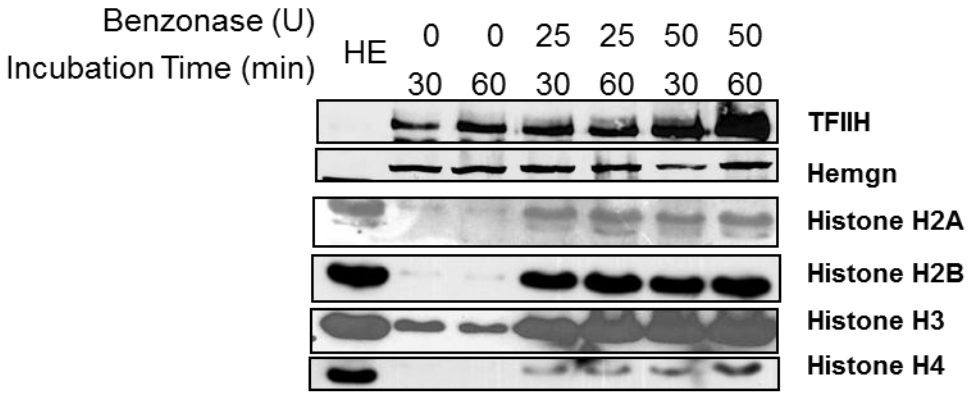


Supplementary Figure 12. Standardization of Benzonase treated Nuclear extract protocol. (A) Nuclear extraction was performed using Differentiated MEL cells (Diff. MEL) in the presence (+) and absence (-) of Benzonase. DNA was extracted from the Nuclear extract and the protein and DNA were quantified for the nuclear extracts. (B) Equal amount of DNA extracted from the Nuclear extract was run on a 1.5 % Agarose gel. The major fraction of the DNA extracted from the Benzonase treated Nuclear extract was below 300bp. (C) Histone extract from Differentiated MEL cells and the nuclear extract performed with and without Benzonase treatment were loaded onto a gel and Coomassie staining was performed. Proteins smaller than 20 kDa were enriched more in the Benzonase treated Nuclear extract. (Most histone proteins are smaller than 20 kDa)



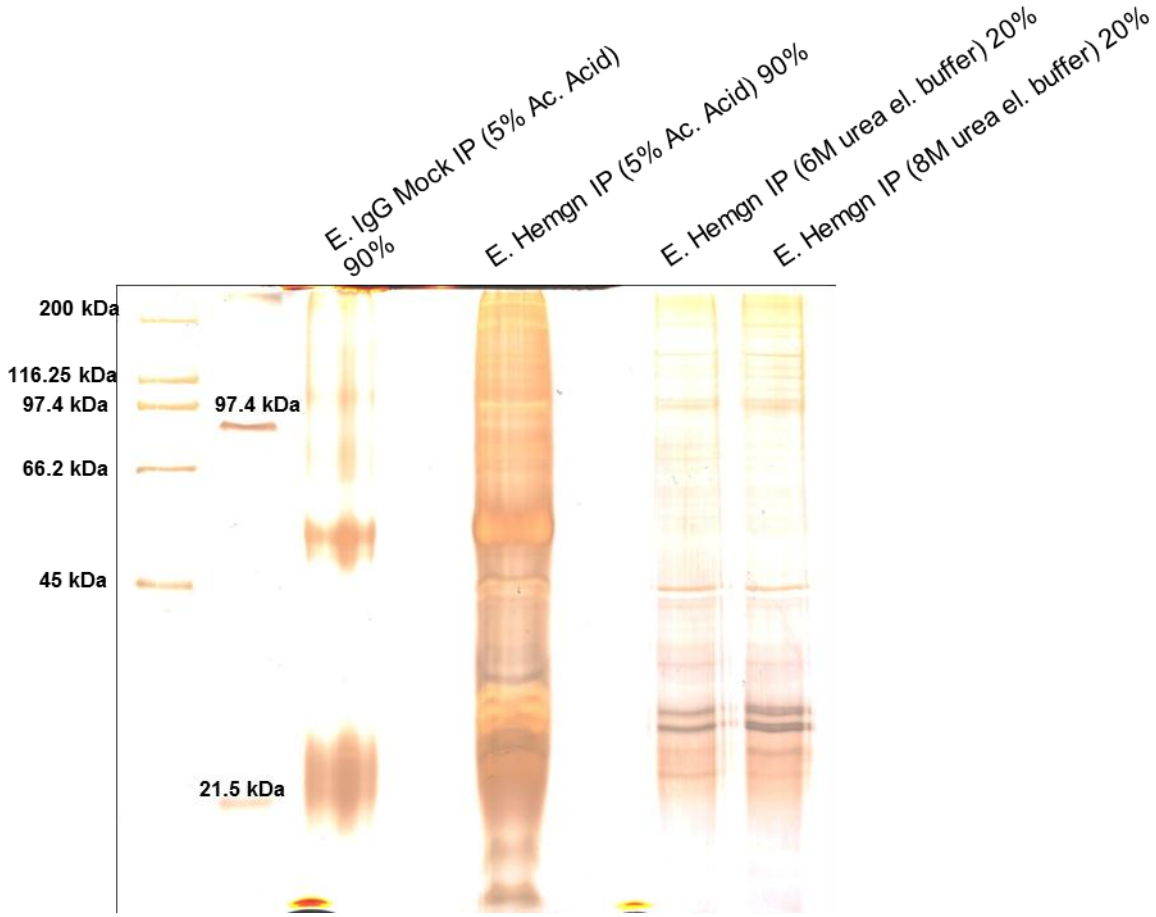
Supplementary Figure 13. Standardization of Benzonase treated Nuclear extract protocol. The time of incubation in Benzonase and the amount of enzyme to be used for the experiment was standardized by performing a time profile with different concentrations of Benzonase (25 Units and 50 Units for every 10^8 cells used). (A) DNA was extracted from the Nuclear extract and its concentration was quantified using Nanodrop by observing the absorption at 260nm. The Benzonase treated Nuclear extracts had higher DNA content. (B) Equivalent amount of Nuclear extracts treated with different concentrations of Benzonase over different time periods of incubation were loaded onto a 15% SDS Polyacrylamide gel. Proteins smaller than 20kDa (indicated by the black arrow) were significantly enriched when treated with Benzonase. (C) Equivalent amount of DNA extracted from the nuclear extract was loaded onto 1.5% Agarose gel. Major fraction of the DNA extracted from the Benzonase treated Nuclear extract was below

300bp. (D) The enrichment of histones in the nuclear extract over Benzonase treatment with different amounts and periods of incubation was studied using Histone H3 Western blot. Loading equal amount of Nuclear extract, more amount of Histone H3 was observed in the nuclear extract after Benzonase treatment. No significant change in the histone H3 was observed when the time and amount of Benzonase was increased from 30 min and 25 units respectively.



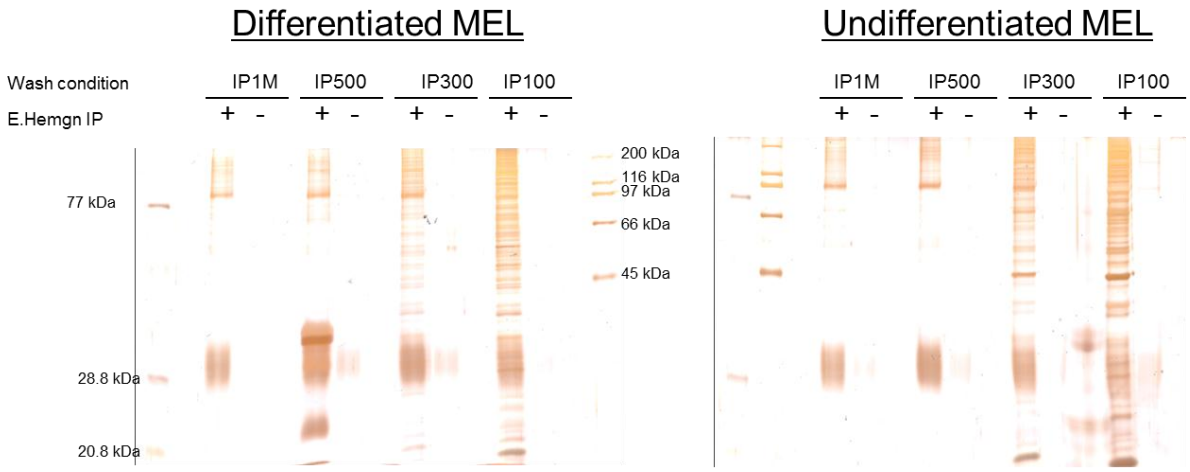
Supplementary Figure 14. Standardization of Benzonase treated Nuclear extract protocol. The time of incubation in Benzonase and the amount of enzyme to be used for the experiment was standardized by performing a time profile with different concentrations of Benzonase (25 Units and 50 Units for every 10^8 cells used). The enrichment of histones in the nuclear extract over Benzonase treatment with different amounts and periods of incubation was studied using Western blot for Histones H2A, H2B, H3 and H4. Hemgn and TFIIH p89 were used as a loading control. Histone extract (HE) was included as a positive control for Histones. Loading equal amount of Nuclear extract, more amount of Histones was observed in the nuclear extract after Benzonase treatment. No significant change in the histone H3 was observed when the time and amount of Benzonase was increased from 30 min and 25 units respectively. No enrichment of

Hemgn was observed with Benzonase treatment. Hence 30 min of incubation with 25 U of Benzonase for every 10^8 cells was used for the nuclear extraction protocol.

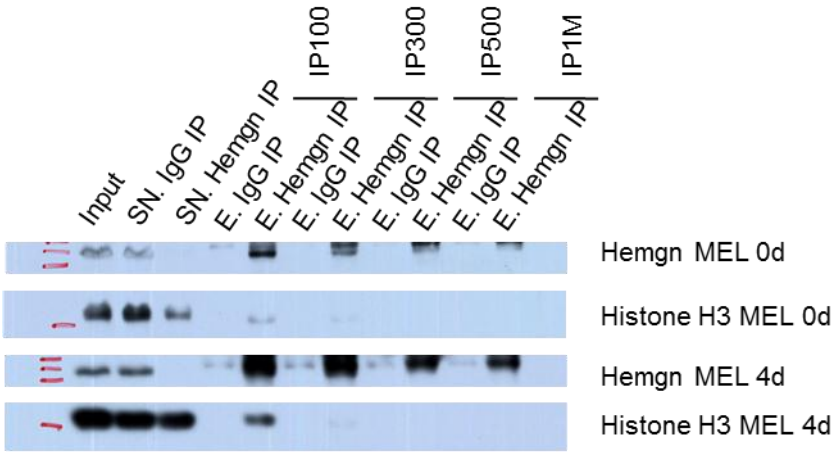


Supplementary Figure 15. Standardization of elution protocol for Hemgn IP. Immunoprecipitation was performed with MEL Nuclear extract and eluted using either 5 % acetic acid, 6 M urea elution buffer or 8M urea elution buffer. The elutions were loaded on a 10% SDS Polyacrylamide gel and silver staining was performed. 6M urea elution buffer contains very less light and heavy chain in comparison to the elution with 5% acetic acid. The silver staining profile appeared similar with both the urea elution

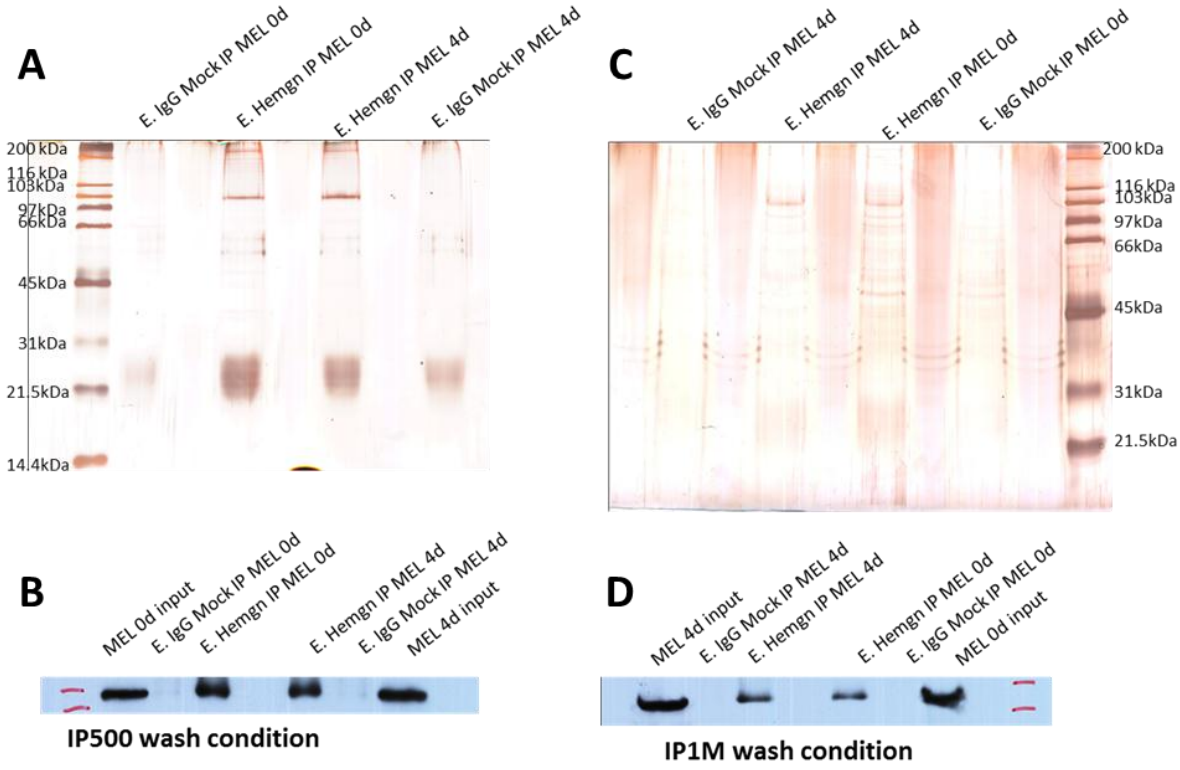
buffers. Hence, 6M urea elution was used for elution for Immunoprecipitations during the course of the study.



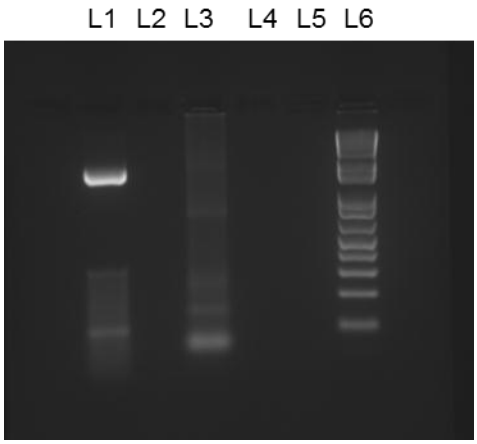
Supplementary Figure 16. Hemgn and Mock IgG Immunoprecipitations were performed with Differentiated (left) and Undifferentiated (right) Benzonase treated Nuclear extract in parallel. After overnight immunoprecipitation, the IP was washed with different salt stringency (100mM, 300mM, 500mM, 1M) and eluted. The elutions were loaded onto a 10% SDS Polyacrylamide gel and Silver staining was performed. The numbers indicated on the side of the gel denote the size of the protein in the standard marker used. '+' denotes elution form Hemgn IP and '-' denotes the elution from Mock IgG IP. The number that follows 'IP' over each lane denotes the concentration of KCl in the IP wash buffer used.



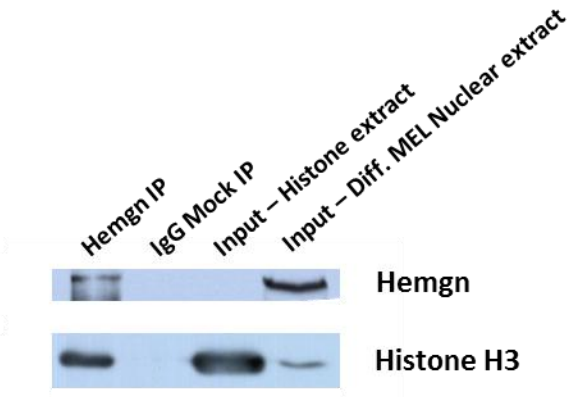
Supplementary Figure 17. Hemgn IP was performed with Benzonase treated MEL Nuclear extract and washed with increasing salt stringency in wash buffer (100mM, 300mM, 500mM and 1M). IP was performed with both differentiated and undifferentiated Nuclear extract. Hemgn histone complex dissociated with higher salt (more than 500mM) strigency wash. ‘E’ denotes elution and ‘SN’ denotes supernatant.



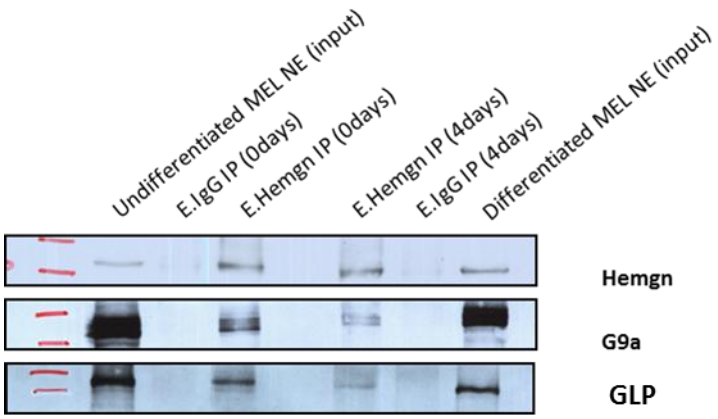
Supplementary Figure 18. Immunoprecipitation was performed with 500mM KCl (IP500, A and B) and 1M KCl (IP1M, C and D) in IP wash buffer. The immunoprecipitates were then eluted and the elutions were loaded on to 10% Polyacrylamide gel. Silerstaining was performed (A and C) using elutions from IP performed with both Undifferentiated and Differentiated Benzonase treated MEL Nuclear extract. Western blot was performed using Hemgn (M180) antibody and Hemgn was observed to be enriched only in the Hemgn IP in both IP500 and IP1M washed Immunoprecipitates.



Supplementary Figure 19. The Benzonase untreated Nuclear extract (Lane L3) and histone extract (Lane L4) used for Histone-Hemgn interaction studies were subjected to DNA extraction. DNA extracted from the Nuclear extract in L3 may be a result of the nucleosomes that is present in the nuclear extract. Hence in the nuclear extract, histones may be present mostly in the nucleosomal conformation. Whereas the histone extract shows no detectable amount of DNA on the agarose gel. Hence, most of histones present in the histone extract are in their DNA free conformation. Lane L2 and L6 are blank and Lane L1 has 10bp marker (Invitrogen) whereas Lane L7 has 1Kbp plus ladder (Invitrogen).

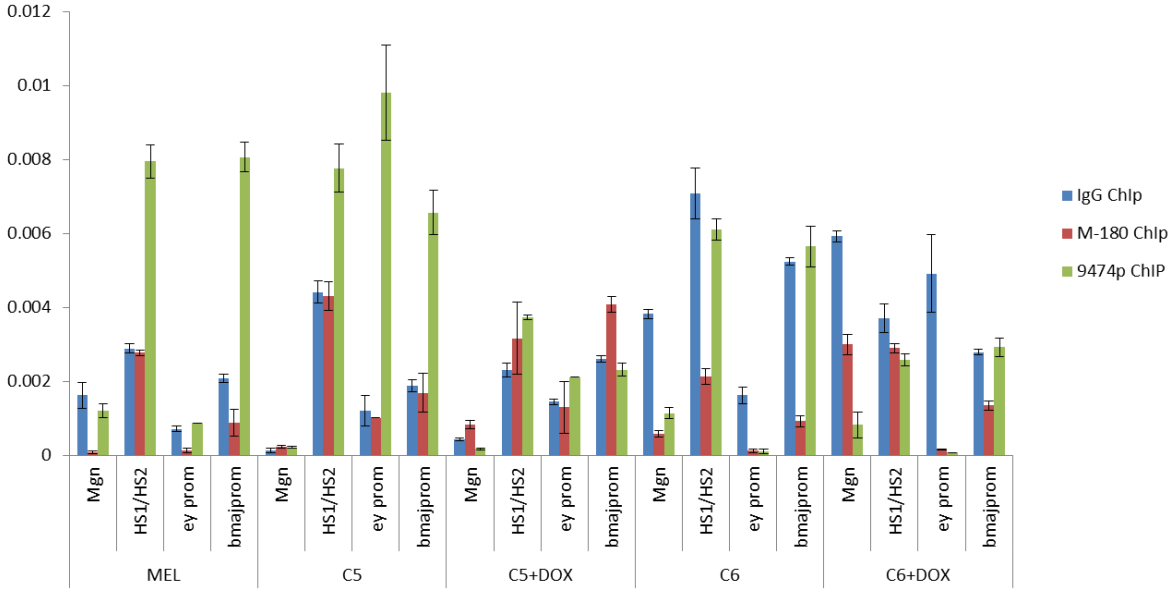


Supplementary Figure 20. Interaction of Histone with Immunopurified Hemgn (trial 2). Histone extract (Lane 3 from left) was incubated with Mock IgG and Hemgn antibody pull down with Differentiated MEL Nuclear extract after high stringency wash (500mM). Histones were enriched only in Hemgn IP (Lane 1 from left) and not in Mock IgG IP (Lane 2 from left). Therefore Histone can interact with immunopurified Hemgn core complex.

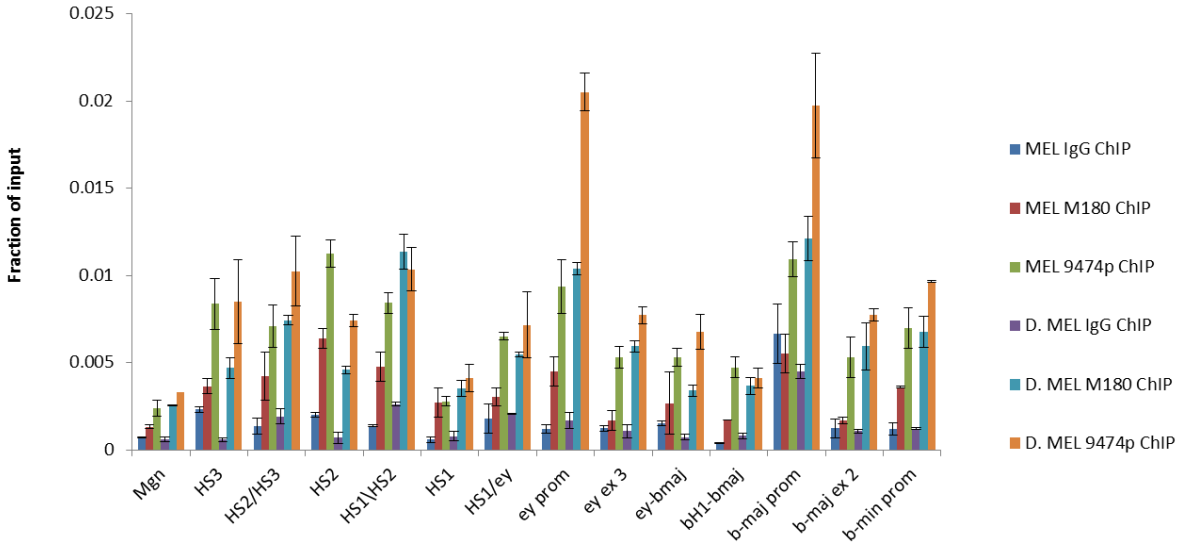


Supplementary Figure 21. Validation of G9a-GLP interaction with Hemgn (trial 2). G9a (Ehmt2) and GLP (Ehmt1) were identified to interact with Hemgn during Mass spectrometry analysis. Hemgn IP was performed with Undifferentiated (Left) and

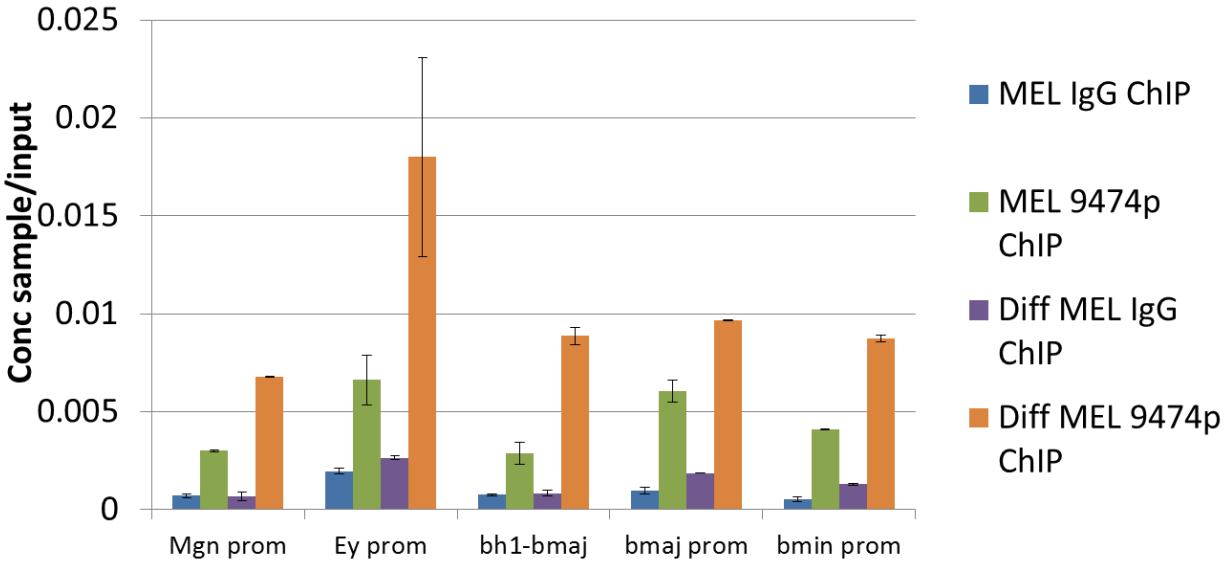
Differentiated MEL Nuclear extract (Right) and the IP input and elutions from Mock IgG IP and Hemgn IP were loaded on the gel and Western Blot was performed for the interacting proteins to validate Mass Spectrometry analysis.



Supplementary Figure 22. Hemgn Chromatin Immunoprecipitation was performed with crosslinked MEL, Hemgn knock down clone C5, C6 with and without DOX induction in growth condition using 9474p Antiserum, M180 polyclonal antibody and Mock IgG ChIP that was performed in parallel. The enrichment of Hemgn over specific locus was studied using specific primers and probes in Realtime-qPCR. Myogenin promoter (Mgn) which is a muscle specific gene was taken as a negative control. The enrichment of Hemgn over different region of β-globin locus (HS1/HS2 is the intermediate region between hypersensitive sites HS1 and HS2 in the 5' region, ey prom represents the promoter region of the embryonic globin gene ey and bmaj prom represents the promoter of the adult globin gene βmaj).



Supplementary Figure 23. Hemgn Chromatin Immunoprecipitation was performed with crosslinked MEL and Differentiated MEL Cells using 9474p Antiserum, M180 polyclonal antibody and Mock IgG ChIP that was performed in parallel. The enrichment of Hemgn over specific locus was studied using specific primers and probes in Realtime-qPCR. Myogenin promoter (Mgn) which is a muscle specific gene was taken as a negative control. The enrichment of Hemgn over different region of β-globin locus (Hypersensitive sites at 5' region represented by HS3, HS2, HS1, embryonic genes, ey and bH1 and adult genes bmaj and bmin, prom represents promoter, ex represents exon and / represents intermediate region) was studied.



Supplementary Figure 24. Hemgn Chromatin Immunoprecipitation was performed with crosslinked MEL and Differentiated MEL Cells using 9474p Antiserum and Mock IgG ChIP was performed in parallel. The enrichment of Hemgn over specific locus was studied using specific primers and probes in Realtime-qPCR. Myogenin promoter (Mgn) which is a muscle specific gene was taken as a negative control. The enrichment of Hemgn over different region of β-globin locus (embryonic gene promoter - ey prom, intergenic region between embryonic and adult genes – bh1-bmaj, adult gene βmaj promoter – bmaj prom and adult gene βmin promoter – bmin prom) was studied.