Identification of the molecular partners that regulate MEIS1A function

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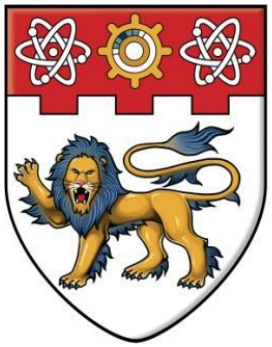

NANYANG TECHNOLOGICAL UNIVERSITY

IDENTIFICATION OF THE MOLECULAR PARTNERS THAT REGULATE MEIS1A FUNCTION

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A thesis submitted to the Nanyang Technological University in partial fulfillment of the requirement for the degree of Doctor of philosophy

2013

To

My parents

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LIST OF ABBREVIATIONS:

aa	amino acids
abd-A	abdominal-A
Abd-B	abdominal-B
AER	Apical ectodermal ridge
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
Antp	Antennapedia
ARE	Autoregulatory element
ASHL2	Absent, small or homeotic-like
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CBX	Chromobox
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
CMP	Common myeloid progenitor
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
CRE	cAMP response element
CREB	CRE-binding protein
CRS1	cAMP response sequence 1
CRTC	CREB-regulated transcription co-activator
CTD	C-terminal domain
DBD	DNA binding domain
dll	distalless
DPP	Decapentaplegic

EMSA	Electrophoretic mobility shift assay
ESC	Embryonic stem cells
EXD	Extradenticle
FGF	Fibroblast growth factor
Flt3	fms-related tyrosine kinase 3
Gata1	Globin transcription factor 1
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte macrophage progenitor
GST	Glutathione S-transferase
h	hour
HAT	Histone acetyltransferase
HCFC	Host cell factor C
HCNR	Highly conserved non-coding region
HDAC	Histone deacetylase
HEK293	Human embryonic kidney 293
Hox	Homeobox
HSC	Hematopoietic stem cells
HTH	Homothorax
H3K4	Histone 3 lysine 4
IgG	Immunoglobulin G
IP	Immunoprecipitation
IRO/IRX	Iroquois
kb	kilobases
kD/kDa	kilodalton
KNOX	Knotted-like homeodomain
LAB	Labial

LEDGF	Lens epithelium-derived growth factor
LT-HSC	Long term repopulating HSC
M-CSF	Macrophage colony stimulating factor
MEIS	Myeloid ecotropic viral integration site
min	minutes
miRNA	microRNA
ml	milliliter
mg	milligram
MLL	Mixed lineage leukemia
MLL1	Mixed lineage leukemia protein 1
MyoD	Myogenic differentiation
ng	nanogram
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
NONO	non-POU domain containing, octamer binding
NOP	Nucleolar protein
NUP98	Nucleophorin 98kD
Pax6	paired box 6
PBX	pre-B cell leukemia homeobox
PCR	Polymerase chain reaction
PcG	Polycomb group
PF4	Platelet factor 4
PHD	Plant homeodomain
PKA	Protein kinase A
PKNOX	PBX/knotted 1 homeobox
r	rhombomere
RA	Retinoic acid

RAR	Retinoic acid receptor
RBBP5	Retinoblastoma binding protein 5
Rlu	Relative light unit
ROS	Reactive oxygen species
rt	room temperature
RT-PCR	Real time PCR
Runx	Runt-related transcription factor
Scr	Sex combs reduced
SDS	sodium dodecyl sulphate
Shh	Sonic hedgehog
ST-HSC	Short term repopulating HSC
TALE	Three amino acid loop extension
TrxG	Trithorax group
Tsh	Teashirt
Ubx	Ultrabithorax
VP16	Viral protein 16
WB	Western blot
WCE	whole cell extract
WDR5	WD repeat domain 5
WG	Wingless
WT	wild type

Abstract

Meis1 encodes a homeodomain-containing transcription factor which performs a vital role during embryonic development as well as in adult physiological processes, particularly hematopoiesis. It is an oncogene in many kinds of leukemia, most notably acute myeloid leukemia (AML) and mixed lineage leukemia (MLL) as well as solid tumors like neuroblastoma. In addition, *Meis1* has been implicated in genetic disorders like restless leg syndrome. MEIS1 functions upstream of many target genes to regulate their expression. HOX and PBX proteins are the most important and well-studied interaction partners of MEIS1. Recently, however, MEIS1 has been shown to interact with CRTC transcription factors and function in a PKA-dependent signaling pathway. Considering the significance and the diversity of the processes regulated by MEIS1, we hypothesized that it could interact with a large repertoire of proteins in order to carry out its functions. Hence my work involved the purification and identification of novel interaction partners of MEIS1A, the widely expressed isoform of MEIS1.

To accomplish this goal, I used a recently described technique for the purification of interaction partners. It involved the *in vivo* biotinylation of MEIS1A followed by a single-step purification of the MEIS1A-interacting proteins using streptavidin-affinity purification. Using mass spectrometry, I identified approximately 40 different proteins that specifically co-purified with MEIS1A. Through co-immunoprecipitation assays, I validated the interaction of MEIS1A with some of these proteins including NONO, NOP56, NOP58, CBX3 and MLL1. I was also able to show that the interaction with NONO, NOP56 and NOP58 is conserved among other MEIS1-related proteins like PKNOX1 and PKNOX2.

NONO is of interest because of its reported interaction with CRTC1. I was able to show that NONO does not mediate the interaction between CRTC1 and MEIS1A. In addition, NONO does not contribute to the transactivation potential of MEIS1A under our experimental conditions. The physiological relevance of the MEIS1A-NONO interaction is therefore yet to be understood. The MEIS1A-MLL1 interaction that I identified was very interesting because both proteins play physiologically important roles during hematopoiesis and leukemia and function in a common pathway. We were able to show that MLL1 and MEIS1 interact endogenously in RS4;11 cells. The MEIS1A interaction is retained by MLL-AF4 which is a leukemogenic fusion protein of MLL1. I was also able to map the interaction of MLL1 to the C-terminal region of MEIS1A. Together, my data provide insight into the

interactome and mechanisms of MEIS1A function, and open multiple avenues for further investigation.

Chapter 1
INTRODUCTION

1.1. Hox genes: An overview

Hox genes encode homeodomain (HD) proteins which are the principal determinants in generating the antero-posterior body plan in most animals (Krumlauf, 1994). Mutation or mis-expression of *Hox* genes can lead to severe morphological changes in the body plan predominantly resulting in homeotic transformations as has been demonstrated vividly through experiments in *Drosophila* (Lewis, 1978; Randazzo et al., 1991). In *Drosophila*, *Hox* genes are present as a single cluster containing 8 genes which are split between the Antennapedia and the Bithorax complexes (Akam, 1989). In mammals there are 39 *Hox* genes in four clusters, each cluster further subdivided into thirteen paralog groups. The clusters are named from *HoxA* through *HoxD*. Because of gene loss, not all clusters retain all the thirteen genes (Holland and Garcia-Fernandez, 1996; Krumlauf, 1991). The mammalian *Hox* genes have clear counterparts in insects indicating a common evolutionary descent (Figure 1.1) (Duboule and Dolle, 1989; Ferrier and Holland, 2001; Finnerty and Martindale, 1998; Graham et al., 1989).

The *Hox* genes exhibit a collinear pattern of expression, i.e. the spatial (antero-posterior) as well as the temporal pattern of expression of the *Hox* genes reflects the order in which they are present in the chromosome. 5' genes are expressed more posteriorly while the 3' genes are expressed more anteriorly in the embryo (Figure 1.1) (Duboule, 1998; Kondo and Duboule, 1999; Kondo et al., 1998).

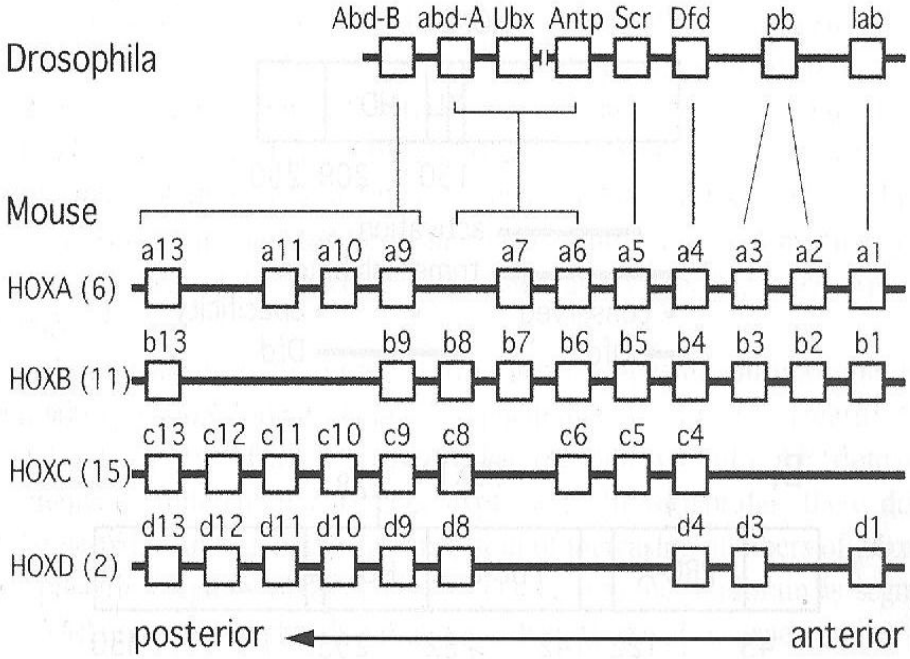


Figure 1.1: *Hox* genes – Genomic organization in *Drosophila* and chordates. Adapted from (Featherstone, 2003). Diagram not to scale.

The DNA binding homeodomain spans 60 amino acids and is organized into a short N-terminal arm followed by three α-helices. Helices one and two adopt an anti-parallel conformation. The primary DNA-binding domain is formed by a helix-turn-helix motif spanning helices two and three. The third helix is the recognition helix and it contacts the major groove of DNA, while the N-terminal arm contacts the minor groove. All homeodomains bind to a similar stretch of 'AT'-rich recognition sequence containing the core binding site 'TAAT'. With some exceptions, the N-terminal arm of the homeodomain in most HOX proteins binds the first two base pairs in the recognition sequence. Another common feature of all HOX proteins is the presence of an arginine at position 51 of the third helix in the homeodomain. Arg 51 binds the third base pair in the recognition sequence. A conserved glutamine at position 50 has also been implicated in DNA binding (Ekker et al., 1994; Featherstone, 2003; Gehring et al., 1994; Hanes and Brent, 1989; Scott, 1999; Treisman et al., 1992; Walter et al., 1994).

1. 2. Specificity of HOX proteins: The need for cofactors:

Since HOX proteins perform vital gene regulation functions during embryogenesis and in adults, the specificity of HOX-DNA binding and the subsequent expression of specific gene targets needs to be tightly controlled. Most of the HOX proteins share the same or similar amino acid residues at positions which bind DNA.

Many functions regulated by *Hox* genes during organ development, like the limb specification in the thoracic segments in *Drosophila*, are paralog specific. For example, all the genes belonging to the Bithorax complex namely *ultrabithorax* (*Ubx)*, *abdominal-A (abd-A)* and *abdominal-B (Abd-B)* regulate limb development by repressing *distalless (dll)* expression. It has been shown that UBX and ABD-A bind to the same DNA binding site in the *dll* enhancer to mediate repression (Estrada and Sanchez-Herrero, 2001; Gebelein et al., 2004; Gebelein et al., 2002; Vachon et al., 1992).

Many other developmental processes are regulated by a single *Hox* gene. The salivary gland specification is regulated only by *Sex combs reduced (Scr)* in *Drosophila* (Bradley et al., 2001; Henderson and Andrew, 2000). Similarly, the development of the gonads in *Drosophila* requires *abd-A* whereas the other bithorax genes, *Ubx* and *Abd-B* are not required (Greig and Akam, 1995). These and many other examples of organ development regulated by *Hox* genes emphasize the importance of specificity in HOX-DNA binding and how accurately they need to be regulated.

Recent studies carried out to elucidate the genome wide binding patterns of the *Drosophila* HOX proteins have shown that despite similar DNA binding specificities, they regulate many distinct target genes. These studies also show that many downstream genes are regulated by a single HOX protein (Hueber et al., 2007). One way by which HOX proteins achieve specificity is by interaction with cofactors which may or may not bind DNA co-operatively. Some studies have reported that HOX proteins also achieve specificity by multiple binding of HOX monomers without the need for cofactors (Galant et al., 2002; Hersh and Carroll, 2005; Lohmann et al., 2002).

The homeodomain containing protein extradenticle (EXD) was the first HOX co-factor to be identified (Rauskolb and Wieschaus, 1994; Rauskolb et al., 1993). Recent evidence shows that EXD unlocks new recognition site preferences in HOX proteins which are hidden in the absence of cofactor binding (Ansari and Peterson-Kaufman, 2011; Slattery et al., 2011). Structural studies have shown that EXD increases the DNA binding specificity and site selectivity of the HOX proteins by altering its structure so that the N-terminal arm could recognize the minor groove of DNA (Joshi et al., 2007; Mann et al., 2009). Differential usage of binding sites apart from the canonical 'YPWM' EXD interaction motif also has been shown to increase specificity of HOX proteins (Lelli et al., 2011)

To date many HOX co-factors have been identified in *Drosophila*, vertebrates, and *C. elegans* and multitude of evidence show that they function co-operatively with HOX proteins to increase their specificity. The main group of these HOX collaborators belongs to the TALE class of homeodomain-containing proteins.

1.2.1. TALE Class of HOX cofactors:

The Three Amino acid Loop Extension (TALE) class of proteins is called so due to the presence of an additional three amino acids between helices 1 and 2 of the canonical homeodomain (Mann and Chan, 1996; Mann et al., 2009; Moens and Selleri, 2006; Mukherjee and Burglin, 2007). The TALE class mainly comprises the PBC and the MEIS/PREP group of HOX cofactors in chordates (Figure 1.2).

A broader classification of the TALE class would also include the TGIF, IRO (Iroquois), KNOX family proteins in plants, and some Mating Type gene products in fungi. But these groups of proteins do not bind HOX proteins and are not considered as HOX cofactors (Burglin, 1997, 1998).

HOX proteins belonging to paralog groups 1 to 8 physically interact with PBC group of proteins using a tetrapeptide tryptophan containing YPWM motif located at the N-terminus of the homeodomain. The ABD-B-like paralog groups 9 and 10 also show interaction with PBX but through a different tryptophan motif, ANW (Chang et al., 1995; Johnson et al., 1995; Neuteboom et al., 1995; Papadopoulos et al., 2011;

Phelan et al., 1995; Shen et al., 1996). HOX proteins belonging to the paralog groups 12 and 13 lack the ability to bind PBX proteins even though they contain tryptophan residues N-terminal to the homeodomain (Shen et al., 1997a).

Mutating the conserved tryptophan motif leads to alteration in some of the HOX protein functions, as has been clearly demonstrated for the *Drosophila* Labial (LAB), ABD-A and Antennapedia (ANTP) and their vertebrate homologs. But these studies also show that there might be other regions apart from the YPWM motif that might also mediate HOX-PBX interaction. This is because, in some instances, mutation of the YPWM motif does not lead to loss of heterodimer formation. (Chan et al., 1996; Galant et al., 2002; Medina-Martinez and Ramirez-Solis, 2003; Merabet et al., 2003). Recent studies examining the UBX-EXD interaction in *Drosophila* also indicate the presence of alternative domains in HOX proteins for cofactor interactions (Saadaoui et al., 2011). A conserved arginine residue present in the flexible N-terminal arm of the homeodomain has also been shown to be important for HOX-PBX interaction and heteromeric DNA binding (Phelan and Featherstone, 1997).

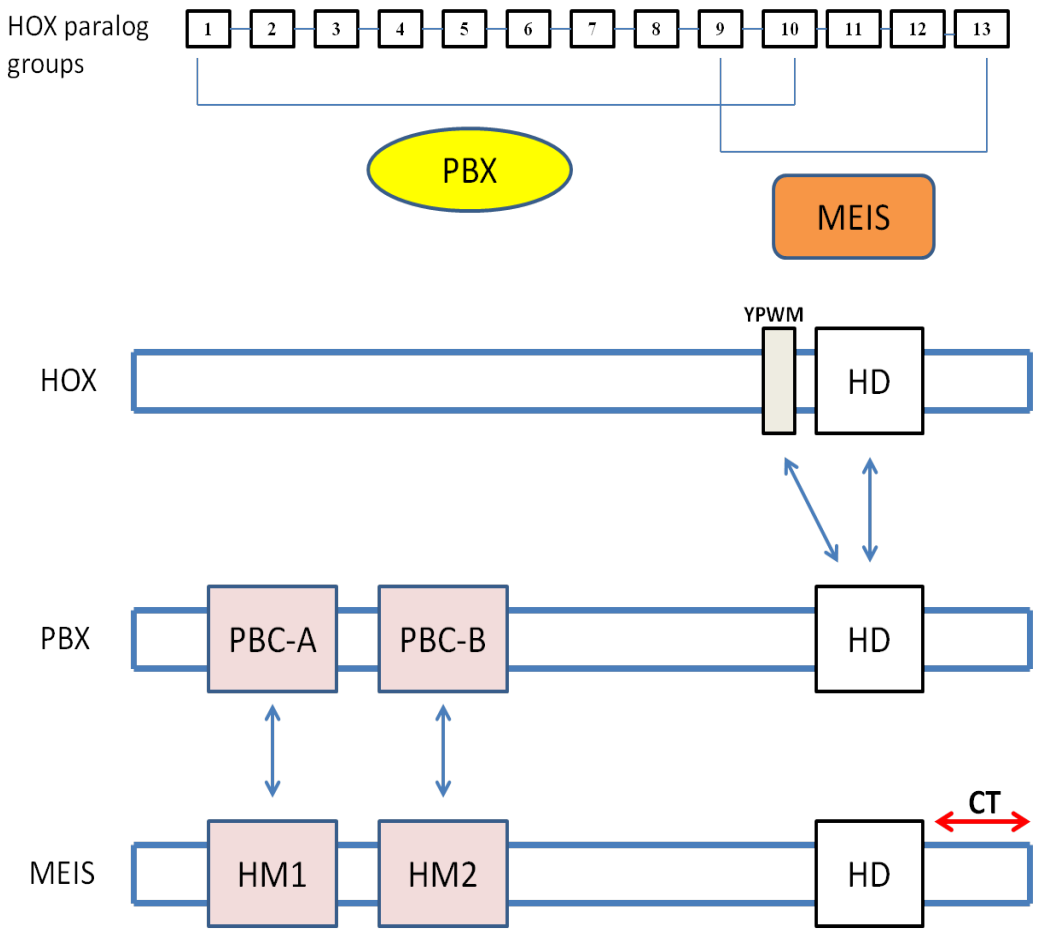


Figure 1.2: HOX and its cofactors

(i) HOX and cofactor interactions: HOX proteins belonging to paralog groups 1-10 interact with PBX and paralogs 9-13 interact with MEIS. HOX proteins bind PBX using a tryptophan containing motif.

(ii) PBX and MEIS proteins interact with each other through their PBC and HM domains, respectively.

(iii) A C-terminal transcriptional activation domain has been identified in MEIS1/2.

The ABD-B class of HOX proteins, which includes the paralog groups 9 to 13 in vertebrates, binds DNA along with MEIS group proteins. Regions at the extreme N-terminus (residues 1-61) as well as the homeodomain and the C-terminal tail of the HOX proteins mediate the stable interaction with MEIS, but neither regions confer stable interaction, alone (Shen et al., 1997b).

1.3. Hox cofactors: The *Drosophila* paradigm

1.3.1. Extradenticle (EXD):

exd was first identified in *Drosophila* as a homolog of *Pbx1*, which had earlier been identified as an oncogene in humans. *exd* was found to show distinct spatio-temporal patterns of expression during development and it was recognized as a factor modulating the activity of many HOX proteins (Peifer and Wieschaus, 1990; Rauskolb and Wieschaus, 1994; Rauskolb et al., 1993). This was soon shown to be caused by a direct interaction between EXD and HOX proteins. EXD was found to increase the specificity of the HOX proteins for their DNA binding sites and also contribute to its transcriptional activation and repression functions (Chan and Mann, 1996; Chan et al., 1996; Chan et al., 1994; Chan et al., 1997; Johnson et al., 1995; Passner et al., 1999; Ryoo and Mann, 1999; Sun et al., 1995; van Dijk and Murre, 1994).

Binding to EXD is vital for the function of many HOX proteins. The competition for binding to EXD has been reported to be the cause for posterior dominance, a feature in which posteriorly expressed HOX proteins suppress the activity of the anterior proteins (Noro et al., 2011). Mutations in *exd* was found to lead to homeotic transformations (ex: ectopic eyes) similar to *Hox* genes implying an essential role for *exd* in patterning the embryonic axes in the *Drosophila* embryo. (Gonzalez-Crespo and Morata, 1995; Rauskolb et al., 1995). Apart from HOX proteins, EXD was found to bind with non-HOX proteins, mainly Engrailed, to modulate its functions (Fujioka et al., 2012; Kobayashi et al., 2003; Peltenburg and Murre, 1996)

EXD cellular localization is highly regulated during development. It being a transcription factor, EXD has to localize mainly to the nucleus for its functions (Aspland and White, 1997; Mann and Abu-Shaar, 1996). An important factor for EXD nuclear localization is the TALE class homeoprotein, Homothorax (HTH). Direct interaction of EXD with HTH is essential for this nuclear import (Abu-Shaar et al., 1999; Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997).

EXD is actively exported out of the nucleus by a CRM1-mediated mechanism. Two nuclear localization signals (NLS) have been identified in the homeodomain of

EXD (Abu-Shaar et al., 1999; Saleh et al., 2000a) which in the absence of HTH are masked by the PBC domains. Furthermore, in the absence of HTH, EXD is retained in the cytoplasm by non-muscle myosin (*zipper* protein in *Drosophila*) (Huang et al., 2003). It is proposed that upon HTH binding, the NLS are unmasked, bound by importin class nuclear translocation proteins and transported to the nucleus (Stevens and Mann, 2007). Concentration gradients of (Decapentaplegic) DPP and (Wingless) WG also contribute to EXD nuclear localization (Gonzalez-Crespo et al., 1998; Mann and Abu-Shaar, 1996).

Interaction with HTH and EXD nuclear localization are needed for important functions like proximo-distal leg patterning (Abu-Shaar and Mann, 1998; Gonzalez-Crespo et al., 1998; Mercader et al., 1999), antenna versus leg development (Casares and Mann, 1998), eye development (Pai et al., 1998) and specification of the peripheral nervous system (Kurant et al., 1998) in *Drosophila*.

1.3.2. Homothorax (HTH):

As described above, *hth* was first identified as a gene encoding a TALE class homeodomain protein involved in the nuclear localization of EXD (Kurant et al., 2001; Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997). HTH can bind DNA in trimeric complexes containing HOX-EXD-HTH proteins in equimolar concentration (Ryoo et al., 1999). DNA binding by HTH is dispensable in these trimeric complexes for gene regulation. Alternative splicing of *hth* transcripts has been shown to generate at least 2 shorter isoforms lacking a homeodomain. Functional studies demonstrated that some HTH functions during embryogenesis, such as the specification of the limb proximodistal axis, do not require the HTH homeodomain, whereas antenna development is homeodomain dependent (Noro et al., 2006).

Apart from modulating the transcriptional regulation function of HOX and EXD proteins, HTH possesses inherent transcriptional activation functions of its own (Inbal et al., 2001). Interactions with EXD/HTH do not always have a positive influence on HOX proteins. For example, ABD-B does not require interaction with EXD/HTH for its functions and in fact EXD/HTH interaction prevents its binding to

DNA (Rivas et al., 2013). HOX independent functions for the EXD-HTH complex have also been described (Aldaz et al., 2005).

1.3.2. A. Physiological function of *hth:*

A well studied role of *hth* is during limb development in *Drosophila*. *hth* is expressed only in the proximal region of the limb bud. *wg* and *dpp* are expressed in the distal region and repress *hth* to demarcate its proximal expression margin. In the proximal region of the limbs, HTH functions to interact with and translocate EXD to the nucleus. The nuclear localization of both HTH and EXD in this proximal region is important for the limb specification (Abu-Shaar and Mann, 1998; Rauskolb, 2001; Wu and Cohen, 1999, 2000). Similarly, HTH specifies the proximal domain namely the 'wing hinge', during wing development in response to WG signaling (Azpiazu and Morata, 2000; Casares and Mann, 2000; Zirin and Mann, 2004).

Apart from limb development, HTH along with EXD is also important for antennae formation in *Drosophila*. Along with *dll, hth* has been identified as an antenna-specific gene. Suppression of *hth* expression in the anterior head regions by Bithorax-group *Hox* genes has been proposed as the reason behind the antenna-to-leg transformation caused by the ectopic expression of these *Hox* genes. Forced expression of *hth* and *dll* in the limb and genital primordia leads to the development of antennae-like structures (Casares and Mann, 1998; Dong et al., 2000; Ronco et al., 2008; Yao et al., 1999). It has been shown that *dll* and *hth* function together to regulate multiple target genes like *Dach*, *ato* and *sal* to regulate antenna formation (Dong et al., 2002). *Antp*, the *Hox* gene responsible for the specification of legs versus antennae in the thoracic regions represses the expression of *hth*. Ectopic expression of *Hth* induced the formation of antennae in abnormal positions in *Drosophila* (Casares and Mann, 1998).

Other important functions of HTH/EXD include the regulation of eye development in *Drosophila* (Heine et al., 2008; Lopes and Casares, 2010; Pai et al., 1998). They function as negative regulators suppressing eye development specifically by inhibiting the marginal front (MF) progression (Lopes and Casares, 2010; Pai et al., 1998). *hth* functions downstream of the developmentally regulated *tsh* genes during

antenna, wing and eye development (Bessa et al., 2002; Wu and Cohen, 2002). In eye primordia, *hth* and *tsh* function alongside the Hippo pathway to regulate downstream genes (Peng et al., 2009; Zhang et al., 2011a).

In the central nervous system (CNS), HTH induces the development of a specific class of photoreceptors located in the "dorsal rim area" which helps in recognizing polarized light (Wernet et al., 2003). HTH/EXD has also been shown to be vital for the formation of the abdominal chordotonal neurons and olfactory projection neurons (Ando et al., 2011; Kurant et al., 1998). Both EXD and HTH are also responsible for specifying fibrillar (flight) muscle identity versus tubular (jump) muscle identity in *Drosophila* by associating with the transcription factor, SALM (Bryantsev et al., 2012). Taken together these studies implicate an important role for HTH in the formation of vital appendages in *Drosophila*.

1.4. TALE cofactor proteins in chordates

Drosophila EXD and HTH proteins are homologous to the mammalian PBC and MEIS/PKNOX family of proteins, respectively (Table 1.1).

Table 1.1: List of TALE class of HOX cofactor homologs in different model organisms.

FAMILY	*Drosophila* Homologs	Vertebrate Homologs	Zebrafish homologs	*C. elegans* homologs
PBC	extradenticle (EXD)	*Pbx1* *Pbx2* *Pbx3* *Pbx4*	*pbx1* *pbx2* *pbx3* *pbx4*	ceh-20 ceh-40 ceh-60
MEIS	homothorax (HTH)	*Meis1* *Meis2* *Meis3*	*meis1.1* *meis2.1,* *meis2.2* *meis3* *meis4*	unc-62
PKNOX	homothorax (HTH)	*Pknox1* *Pknox2*	*pknox1* *pknox2*	psa-3 (homeodomain-less)

1.5. MEIS1:

Meis/Prep genes are the homologs of the *Drosophila hth*. The murine ecotropic viral integration site 1 (*Meis1*) gene was first identified as a prominent site of ecotropic viral DNA insertion in the BXH-2 myeloid leukemia mouse model. Subsequent DNA mapping showed that the *Meis1* gene is present on chromosome 11 and chromosome 2p23-p12 in mouse and humans, respectively (Moskow et al., 1995; Smith et al., 1997b). A similar study to identify proviral integration sites in BXH-2 mice identified *Meis1* together with *HoxA7* and *HoxA9* genes as important insertion sites indicating a collaborative role for these genes in oncogenesis (Nakamura et al., 1996b). *Meis1* was found to encode a DNA binding protein with a TALE class homeodomain motif which is similar to PBX proteins (Moskow et al., 1995). Based on homeodomain similarity, *Meis1* related genes *Meis2 and Meis3* have also been identified (Nakamura et al., 1996a; Steelman et al., 1997).

1.5.1. Isoforms of *Meis1*:

Due to alternative splicing, the *Meis1* gene encodes many isoforms, of which MEIS1A and MEIS1B have been known to play physiologically important functions. MEIS1A is the predominantly expressed isoform of MEIS1 in chordates (Figure1.3). These isoforms vary at their C-termini, with MEIS1A being shorter than MEIS1B. The predicted molecular weights for MEIS1A and MEIS1B are 43 kD and 51 kD, respectively (Irimia et al., 2011; Moskow et al., 1995; Sanchez-Guardado et al., 2011b). Another isoform, MEIS1C, containing a 48 amino acid deletion between the HM2 domain and the homeodomain was isolated from embryonic mouse tissue. This isoform is also predicted to arise due to alternative splicing (Knoepfler et al., 1997).

MEIS1A is the predominantly expressed isoform in chordates

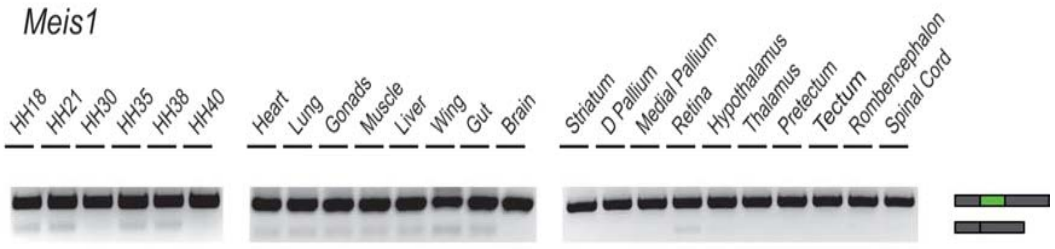


Figure 1.3: Comparison of the expression levels of the alternatively spliced isoforms of *Meis1*, *Meis1a* and *Meis1b* in chicken: RT-PCR for *Meis1a* (top band) and *Meis1b* (bottom band) in different tissues and embryonic stages in chicken. *Meis1a* because of the inclusion of exon 12a has a longer mRNA transcript compared to *Meis1b*. The *Meis1a* isoform is expressed predominantly in all the growth stages and the tissue types examined. Reproduced from (Sanchez-Guardado et al; 2011).

Similar to *hth*, *Meis1* also has been shown to encode homeodomain-less isoforms through alternative splicing. Recently, two novel HD-less isoforms of 32 and 27 kD were found to be expressed specifically in the colon. Exon 8 skipping leads to a premature termination of translation resulting in truncated products (Crist et al., 2011).

1.5.2. Cooperative Binding of MEIS1 with PBX-HOX:

MEIS1 binds to DNA primarily with the ABD-B (posterior) class of HOX proteins and PBX proteins as partners (Chang et al., 1997b; Shen et al., 1997b; Williams et al., 2005). ABD-B class HOX proteins have been found to stabilize the DNA binding activity of MEIS1 (Shen et al., 1997b). Shen et al (1997b) showed that HOX proteins belonging to the non-ABD-B class which includes paralog groups 1-8 in vertebrates do not bind MEIS1 directly, however other studies have shown that MEIS1 could also bind to these anterior HOX proteins *in vitro* (Shen et al., 1997b; Williams et al., 2005). The interaction between MEIS1 and HOX proteins is mediated by the C-terminal region in MEIS1A and MEIS1B and multiple domains in HOX (Figure 1.4) (Williams et al., 2005).

PBX1 interaction with MEIS1 is mediated by the extreme N-terminal domain of PBX and is lost in fusion complexes of PBX1 as in the case of E2A-PBX1 fusion protein. The PBX1 interaction domain on MEIS1 has been mapped to homology motifs 1 and 2 (HM1 and HM2) in the N-terminus of MEIS1 (Figure 1.2) (Chang et al., 1997b; Knoepfler et al., 1997). As described previously, studies in *Drosophila* also identified an interaction between the MEIS and PBX homologs HTH and EXD, respectively (Figure 1.4) (Pai et al., 1998; Rieckhof et al., 1997).

MEIS1 has been shown to form stable heterodimeric interactions with PBX even in the absence of DNA binding (Chang et al., 1997b; Jacobs et al., 1999; Shanmugam et al., 1999). A functional trimeric complex between MEIS, PBX and HOX was first described on the *Hoxb2* r4 enhancer. In this enhancer, MEIS1 and PBX1 interact with HOXB1 to up-regulate the expression of *Hoxb2* in rhombomere 4 of the mouse embryo (Jacobs et al., 1999). Many cross and auto-regulatory functions by HOX proteins are mediated by such trimeric complexes. The r4 expression of *Hoxa2* is also mediated by a HOXB1-MEIS-PBX complex (Tumpel et al., 2007). It has been shown in Zebrafish that MEIS proteins contribute to transcriptional activation by the trimeric complex. It does this by competing with histone deacetylases (HDAC) to bind to PBX and by inducing histone 4 (H4) acetylation at target promoters (Choe et al., 2009). During axial patterning activation of *Krox20* in rhombomere 3 is induced co-operatively by HOXB1A, PBX and MEIS1/2. In zebrafish, it has been shown that *meis1.1* genetically interacts with Iroquois (*irx7*) protein to activate the expression of *krox20* (Stedman et al., 2009; Wassef et al., 2008).

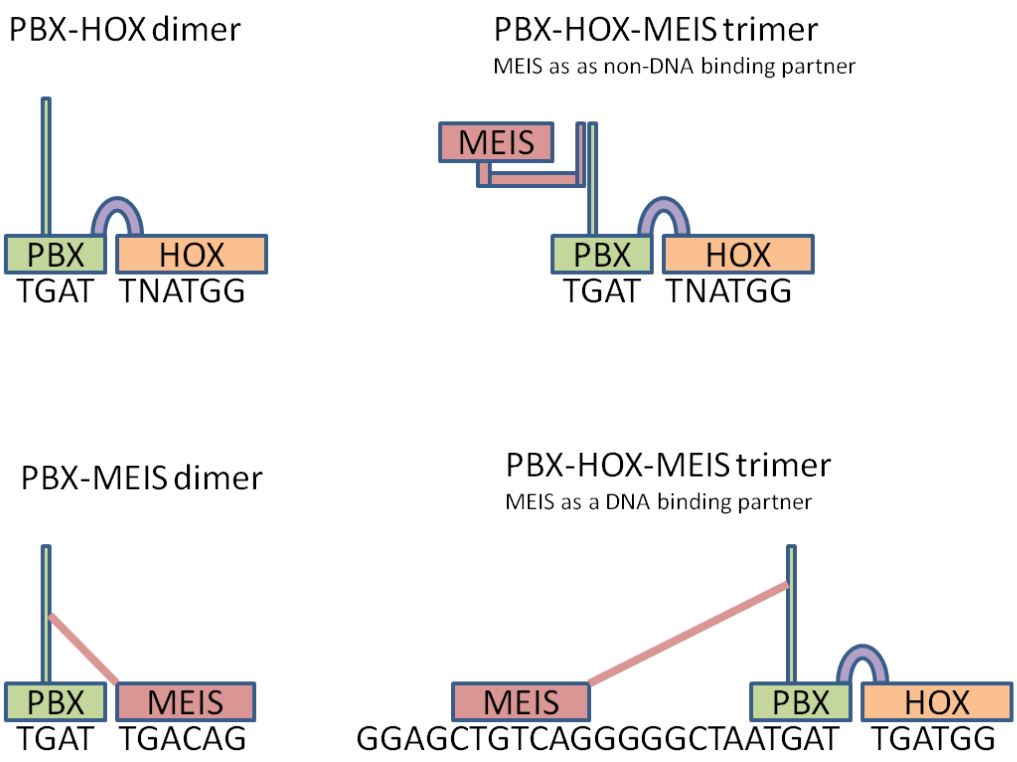


Figure 1.4: DNA binding complexes of HOX proteins and their cofactors
Cooperative binding of HOX, PBX and MEIS to recognition sequences. HOX-PBX heterodimers can bind DNA together where both proteins make sequence specific contacts.

In HOX-PBX-MEIS heterotrimers MEIS can be either DNA binding or non-DNA binding. PBX can also function as a non-DNA binding partner in these trimeric complexes. Adapted from (Featherstone, 2003).

Trimer formation of MEIS-PBX-HOX with MEIS functioning as the non-DNA binding partner has also been described. One interesting observation in these trimers is that the HOX partner could be a non-ABD-B class protein (Jacobs et al., 1999; Shanmugam et al., 1999; Shen et al., 1999). Shanmugam et al (1999) have also shown a second trimer complex with PBX as the non-DNA binding partner. In this case, PBX is tethered to DNA-bound MEIS and HOXA9 (Figure 1.4) (Shanmugam et al., 1999).

1.5.3. Regulation of *Meis1* expression:

Hox gene products are the best described regulators of *Meis1* expression. Many HOX proteins are known to directly and indirectly affect *Meis1* expression during development and cancer. HOXB4 and HOXA9 are upstream activators of *Meis1* in leukemia and hematopoiesis (Faber et al., 2009; Hu et al., 2009; Oshima et al., 2011). In mice, HOXD13 has been shown to directly bind and activate the expression of *Meis1* during limb development in the proximal regions of the limb bud (Salsi et al., 2008).

Meis1 is activated in response to retinoic acid (RA) signaling both *in vitro* and *in vivo*. In the proximal limb bud, *Meis1* expression is positively regulated by RA and repressed by Fibroblast growth factor (FGF) signaling (Mercader et al., 2000). In the embryonic stem cells (ESCs) the RA responsiveness of *Meis1* is mediated by RARγ and knockout of RARγ reduces the expression of *Meis1* (Kashyap et al., 2012).

During hematopoiesis *Meis1* plays a vital role in hematopoietic stem cell (HSC) survival and differentiation. Consequentially its expression is tightly regulated in hematopoietic tissues. Wild type Mixed lineage leukemia 1 protein (MLL1) which is a histone methyltransferase and MLL1 fusion proteins are important upstream activators of *Meis1* expression in normal hematopoiesis and leukemogenesis (Armstrong et al., 2002; Dou and Hess, 2008; Milne et al., 2005a; Wang et al., 2011b; Wong et al., 2007; Zeisig et al., 2004). A study also shows that AML1a, a short isoform of AML1 (RUNX1) might function upstream of *Meis1* and *Hox* genes to regulate hematopoiesis (Tsuzuki and Seto, 2012). ELF1 which belongs to the ETS family of transcription factors is also an upstream activator of *Meis1* expression during hematopoiesis. ELF1 was found to bind to a conserved sequence upstream of the *Meis1* start site and activate its expression in leukemic cell lines (Xiang et al., 2010).

In hematopoietic cells, *Meis1* has also been found to be a target of GFI1B and its cofactors LSD1 and RCOR1. This complex functions to repress *Meis1* expression in the erythroid lineage (Chowdhury et al., 2013; Horman et al., 2009; Sprussel et al., 2012). CREB1 has been shown to highly induce the expression of *Meis1* in hematopoietic cells consistent with the observation that the *Meis1* enhancer carries a

cAMP responsive element (CRE) which is specific for CREB binding (Esparza et al., 2008; Zhang et al., 2005). It has also been shown that HOXA9 induces the expression of *Meis1* indirectly by binding to the upstream enhancer sequences of *Creb1* and *Pknox1* and activating their expression. *Meis*$^{+/-}$ *Hoxa*9$^{-/-}$ compound mutant mice exhibit various defects in the hematologic lineage concomitant with the decrease in the levels of *Meis1* (Hu et al., 2009).

In a recent study, highly conserved non-coding regions (HCNRs) spanning the human *Meis1* genomic locus was screened for enhancer functions. Through this study PAX6 was found to bind to an enhancer to regulate retinal and tectum specific expression of *meis1.1* in zebrafish (Royo et al., 2012).

Many microRNAs have been found to regulate the expression of *Meis1* in cancer and hematopoiesis. During megakaryocyte differentiation *Meis1* is regulated by miR-155. Loss of miR-155 is needed for Meis1-mediated megakaryopoiesis (Romania et al., 2008). In infant leukemia, *Meis1* is the prime target of miRNAs miR-495 and miR-196b (Jiang et al., 2012; Li et al., 2012b).

1.5.4. Physiological roles of MEIS1:

MEIS1 is an essential transcription factor involved in many physiological and oncological processes. It is expressed very early during development, performing indispensable roles during embryogenesis and later on during adult life. In mouse embryos, there is indication that *Meis1* is expressed as early as the blastocyst stage (Sonnet et al., 2012). Hisa et al (2004) conducted an extensive study on the importance of *Meis1* during mouse embryonic development. *Meis1* is vital for the survival of the embryo since knock-out homozygous mice perish around embryonic day (E) 14.5. The *Meis1* null and heterozygous mice were created by the insertion of a cassette containing the β-galactosidase gene into the *Meis1* homeodomain encoding region. A β-galactosidase gene knocked into the *Meis1* locus and thus driven by the *Meis1* promoter shows extensive staining in the nervous system and the sensory regions in the face (Hisa et al., 2004).

In particular, expression was observed in the eye, which was severely affected in homozygotic knockouts, as displayed by the degeneration of the lens and retina (Hisa et al., 2004). This could be due to the role of *Meis1* in regulating the expression of *Pax6* during eye morphogenesis. It has been shown that both MEIS1 and MEIS2 directly bind an upstream enhancer and activate the expression of *Pax6* (Zhang et al., 2002). Apart from *Pax6,* MEIS1 and 2 have also been found to regulate the expression of *cyclin d1* and *c-myc* during vertebrate eye development. Both *Meis1* and *Meis2* are expressed specifically in the retinal progenitor cells and induce their proliferation (Heine et al., 2008).

Similar to eye morphogenesis, *Pax6* has been found to be directly regulated by PBX and MEIS family proteins during pancreatic development as well. MEIS1, MEIS2 and PKNOX1 were found to be expressed in the developing pancreas and along with PBX2 they upregulate *Pax6* expression (Zhang et al., 2006). In the pancreatic duct cells, a trimeric complex of MEIS1, PBX1 and PDX1 which is a homeodomain transcription factor has been found to regulate the expression of Keratin 19 (Deramaudt et al., 2006). *Meis1* expression is also seen in the ear primordia and the cochlear epithelium (Hisa et al., 2004). Expression studies in chicken have also shown staining for *Meis1/2* in specific regions of the otic epithelium indicating a possible role for *Meis* genes in the morphogenesis and development of the auditory system (Sanchez-Guardado et al., 2011a).

Similar to HTH-EXD mediated limb development in *Drosophila,* MEIS1 is expressed in the proximal limb regions during vertebrate embryogenesis were it functions to translocate PBX1 to the nucleus. Retroviral mediated ectopic expression of MEIS1 in chicken embryos leads to the proximalization of the limb structures (Mercader et al., 1999). It was further shown that MEIS1 inhibits the formation of the distal portion of limbs by affecting the proliferation and the differentiation of the cells in the apical ectodermal ridge (AER) of the dorsal limb bud in mice (Mercader et al., 1999; Wu and Cohen, 2000). *Meis1* is activated in response to RA signaling in the proximal regions of the limbs (Mercader et al., 2005). FGFs expressed in the distal portions of the limb bud repress RA signaling, thereby inhibiting MEIS1 production in the distal regions (Mercader et al., 2000). Some studies have shown that functions other that PBX nuclear localization might be important during MEIS1-mediated limb

development. For example, PBX1 deficiency does not negate or reduce the proximalization effect of ectopic *Meis1* expression (Mercader et al., 2009).

The midgut, mediastinum, spinal cord as well as vital internal organs like heart and lungs, liver, kidney (Hisa et al., 2004) and epididymis (Bomgardner et al., 2003) also stain extensively for MEIS1. In particular *Meis1* in conjunction with *Pbx1/2/3* play essential roles in heart development in mouse. *Meis1* deficiency leads to congenital heart malformations in mouse similar to but less severe than those seen in *Pbx1/2/3* deficient mice implying that the defects are mediated by PBX-MEIS1 interaction (Stankunas et al., 2008).

Meis1 is expressed in the olfactory epithelium where it identifies a distinct class of self-renewing cells. It functions in a genetic pathway involving the transcription factors *Sox2* and *Ascl1* to pattern the olfactory epithelium (Tucker et al., 2010). Another study showed that *Meis1* is expressed in the telencephalon area of the forebrain and contributes to PBX1 nuclear localization there (Toresson et al., 2000). *Meis1* expression is also found in the uteral endometrium in human samples were it functions to regulate target genes including *itgb3* in cooperation with *HoxA10.* Furthermore, siRNA-mediated knockdown of *Meis1* resulted in a decreased incidence of embryo implantation in target mice (Xu et al., 2008).

Meis1 is thus widely expressed in many tissues during development. This is the reason behind the pleiotropic phenotype observed with the *Meis1* null mice (Hisa et al., 2004). Similar pleiotropic effects were also observed in mutants of the orthologous *unc-62* gene in *C. elegans* embryos suggesting evolutionarily conserved roles for *Meis1* in regulating animal embryogenesis (Hisa et al., 2004; Van Auken et al., 2002). In *Xenopus*, the *Meis1* homolog *XMeis1* is expressed in the neural plate and may be involved in the specification of neural crest cells (Maeda et al., 2001; Maeda et al., 2002). The zebrafish homolog, *meis1.1*, similar to its mouse counterparts, is important for Hox function. *meis1.1* is expressed mainly in the central nervous system and eyes and plays important roles in hindbrain patterning in conjunction with *Hox* genes in zebrafish (Choe and Sagerstrom, 2004; Choe et al., 2002; Waskiewicz et al., 2001).

Since *MEIS1* plays such a vital role in many tissues in the body its mutation leads disruption in normal physiological functions in humans. For example, it has

been implicated as a causative gene for some genetic disorders. Mutations in *MEIS1* are involved in restless leg syndrome (RLS). This could probably be due to its role in regulation genes important for iron metabolism since iron homeostasis has been found to be disrupted in RLS patients (Catoire et al., 2011; Schormair et al., 2011; Schulte et al., 2011; Yang et al., 2011). Linkage studies show that *MEIS1* is also a candidate gene for the PR interval duration measured using an electrocardiogram. The PR interval measures the electrical conduction in the atrium and the antrioventricular node and is an inherited phenomenon (Smith et al., 2011).

1.5.5. MEIS1 in Hematopoiesis:

As described above, gene knock-out studies in mice reported in two separate studies indicate a prominent role for *Meis1* in normal hematopoiesis (Azcoitia et al., 2005; Hisa et al., 2004). Homozygous *Meis1* mutants show hypoplasia of the liver, which is the primary site for hematopoiesis in the developing embryo. The hematopoietic stem cell population in the fetal liver is grossly reduced in the mutants. Loss of MEIS1 function also results in concomitant decrease in the expression of the hematopoietic markers *Runx1* and *Gata1*. *Meis1* mutant mice show extensive hemorrhaging along the trunk and limbs. This is attributed to defective angiogenesis and incorrect vasculature formation in these mice. Concomitantly, the expression of PECAM, a marker for endothelial cells, is also reduced and the capillary network is altered in the *Meis1* mutant mice (Azcoitia et al., 2005; Hisa et al., 2004).

Among the differentiated hematopoietic cells, *Meis1* is important for the survival and the function of platelets (Carramolino et al., 2010). High levels of CD41 expression is a marker for megakaryocytes and platelets. Staining for CD41 in heart sections of *Meis1* mutants revealed the complete absence of megakaryocytes or platelets. When compared to wild-type mice, megakaryocytic colonies failed to form when fetal liver cells from *Meis1* mutant mice were cultured in appropriate growth factors (Azcoitia et al., 2005; Hao et al., 2006; Hisa et al., 2004). These observations, along with the identification that both MEIS and PBX activate the expression of

Platelet Factor 4 (PF-4) in megakaryocytes (Okada et al., 2003), establish *Meis1* as an important regulator of megakaryocyte differentiation.

When embryonic stem cells are allowed to differentiate along the hematopoietic lineage in culture, *Meis1* over-expression leads to an increase in the number of hematopoietic progenitors. But whereas there is a manifold increase in the number of CD41+ CD42d+ cells (megakaryocyte precursors), CD71+ cells (erythroid progenitors) are reduced substantially. MEIS1 activates various genes which have been shown to be expressed in megakaryocytes and could play a role during their differentiation. MEIS1 also reduces the expression of various erythrocyte-specific genes such as hemoglobin α (*Hba-α1/2*) and glycophorin A (*Gypa-a*). Thus MEIS1 might function at the crossways between megakaryocyte and erythroid differentiation (Cai et al., 2012).

Apart from the role it plays in differentiated progenitors in the hematopoietic lineage, recent evidence suggest that MEIS1 could perform important roles in HSCs. MEIS1 is important for the survival and the quiescence of long term repopulating-HSCs (LT-HSCs). The HSC niche is a hypoxic environment which is important to maintain the quiescent nature of these cells. The LT-HSCs in *Meis1* null mice (Azcoitia et al., 2005) lose their stem-cell character, start proliferating, enter the blood stream and eventually undergo apoptosis. Conditional tissue-specific knock-out of *Meis1* in HSCs has a deleterious effect on the development of the blood lineage cells. Loss of *Meis1* in LT-HSCs leads to reactive oxygen species (ROS) accumulation and apoptosis (Kocabas et al., 2012; Unnisa et al., 2012). MEIS1 directly regulates the expression of the hypoxia signaling factors *Hif1α* and *Hif2α*. HIF1α is a general regulator of metabolism in cells and helps them survive in hypoxic conditions by switching energy production from aerobic mitochondrial respiration to anaerobic glycolysis. HIF2α has been shown to have a protective effect against oxidative stress. Thus, by functioning upstream of these factors, MEIS1 plays a vital role in the survival and stemness of the LT-HSCs (Kocabas et al., 2012; Simsek et al., 2010).

Two recent papers have explored the role of *meis1* during zebrafish hematopoiesis. Interestingly, the papers present a contradictory picture of the role of *meis1* during hematopoiesis. Whereas Pillay et al (2010) use morpholino-mediated knockdown to suggest that *meis1* is important for primitive hematopoiesis by acting

upstream of s*cl* and *gata1*, Cvejic et al (2011) using similar studies observe that *meis1* functions downstream of *scl* and independent of *gata1* during hematopoiesis (Cvejic et al., 2011; Pillay et al., 2010). The papers also differ on the role of *meis1* during myelopoiesis in zebrafish. Based on the observation that the expression of PU.1, a marker for myelopoiesis, is increased in *meis1* morphants, Pillay et al (2010) conclude that MEIS1 represses myeloid differentiation in zebrafish. Cvejic et al (2011) do not observe such changes.

1.5.6. MEIS1 and Cancer:

Meis1 has been identified as an oncogene and found to be over-expressed in many cancers including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), mixed lineage leukemia (MLL), neuroblastoma, ovarian cancer (Crijns et al., 2007) Wilms' tumor (Dekel et al., 2006) and breast cancer (Doolan et al., 2009). *Hoxa9* over-expression transforms normal bone marrow cells into leukemic cells (Argiropoulos and Humphries, 2007; Bach et al., 2010; Calvo et al., 2000; Drabkin et al., 2002; Eklund, 2006; Kroon et al., 1998; Lawrence et al., 1999). *Meis1* has been shown to have the potential to transform bone marrow cells into leukemic cells when over-expressed along with HOXA9. The transformed bone marrow cells cause myeloid leukemia when transplanted into mice (Drabkin et al., 2002; Kroon et al., 1998; Lawrence et al., 1999). The transforming potential of HOXA9 is greatly increased by its complex formation with MEIS and PBX and the TALE interaction site at the HOXA9 N-terminus is essential for its oncogenesis (Schnabel et al., 2000).

Hoxa9 over-expression mediates a *Meis1*-independent block to differentiation mediated by granulocyte macrophage colony stimulating factor (GM-CSF) (Calvo et al., 2000) but *Meis1* is important for the differentiation block mediated by granulocyte (G-CSF) and Macrophage (M-CSF) -colony stimulating factors (Calvo et al., 2001), respectively. MEIS1 also had a profound activating effect on stem cell factor (SCF)-mediated proliferation of the *HoxA9* immortalized cells (Calvo et al., 2000; Calvo et al., 2001). It is also essential for *Hoxa7*-induced AML (Afonja et al., 2000). Hence, *Meis1* along with certain *Hox* genes seems to be important for maintaining self-renewal and inhibiting differentiation of hematopoietic progenitors.

Meis1 by itself lacks the ability to cause AML when over-expressed but it accelerates the onset of leukemia caused by *Hox* genes. This feature is observed primarily for *Meis1* whereas other TALE class genes like *Pbx1* and *Pknox1* lack this ability. In fact, over expression of *Pknox1* along with *Hox* genes delays the onset of leukemia in mice (Thorsteinsdottir et al., 2001).

Meis1 is also upregulated in some acute lymphoblastic leukemia (ALL) and AML caused by the translocation of the *MLL1* gene (Imamura et al., 2002; Rozovskaia et al., 2001). MEIS1 also accelerates AML caused by NUP98-HOXD13 (ND13) (Pineault et al., 2003) and NUP98-HOXA9 (Kroon et al., 2001) fusion proteins. It has been shown that the NUP98-HOX fusions activate the expression of *Meis1* and *Hox* genes thereby causing increased self-renewal and proliferation of myeloid progenitors (Calvo et al., 2002) The NUP98-HOX fusions by themselves induce a mild form of myeloproliferative-like disease. But in conjunction with MEIS1, they induce an aggressive form of AML (Pineault et al., 2003). Indeed, MEIS1 has the potential to collaborate with a non-leukemogenic NUP98-HOXB4 fusion and convert it into an AML-causing oncogene (Pineault et al., 2004). In addition, MEIS1 is also important for the survival and expansion of leukemic stem cells in AML caused by nucleophosmin 1 (*NPM1*) mutations (Woolthuis et al., 2012).

MEIS1 along with ABD-B class of HOX proteins HOXA9 or HOXA10 is able to confer leukemogenic activity on meningioma 1 (MN1) protein. In blood progenitors, retrovirally introduced *Mn1* transforms common myeloid progenitors (CMPs) but not granulocyte macrophage progenitors (GMPs) and HSCs. When introduced along with *Meis1* and either *Hoxa9* or *Hoxa10*, *Mn1* was able to transform GMPs. MEIS1 alters the transcriptomic profile of MN1-expressing cells to upregulate many oncogenes. Hence by altering the transcriptome and/or epigenome, MEIS1/ABD-B HOX proteins are able to convert cells which are normally not susceptible to transformation into readily transformable cells (Heuser et al., 2011).

1.5.7. MEIS1 in mixed lineage leukemia (MLL):

Comparison of the expression levels of *Hox* genes, *Meis1* and *Mll1* in normal hematopoietic cells versus AML cells first revealed a causative role for these genes in

leukemia (Kawagoe et al., 1999). *Meis1* along with *HoxA9*, *HoxA7* and *HoxA10* were then identified to be highly over-expressed in mixed lineage leukemia, also called infant leukemia, by DNA microarray studies and RT-PCR analysis of patient samples and cell lines (Ferrando et al., 2003; Imamura et al., 2002; Li et al., 2009; Poppe et al., 2004; Rozovskaia et al., 2001; Wang et al., 2011b; Zeisig et al., 2004). This has led to the understanding that the deregulation of *Hox* genes and their cofactors form the primary mechanism by which infant leukemia is established. In fact, sustained over-expression of *Hox* and *Meis1* is able to substitute for MLL-fusion proteins in causing leukemia in experimental models (Zeisig et al., 2004). Knockdown of the expression of MLL fusion proteins impairs leukemogenicity due to a reduction in expression of *Hox* and *Meis1* (Thomas et al., 2005), and similarly knockdown of *Hoxa9* in MLL-rearranged leukemic cells leads to increased apoptosis and decreased leukemogenicity (Faber et al., 2009). MLL1 wild-type as well as fusion proteins bind to the *Hoxa9* and *Meis1* upstream regions and modify histones extensively. The histone modification leads to higher transcriptional levels and expression of *Hoxa9, Hoxa7* and *Meis1* genes (Milne et al., 2005a).

Knockdown of *Meis1* in MLL-rearranged leukemic cells leads to decreased proliferation and reduced leukemogenicity in transplanted mice. A mechanism by which MEIS1 operates in leukemic cells is through the activation of important cell cycle regulators (Kumar et al., 2009). Knockdown of *Meis1* also decreases the efficiency of engraftment of MLL rearranged leukemic cells when they are transplanted into mice (Orlovsky et al., 2011). In fact, *Meis1* is a natural target of miR-495 in MLL leukemia and over-expression of this tumor suppressor miRNA reduces the viability of the leukemic cells (Jiang et al., 2012). One of the important targets of HOXA9 and MEIS1 in MLL1-translocation-mediated leukemia is *c-myb* (Hess et al., 2006). The targets of MEIS1 in MLL-leukemia include genes which normally exhibit an embryonic stem cell-like profile, thereby suggesting that *Meis1* might be important for the stem cell character of MLL leukemic cells (Kumar et al., 2010)

Wong et al (2007) have conclusively shown that *Meis1* is indispensable for the induction and maintenance of MLL leukemia. *Meis1* is important for shortening the latency period in mouse models for MLL leukemia. MLL fusion proteins were unable to transform fetal liver cells derived from *Meis1*-knockout mice, whereas they readily transformed wild-type cells. These activities of MEIS1 require DNA-binding, PBX

interaction and transcriptional activation since the deletion of the corresponding functional regions impairs its ability to facilitate MLL-mediated leukemia (Wong et al., 2007).

Together, these studies establish a vital causative role for MEIS1 in the induction of leukemia and possibly other cancers.

1.5.8. Mechanism of MEIS1 cancer induction:

Transcriptional activation of potential oncogenes is the mechanism by which MEIS1 causes AML. The C-terminal domain (CTD) of MEIS1 has the transcriptional activation function. By using various deletion mutants and a VP16-activation domain substitution of the MEIS1 C-terminus, it was proved that the leukemogenic property of MEIS1 is dependent on the transcriptional activation function of this domain. Interaction with PBX was similarly found to be indispensible for MEIS1-mediated AML induction (Mamo et al., 2006). VP16-MEIS1 is able to induce AML by the activation of leukemogenic genes without the need for over-expressing HOX proteins; however co-expression of *Hox* genes further accelerates this effect (Wang et al., 2006).

The CTD of MEIS1 confers oncogenic properties to PKNOX1, another Meis family protein (Bisaillon et al., 2011). Previous studies have shown that PKNOX1 has tumor suppressor functions (Longobardi et al., 2010). A PKNOX1 mutant carrying the MEIS1 CTD adjacent to its own CTD, transforms bone marrow cells and subsequently causes an accelerated leukemia in mice, in association with HOXA9 whereas wild-type PKNOX1 delays oncogenesis. Both MEIS1 and a chimeric PKNOX1 carrying a MEIS1 CTD were found to activate similar genes in the transformed cells (Bisaillon et al., 2011). One of most important oncogenes up-regulated by MEIS1 is the tyrosine-kinase, *Flt3*. It also activates many genes of the short-term hematopoietic stem cell (ST-HSC) lineage. Other known genes involved in HOXA9- and MEIS1-induced AML are *Trib1* and *Evi1* (Jin et al., 2007).

Huang et al (2005) showed that the MEIS1A C-terminus is responsive to protein kinase A (PKA) signaling. Deletion of amino acids 335-390 of the C-terminus abolished the PKA responsiveness of MEIS1, thereby inhibiting its transcriptional

activation function. Even though PKNOX1 belongs to the same family and can substitute for MEIS1 in many *in vitro* assays, it does not respond to PKA signaling (Huang et al., 2005). Apart from the transcriptional activation which is mediated directly by its CTD, MEIS1 also increases histone H4 acetylation at target gene promoters. MEIS1 inhibits HDAC mediated repression by competing with it for PBX binding and also induces CBP recruitment to the target genes (Choe et al., 2009).

Meis1 is expressed highly in many neuroblastoma cell lines and in the case of IMR-32 cells it is amplified and present as multiple copies (Geerts et al., 2003; Jones et al., 2000; Spieker et al., 2001). Some *Pbx* and *Tgif* family genes were also found to be expressed in some neuroblastoma cell lines (Geerts et al., 2003). There is also evidence of *Meis1* over-expression in many neuroblastoma patient samples (Spieker et al., 2001). The MEIS1 protein may play an important role in maintaining the proliferation and survival of these tumors as revealed by the growth arrest and differentiation of neuroblastoma cell lines transfected with a dominant negative form of MEIS1 (Geerts et al., 2003).

Hence, *Meis1* is an important oncogene for the establishment of leukemia and other cancers. It functions upstream of many oncogenes and activates their expression. Furthermore, evidence shows that it induces cell proliferation and survival which are two key features required for oncogenesis. However, contrary to the standard view that *Meis1* is involved in self-renewal and proliferation of progenitor cells and cancer stem cells, Wermuth et al (2005) have shown that *Meis1* over-expression in lymphoblast and fibroblast cell lines leads to apoptosis mediated by caspase 3 and 8 (Wermuth and Buchberg, 2005). This remains a sole observation and is yet to be substantiated by other studies.

1.5.9. MEIS1 targets:

MEIS1 regulates the expression of many downstream genes which function as effectors to modulate cellular function. Many of these targets encode transcription factors which further activate other genes in the cascade. Cell cycle regulators like cyclins are also important targets of MEIS1. This is important because MEIS1 is involved in activating cell proliferation in many cell types and maintaining self

renewal in HSCs. Tissue specific targets of MEIS1 have also been identified. For example, Dynamin 3, a GTPase involved in endocytosis is expressed mainly in monocytes and platelets and is regulated by MEIS1.

MEIS1 along with PBX proteins is involved in the regulation of metabolic enzymes in the liver and pancreas. Activation of the expression of *cyp17* gene which encodes Cytochrome P450 17 α-hydroxylase/17, 20 lyase, by cAMP signaling is mediated through PBX and MEIS1. Both PBX and MEIS1 co-operatively bind the cAMP response sequence (CRS1) of the *cyp17* promoter (Bischof et al., 1998a; Bischof et al., 1998b). PBX1 and MEIS1/PKNOX1 also enhance the expression of the malic enzyme genes in hepatocytes in response to thyroid hormone signaling. The PBX-MEIS heterodimer has been shown to bind to region E of the malic enzyme promoter co-operatively with the thyroid hormone alpha (TRα) receptor and retinoid X receptor (RXR) (Wang et al., 2001).

One study shows that MEIS1 is an upstream regulator of transcription in the mitochondrion. Microarray and qRT-PCR analysis after the knockdown of *Meis1* showed a decrease in expression of many mitochondrial genes. Furthermore, Chromatin immunoprecipitation ChIP experiments also showed the binding of MEIS1 to mitochondrial DNA (Tomoeda et al., 2011).

Chromatin immunoprecipitation and DNA binding assays have shown direct binding of MEIS1 to the enhancers of many targets. Other targets have been identified by gene expression profiling using microarrays and luciferase assay. Table 1.2 summarizes the downstream target genes of MEIS1 identified thus far.

Table 1.2: Downstream targets of Meis1. The method used to identify these targets have also been summarized

Name of target gene	Tissue or Cell type used	Methods used for identification	References
C-myb	Bone marrow cells over-expressing Hoxa9 +Meis1 (1) and HPC7 and FMH9 cells (2)	Microarray, qRT-PCR (1) and ChIP (2)	(Hess et al., 2006) (1) (Dasse et al., 2012) (2)
Pf4	HEL megakaryocyte cell line	EMSA with nuclear extracts on a target promoter	(Okada et al., 2003)
Cyclind1 and C-myc	Zebrafish	In-situ hybridization in zebrafish embryos injected with (1) meis1 morphants (2)meis1 inactivating constructs	(Bessa et al., 2008) (Heine et al., 2008)
Trib2,Ccl3, Flt3,Dlk1, Ccl4, Rgs1, Pf4	Nup98-HoxD13 Leukemic cells	Microarray and ChIP	(Argiropoulos et al., 2008)
Flt3, Cd34 and Erg1	Bone marrow cells	Microarray analysis and qRT-PCR	(Wang et al., 2005) (Wang et al., 2006)
Cyp17 (bovine)	Y1 mouse adrenal cell nuclear extract	EMSA using a cAMP responsive sequence in the enhancer of Cyp17	(Bischof et al., 1998b) (Bischof et

			al., 1998a)
p21	U937 cells	Luciferase assay on a 35 bp p21 enhancer	(Bromleigh and Freedman, 2000)
Meis1	Forskolin treated P19 cells	ChIP	(Goh et al., 2009)
Hoxb1	P19 cells	ChIP	(Huang et al., 2005)
Hoxb2	COS7 cells	EMSA using Hoxb2 R4 enhancer with in vitro translated proteins, Luciferase assay	(Jacobs et al., 1999)
Ephrin B2	MAE cells	ChIP	(Sohl et al., 2009)
Hif-1α	LT-HSCs and Kasumi1 cells (1), whole bone marrow and Meis1 knockout bone marrow	ChIP using Kasumi1 (1), Western blot for Hif1α using Meis1 knockout bone marrow (2)	(Simsek et al., 2010) (1) (Unnisa et al., 2012) (2)
Hif-2α	LT-HSCs and Kasumi1 cells	ChiP using Kasumi1 cells, Luciferase assay using Hif2α promoter sequences	(Kocabas et al., 2012)
Plac8, Ptger3, Serpinb2	ES cells with inducible Meis1 expression	Microarray analysis and qRT-PCR	(Cai et al., 2012)

	differentiating towards the hematopoietic lineage.		
Pax6	Chicken embryonic lens cDNA library, In vitro translated proteins	Identification of proteins binding to the 26bp Pax6 enhancer from cDNA library, EMSA for the 26bp fragment using in vitro translated MEIS1 protein	(Zhang et al., 2002)
Keratin 19	WT-PDC cells	EMSA with a 16 bp region of the keratin19 promoter and luciferase assays	(Deramaudt et al., 2006)
Sox3	NT2/D1 neuronal cells	EMSA and Luciferase assay	(Mojsin and Stevanovic, 2010)
Gli3	Chick and E10.5 mouse embryos	ChIP	(Coy et al., 2011)
Dnm3	CHRFmegakaryo-cytic cell line	ChIP-Seq	(Nurnberg et al., 2012)

1.6. MEIS2:

Meis2 was identified due to its high sequence similarity with *Meis1* (Nakamura et al., 1996a; Smith et al., 1997a; Steelman et al., 1997). The gene is highly responsive to RA induction in P19 cells (Oulad-Abdelghani et al., 1997). *Meis2* shows predominant expression in the central nervous system during mouse embryogenesis (Cecconi et al., 1997) and in the brain and female genital tract in adult mouse (Oulad-Abdelghani et al., 1997). *Meis2* is also expressed in the telencephalon during mouse development, in regions both overlapping and distinct from *Meis1*. miR-9 regulates the expression of *Meis2* thereby controlling the MEIS2-PAX6 pathway in the telencephalon (Gunhaga et al., 2003; Shibata et al., 2011; Toresson et al., 2000). *Meis*2 is also expressed during development in the mesencephalon where along with *Pbx* it has been shown to regulate Ephrin-a8 expression (Shim et al., 2007) In the mesencephalon, MEIS2 collaborates with the homeodomain transcription factor OTX2 to specify the tectum.

Further evidence supports a functional role for MEIS2 in the CNS. MEIS2 regulates dopamine receptor expression (Yang et al., 2000b), and during hindbrain patterning, MEIS2 defines the expression of *Krox20* in rhombomere 3 (Wassef et al., 2008). RA production in the hindbrain is also controlled by MEIS2 by regulating the expression of the enzyme Retinaldehyde dehydrogenase 2 (RALDH2) (Vitobello et al., 2011). The *Meis2* orthologs in zebrafish, *meis2.1* (Zerucha and Prince, 2001) and *meis2.2* (Biemar et al., 2001; Waskiewicz et al., 2001) also have major sites of expression in the CNS.

Similar to MEIS1, MEIS2 forms trimeric complexes with PBX1 and PDX1 in pancreatic cells. A PDX1-PBX1B-MEIS2 complex was found to regulate the tissue-specific expression of the *elastase1* gene in pancreatic acinar cells (Liu et al., 2001; Swift et al., 1998). Like MEIS1, MEIS2 is expressed during limb development and contributes to the proximalization of the limb bud in chick embryos (Capdevila et al., 1999; Mercader et al., 2000; Mic et al., 2004). MEIS2 regulates *Pax6* expression during eye morphogenesis, again similar to MEIS1. The MEIS2-PAX6 pathway is modulated by miR-204 in the retina. miR-204 induces the differentiation of the lens epithelium by relieving the differentiation block caused by MEIS2 (Conte et al., 2010). Retinal expression of *Meis2* is also maintained by RA and Shh signaling (Bumsted-

O'Brien et al., 2007; Heine et al., 2009). In chick embryos, MEIS2 collaborates with MEIS1 to maintain the proliferative state of the retinal progenitor cells (Bumsted-O'Brien et al., 2007; Heine et al., 2008; Zhang et al., 2002).

Like MEIS1, MEIS2 has a transcriptional activation function at its C-terminus and its binding to OTX2 increases the transcriptional activation by the later (Agoston and Schulte, 2009; Hyman-Walsh et al., 2010). Interestingly, Hyman-Walsh et al (2010) have also proposed that the HM domains of MEIS2 possess an auto-inhibition function. In the absence of PBX binding, the HM domain is proposed to fold and contact the transcriptional activation domain thereby inhibiting its function. Even though auto-inhibitory domains have not been identified in MEIS1, based on sequence similarity the authors propose that such domains may exist in all MEIS proteins (Hyman-Walsh et al., 2010).

Other observed functions for *Meis2* are in cardiac development (Paige et al., 2012) and ovarian and follicular gene regulation. *Meis2* is expressed in ovarian tissues (Sarno et al., 2005; Villaescusa et al., 2004) and may be involved in causing ovarian cancer (Crijns et al., 2007).

1.7. PBX proteins:

Pbx1 was the first gene of the PBC family of TALE cofactors to be identified. It was first recognized as a homeobox gene fused to the gene encoding the E2A bHLH transcription factor as a result of a t(1;19) chromosomal translocation. Different variants of the fusion protein occur as a result of alternative splicing and different chromosomal breakpoints (Hunger et al., 1991; Izraeli et al., 1992; Kamps et al., 1991; Kamps et al., 1990; Nourse et al., 1990; Numata et al., 1993; Privitera et al., 1992). Subsequently, two other members of the PBC family, *Pbx2* and *Pbx3*, were identified based on sequence homology with *Pbx1* (Monica et al., 1991). A fourth vertebrate Pbx gene, *lazarus* or *Pbx4* was identified in zebrafish and was shown to perform important roles in hindbrain segmentation (Popperl et al., 2000). As described above, *Drosophila exd* is a homolog of *Pbx1* (Rauskolb and Wieschaus, 1994; Rauskolb et al., 1993).

The *E2a-Pbx1* fusion gene has the ability to cause leukemia in mice transplanted with transfected bone marrow cells (Dedera et al., 1993; Kamps and Baltimore, 1993; Uckun et al., 1993). PBX1 was subsequently determined to be a DNA-binding transcription factor which acquires an activation function when fused with E2A (Kamps et al., 1996; LeBrun and Cleary, 1994; Lu et al., 1994; Monica et al., 1994; Van Dijk et al., 1993).

PBX proteins bind DNA in co-operation with HOX family proteins and this property is retained in the E2A-PBX1 fusion (LaRonde-LeBlanc and Wolberger, 2003; Lu et al., 1995; Phelan and Featherstone, 1997; Phelan et al., 1995; van Dijk et al., 1995). Interaction with PBX has been shown to increase the DNA binding specificity of HOX proteins (Neuteboom and Murre, 1997). As with EXD, PBX also interacts with HOX proteins. The interaction domain on the HOX partner has been mapped to a small tryptophan-containing motif with the consensus F/YPWM or in the case of ABD-B HOX proteins, the consensus ANW, and its flanking residues N-terminal to the homeodomain. (Chang et al., 1996; Knoepfler and Kamps, 1995; Knoepfler et al., 1996; Neuteboom et al., 1995; Passner et al., 1999; Phelan and Featherstone, 1997; Phelan et al., 1995; Piper et al., 1999; Shanmugam et al., 1997; Shen et al., 1997a). The homeodomain and GKFQ residues C-terminal to the HD in PBX are sufficient for binding to the HOX partner and they have been shown to be important for E2A-PBX1 mediated leukemogenesis (Chang et al., 1997a; Green et al., 1998; Lu and Kamps, 1996; Passner et al., 1999; Piper et al., 1999; Sprules et al., 2000).

The HOX-PBX interaction is important for their transcriptional activities. The oncogenic properties of HOX proteins require their interaction with PBX1 (Knoepfler et al., 2001; Krosl et al., 1998; Schnabel et al., 2000). The heterodimeric complex has been shown to recruit both transcriptional activators and repressors to regulate transcription. HOX-PBX complexes under basal conditions recruit HDAC1 and NcoR/SMRT complexes via direct interaction with PBX to repress transcription But when induced by PKA signaling or RA induction they replace the repressive marks with active marks like CREB which binds HOX proteins (Asahara et al., 1999; Saleh et al., 2000b). Hematopoietic PBX-interacting protein (HPIP), found only in hematopoietic cells, has been shown to bind PBX through the HOX-interaction domain and inhibit PBX-HOX interaction (Abramovich et al., 2000).

Interestingly, non-homeotic homeodomain-containing proteins like Engrailed (Peltenburg and Murre, 1996, 1997), PDX-1 (Dutta et al., 2001), MEOX (Thiaville et al., 2012), EMX2 (Capellini et al., 2010) and CDX2 (Liu et al., 2006) have also been shown to bind PBX. PBX-PDX interaction is required for normal pancreatic function (Dutta et al., 2001) In ovarian cancer cells, PBX1 has been shown to function upstream of *Meox,* activating its expression (Thiaville et al., 2012). During scapula development in mouse, PBX1 regulates the expression of *Alx1* by cooperatively binding to its enhancer as a heterodimer with EMX2 (Capellini et al., 2010). PBX1 also functions together with estrogen receptor alpha to regulate its downstream genes. In breast cancer, PBX1 has vital contributions to estrogen signaling and functions to activate oncogenes (Magnani et al., 2011).

MEIS family proteins are indispensable partners for the functions of wild-type PBX. E2A-PBX1 fusions lack the ability to bind MEIS proteins because the PBX component of these fusion do not retain the MEIS1-interaction domains (Chang et al., 1997b; Knoepfler et al., 1997). Additionally, HOX-PBX-MEIS heterotrimers have also been described *in vivo* and are important for many normal developmental and physiological processes and oncogenic transformation. In these complexes PBX and MEIS can be either DNA-binding or non-DNA-binding. PBX and MEIS homodimers have also been observed (Ferretti et al., 2000; Ferretti et al., 2005; Jacobs et al., 1999; Penkov et al., 2000; Schnabel et al., 2000; Shanmugam et al., 1999; Shen et al., 1999).

PBX is actively exported out of the nucleus by a CRM1-mediated mechanism and its interaction with MEIS is required for the nuclear localization of both the proteins whereas binding with non-muscle myosin (NMHCB) is needed for its cytoplasmic anchoring. Two nuclear localization signals (NLS) within the homeodomain of PBX are also important for this process. In the absence of MEIS/PREP, these NLS are masked by binding of the N-terminal domain of PBX. MEIS's interaction with PBX unmasks these NLS and subsequently leads to its nuclear localization (Berthelsen et al., 1999; Huang et al., 2003; Saleh et al., 2000a). PKA phosphorylation has also been shown to play a role in its nuclear localization (Huang et al., 2003; Kilstrup-Nielsen et al., 2003).

Multiple upstream genes and downstream targets have been identified for *Pbx1*. Like *Hox* and the related *Meis* genes, *Pbx1* is also regulated by RA signaling.

But in contrast to the transcriptional upregulation of many *Hox* genes by RA, PBX proteins are upregulated by RA predominantly post-transcriptionally in P19 cells. Consequently, PBX proteins are required for the neuronal differentiation of P19 cells primarily by regulating *Bmp4* and *Decorin* following RA treatment (Knoepfler and Kamps, 1997; Qin et al., 2004a; Qin et al., 2004b). PBX1 has been shown to bind upstream enhancers like the CRS of *Cyp17*. Likewise, the E2A-PBX1 fusion binds the CRS and is able to activate gene expression in response to PKA signaling (Bischof et al., 1998a; Bischof et al., 1998b; Kagawa et al., 1994; Ogo et al., 1997). Similarly, *in vitro* studies have shown that PBX1 in association with MEIS/PKNOX1 regulates the genes encoding somatostatin (Goudet et al., 1999), glucagon (Herzig et al., 2000), malic enzyme (Wang et al., 2001) and UDP-glucuronosyltransferase 2B17 (Gregory and Mackenzie, 2002). Intronic PBX1 binding sites have been shown to regulate the expression of *Fgf8* (Gemel et al., 1999). It has also been shown to regulate myogenesis by interacting with the muscle-specific transcription factor MYOD and regulating muscle specific genes. In muscles, PBX1 functions as a 'pioneer' factor to mark the genes involved in muscle differentiation. These PBX marks then recruit the muscle specific transcription factor MYOD to downstream target genes including Myogenin (Berkes et al., 2004; Heidt et al., 2007; Sagerstrom, 2004).

1.7.1. Physiological functions of PBX:

The *Pbx1* gene encodes two alternatively spliced isoforms named PBX1A and PBX1B which differ at their C-termini, PBX1B being shorter than PBX1A. PBX1B does not contain a C-terminal transcriptional activation domain found in PBX1A (Asahara et al., 1999; Di Rocco et al., 1997; Moens and Selleri, 2006). Both isoforms differ in their expression patterns and their transcriptional properties. PBX1B is the predominantly expressed isoform. It is expressed early during mouse development and expression is seen in all germ layers and vital organs like lungs, kidney and heart (Schnabel et al., 2001) and hematopoietic tissues (DiMartino et al., 2001). PBX1B like its homolog EXD, has been shown to be important in proximo-distal limb patterning. *Pbx1b* is expressed in the proximal limb bud in mouse and heterozygous knockout mice display abnormalities in the proximal part of the limbs but not the distal portions (Selleri et al., 2001). Further evidence of PBX1 importance for the

formation of proximal limb structures is shown by its role during scapula development (Capellini et al., 2010).

The homozygous knock-out for *Pbx1* results in early embryonic mortality (DiMartino et al., 2001). *Pbx1* is indispensable for definitive hematopoiesis in the mouse embryo since knock-out mice display severe anemia due to defects in the myelo-erythroid lineage caused by reduced proliferation of these cells. *Pbx1* conditional knockout in the hematopoietic lineage results in the reduction of myeloid and lymphoid progenitors. Furthermore, the LT-HSCs have been shown to lose their capacity to self-renew and leave their quiescent state (Ficara et al., 2008). These features are very similar to what is observed in *Meis1* conditional knockout mice indicating a collaborative role for *Pbx1* and *Meis1* in normal hematopoiesis

Apart from the hematopoietic defects, *Pbx1* mutant mice display abnormalities in other tissues and organs including smaller size, internal organ hypoplasia, edema and skeletal defects (DiMartino et al., 2001; Selleri et al., 2001), defects in spleen development (Brendolan et al., 2005) and cardiac malfunctions resulting from defective artery formation (Chang et al., 2008). Particularly the skeletal formation is severely affected in the *Pbx1* mutants and cartilaginous tissue formation is also defective due to impaired chondrocyte differentiation (Selleri et al., 2001).

Other significant defects observed in *Pbx1* deficient mice are defects in pancreatic development, kidney malformations and hindbrain development. In the pancreas insulin secretion is impaired. *Pbx1* functions along with PDX1 to regulate important downstream genes like *Isl1* and *Atoh5* in the pancreas (Kim et al., 2002). The kidney defects observed could be possibly due to defects in kidney mesenchyme formation and abnormal ureteric branching (Schnabel et al., 2003). In zebrafish, PBX family proteins have also been implicated in hindbrain development (Waskiewicz et al., 2002). Hence PBX1 is important for the normal functions of a multitude of tissue and organ types as is evidenced by the pleiotropic phenotype observed in *Pbx1* mutant mice.

Compared to PBX1, PBX3 is mainly expressed in the CNS of the developing mouse embryo. Mice deficient for PBX3 exhibit normal gestational development but die due to respiratory problems post-birth. It has been shown that PBX3 interacts with the RNX transcription factor and regulates respiratory control (Di Giacomo et al.,

2006; Rhee et al., 2004). Mice in which the *Pbx3* gene is conditionally knocked-out in the regions posterior to the hindbrain and hence showing *Pbx3* expression only in the CNS display defects in locomotion (Rottkamp et al., 2008). A recent study showed that *Pbx3*, but not *Pbx1*/2, is important for *Hoxa9*-mediated leukemic transformation. In MLL-leukemia cells, *Pbx3* along with *Hoxa9* and *Meis1* is over-expressed and knockdown of the *Pbx3* leads to apoptosis (Li et al., 2012a; Li et al., 2012c).

While *Pbx1* and *Pbx3* perform essential roles during embryonic development, *Pbx2* is dispensable for embryogenesis and postnatal development. Despite the fact that *Pbx2* is extensively expressed in many tissues, deficient mice exhibit a normal life span without defects in organogenesis or other abnormalities (Selleri et al., 2004). Recently however both *Pbx1* and *Pbx2* were found to perform non-redundant functions during limb development in mice. Both *Pbx1* and *Pbx2* are expressed early during limb specification. However, later in development *Pbx1* expression is restricted to proximal regions while *Pbx2* expression extended distally as well. Furthermore, compound mutants of *Pbx1* and *Pbx2* displayed no limbs structures indicating an indispensable role for *Pbx2* as well in limb development (Capellini et al., 2008).

1.8. PKNOX proteins:

1.8.1. PKNOX1:

PKNOX1 belongs to the MEINOX family of proteins and was first identified as a protein binding an enhancer element of the Urokinase genes. PKNOX1, like MEIS1, interacts with PBX both as DNA-bound and DNA-free forms (Berthelsen et al., 1998a; Berthelsen et al., 1999; Berthelsen et al., 1998c; Ferretti et al., 1999). This interaction has been implicated in the activation of many genes like glucagon (Herzig et al., 2000), UDP-glucoronosyltransferase 2B17 (Gregory and Mackenzie, 2002) and FABP7 (Sanchez-Font et al., 2003), which has been implicated in Down syndrome. Interestingly murine leukemia viruses have been found to contain PBX binding elements and PBX1-PKNOX1 dimers enhanced transcription of the viral DNA (Chao et al., 2003). PKNOX1-PBX-HOX trimers have also been described and have shown

to perform important roles during development (Berthelsen et al., 1998b; Ferretti et al., 2000; Ferretti et al., 2005).

In zebrafish, *pknox1* is widely expressed throughout the embryo (Choe et al., 2002; Deflorian et al., 2004) and knockdown using morpholinos leads to hindbrain patterning and cartilage formation defects (Deflorian et al., 2004). In mice, *Pknox1* is highly expressed in the thymus (Ferretti et al., 1999) and reduction in the levels of PKNOX1, as found in hypomorphic mice, leads to impaired T-cell development (Penkov et al., 2008; Penkov et al., 2005) as well as defects in the entire embryonic hematopoietic system due to reduced levels of LT-HSCs (Di Rosa et al., 2007). *Pknox1* hypomorphic mice also exhibit pleiotropic phenotypes including smaller body size, organ hypoplasia, eye defects and angiogenesis. Furthermore, the expression of other TALE proteins, MEIS and PBX are reduced in these mice (Ferretti et al., 2006).

In the case of *Pknox1* null mice, the severity of the phenotype described above is more pronounced. Embryos do not undergo gastrulation as a result of the apoptosis of epiblast cells by a p53-mediated mechanism (Fernandez-Diaz et al., 2010). In this regard it is interesting to note that *Pknox1* seems to have both pro- and anti-apoptotic roles. PKNOX1 can prevent apoptosis by maintaining the expression of BXL-X_L but also induces apoptosis by activating p53 (Micali et al., 2009; Micali et al., 2010).

A proteomic study identified PKNOX1 as a partner of β-actin, non-muscle myosin heavy chain II A and p160 myb binding protein. Other studies have provided evidence for PKNOX1 interaction with NONO/PSF, RNA POL II, N-WASP (Diaz et al., 2007a; Diaz et al., 2007b; Villaescusa et al., 2009) and nuclear β-actin. The nuclear β-actin interaction is important for the PKNOX1-mediated transcription of the *HoxB* genes (Ferrai et al., 2009). Specifically, p160 competes with PBX1 for binding to PKNOX1, and p160 binding reduces the transcriptional activity of PKNOX1 (Diaz et al., 2007b; Oriente et al., 2008). This interaction is responsible for the maintenance of glucose metabolism since PKNOX1 was found to stabilize p160 protein and the concomitant downregulation of GLUT4 transporter (Oriente et al., 2008). An interesting observation by Villaescusa et al (2009) shows that PKNOX1 interacts with eIF4E translation factor and binds *Hoxb4* mRNA to regulate its translation (Villaescusa et al., 2009).

Pknox1 has been implicated as a tumor suppressor in many studies possibly due to its role in preventing DNA damage. In an Eμ-Myc tumor model, *Pknox1* deficiency leads to the development of tumors in mice. Furthermore, *Pknox1* expression was found to be low in many cancers (Iotti et al., 2012; Iotti et al., 2011; Longobardi et al., 2010). The pro-apoptotic role of PKNOX1 described above may play role in this tumor suppression.

1.8.2. PKNOX2:

Pknox2 was first identified to encode another PBX-binding protein based on sequence similarities with *Pknox1*. It was shown to be expressed highly in different human tissues including skeletal muscle, brain and ovary (Fognani et al., 2002; Haller et al., 2002; Imoto et al., 2001). After synthesis, PKNOX2 is relocated to the cytoplasm by CRM1-mediated nuclear export and anchored in the cytosol by binding with actin and tubulin (Haller et al., 2004). It has been shown that alternative splicing generates five different isoforms of PKNOX2 that exhibit different sub cellular localization. A homeodomain-less isoform of PKNOX2 has also been identified (Haller et al., 2004). Like PKNOX1, it requires PBX interaction for nuclear localization and DNA binding and it is ubiquitously expressed in the mouse embryo (Fognani et al., 2002; Haller et al., 2002). Functional studies on the role of PKNOX2 during embryogenesis or postnatal processes have not yet been done extensively. However, indications that PKNOX2 might perform important roles in neurological disorders and behavior is shown by the recent linkage of *PKNOX2* with substance (alcohol, cigarette smoking etc) dependence (Chen et al., 2011; Wang et al., 2011a) and schizophrenia (Wang et al., 2012b).

1.9. CRTC (CREB-regulated transcription coactivator):

CRTC transcription factors have been shown to interact with MEIS1 and function together in a PKA signaling pathway to activate downstream genes (Goh et al., 2009). *Crtc1* was first identified in a cDNA library screen for transcription factors that could activate IL-8 expression. It was found to activate target gene expression by interacting with CREB (cAMP response element (CRE) binding protein). CRTC1 has a transcriptional activation domain and functions as a co-activator for CREB (Iourgenko et al., 2003; Luo et al., 2012). In the presence of CRTCs, CREB shows increased binding to $TAF_{II}130$, also known as TAF4 (Conkright et al., 2003). CRTC1 also augments transcription by binding with CBP and p300 proteins and inducing their recruitment to CREB target genes (Ravnskjaer et al., 2007).

Another study to identify modulators of CRE revealed the presence of two other homologs of CRTC1, named CRTC2 and CRTC3. With respect to tissue distribution, while CRTC2 and CRTC3 are expressed ubiquitously, CRTC1 shows high tissue specific expression in the brain (Wu et al., 2006). Intracellular localization of CRTCs is regulated by cAMP and calcium signaling which are mediated by PKA and calcineurin, respectively (Figure 1.5) (Bittinger et al., 2004).

A translocation of CRTC1 (t11;19) results in the formation of a fusion protein with MAML2 (Mastermind-like 2). CRTC3-MAML2 fusions have also been described. The CRTC-MAML2 fusions are responsible for cause mucoepidermoid carcinoma, a malignant tumor of the salivary gland in humans. CRTC1-MAML2 fusions also interact with CREB (Conkright et al., 2003).

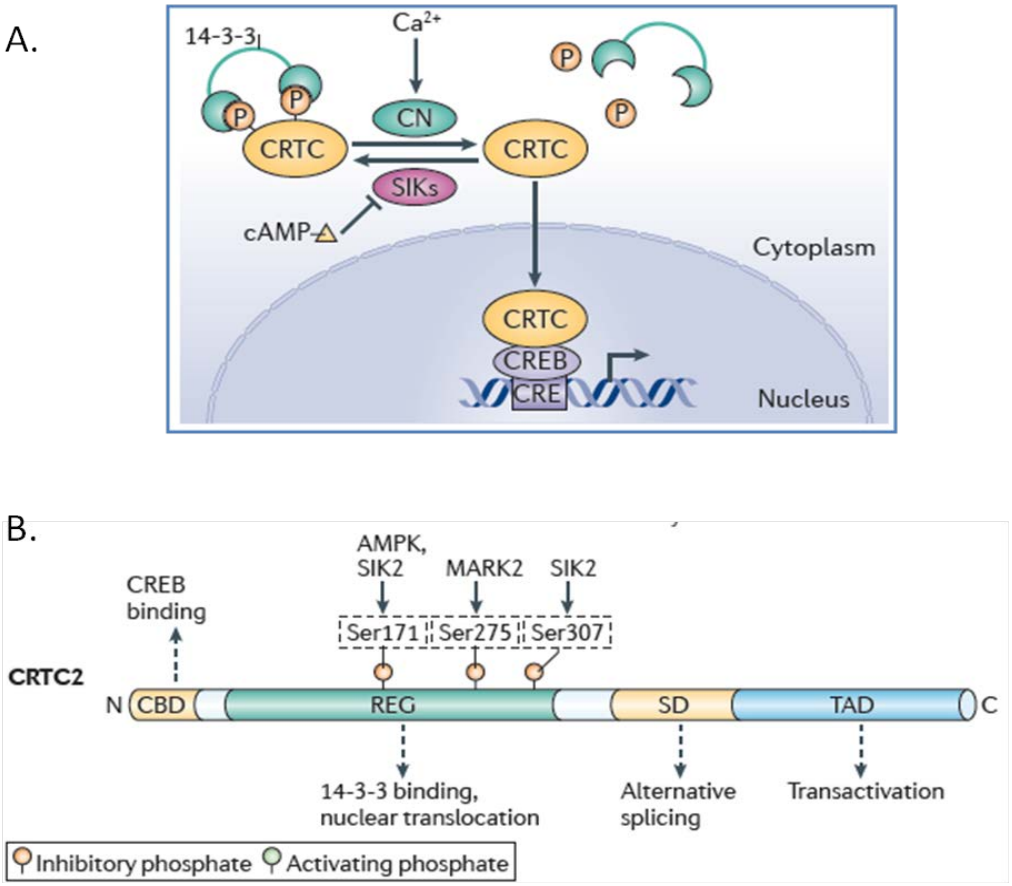


Figure 1.5: Regulation of the intracellular localization of CRTC: A. CRTCs, under normal conditions are phosphorylated and anchored to the cytoplasm by 14-3-3 protein. cAMP and Calcium signaling activate the nuclear localization of CRTCs. PKA which is a mediator of cAMP signaling phosphorylates and inhibits SIK kinases which are inhibitors of CRTC. Calcium signaling on the other hand activates Calcineurin, a CRTC phosphatase which removes the inhibitory phosphates. After entering the nucleus CRTCs interact with CREB to activate downstream genes.

B. The figure shows the phosphorylation sites on CRTC2 mediated by different kinases. CBD- CREB binding domain, REG- central regulatory region, SD- Splicing domain, TAD- Transcriptional activation domain

Adapted from (Altarejos and Montminy, 2011)

1.9.1. Physiological roles of CRTC:

CRTC2 along with CREB has been shown to be an important modulator of gluconeogenesis. Under normal feeding conditions, SIK2 kinase phosphorylates CRTC2, thereby provoking the retention of CRTC2 in the cytoplasm through binding to 14-3-3 protein. Under conditions of fasting, cAMP and calcium signaling initiated by glucose and glucagon relieves this cytoplasmic retention and CRTC2 translocates into the nucleus and activates transcription of target genes (Koo et al., 2005; Screaton et al., 2004; Takemori et al., 2007). AMPK and its phosphorylation-mediated activator LKB1 also function in the CRTC/CREB gluconeogenic pathway by repressing CRTC2 nuclear localization (Al-Hakim et al., 2005; Katoh et al., 2006; Shaw et al., 2005). MARK2, another Serine/Threonine kinase, phosphorylates CRTC2 and negatively regulates CRTC2-mediated transcription in islet cells of the pancreas (Jansson et al., 2008). Contrary to the kinase-induced repression of CRTC activity discussed above, MEKK1-induced phosphorylation of CRTC1 is needed for its nuclear translocation and transcriptional activation. Hence, phosphorylation of distinct residues on can play opposing roles in modulating CRTC activity (Siu et al., 2008). In the liver, PIM1 protein is important for the cytoplasmic retention of CRTC2. PIM1 binds to phosphorylated CRTC2 and prevents its entry into the nucleus (refer figure 1.5 for the different phosphorylations which regulate CRTCs) (Nakatsu et al., 2010)

CRTC and CREB, apart from activating gluconeogenic genes, also function in the insulin signaling pathway in the liver by activating the expression of IRS2 (insulin receptor substrate 2). IRS2 functions antagonistically to CRTC2 to inhibit CRTC2-mediated gluconeogenesis (Canettieri et al., 2005). In fact, during re-feeding, insulin activates the SIK2 kinase leading to phosphorylation and redistribution of CRTC2 to the cytoplasm. The redistributed CRTC2 is proteasomally degraded by an insulin-mediated pathway (Dentin et al., 2007). Thus, by activating both insulin signaling and gluconeogenesis genes, and by being modulated by both insulin and glucagon, CRTCs and CREB maintain the balance between glucose production and uptake in the body (Canettieri et al., 2005).

CRTC2 also responds to endoplasmic reticulum ER stress-induced gluconeogenesis by collaborating with ATF6α to activate stress responsive genes

(Wang et al., 2009b). In *Drosophila*, which has only a single *Crtc* gene, its product has been shown to induce resistance to starvation induced stress. Neuronally expressed CRTC activates many CREB target genes like TrtX and CAT which function to overcome stress. Neuronal CRTC is also regulated by insulin signaling (Wang et al., 2008). Since CRTCs play such important roles in glucose homeostasis, any changes in their intracellular localization or transcriptional activity lead to metabolic disorders. In disorders like chronic hyperglycemia, CRTC2 is aberrantly activated and translocated to the nucleus by an O-linked glycosylation mechanism. The O-glycosyl transferase enzyme has been shown to associate with and glycosylate CRTC2 (Dentin et al., 2008). It is important to note that CRTC2 might not be an indispensable factor for the maintenance of glucose homeostasis. This is because *Crtc2* null mice survive and are not hypoglycemic (Le Lay et al., 2009).

A central nervous system role for CREB-CRTC has also been proposed. CREB and CRTC1 have been shown to be expressed in the brain and they induce the expression of BDNF (brain derived neurotropic factor) and hence influence the synaptic plasticity of the hippocampus (Finsterwald et al., 2010; Kovacs et al., 2007). It has been shown that CRTC1 mediates the synaptic activity of individual neurons. Upon activation of a neuron, the cytoplasmic CRTC1 is translocated to the nucleus to activate CREB target genes (Ch'ng et al., 2012). Also in the hypothalamus, CRTC1 activates the transcription of genes like *Cartpt* and *Kiss1* which function in the leptin hormone-mediated pathway to regulate energy balance (Altarejos et al., 2008).

Other established roles of CRTCs include (i) TCL1 regulation and the resulting inhibition of apoptosis of B cells by CRTC2 (Kuraishy et al., 2007); (ii) Induction of cell growth in a c-JUN- and AP-1-dependent manner by CRTC1 (Canettieri et al., 2009); (iii) Regulation of alternative splicing of target genes (Amelio et al., 2009).

1.10. MLL1 (Mixed lineage leukemia 1) or HRX:

MLL1 is a histone methyltransferase important for mono, di and tri-methylation at histone H3K4 and is an ortholog of the *Drosophila* trithorax complex. In *Drosophila*, the *Trithorax* group (*trxG*) proteins together with the *Polycomb* group (*PcG*) regulate the expression of *Hox* genes by epigenetic mechanisms. While the *trxG* group methyltransferases activate *Hox* gene expression, the *PcG* methyltransferases function to repress *Hox* genes. MLL1 also called human trithorax (HRX) also performs similar functions as the *Drosophila Trithorax.* Histone 3 lysine 4 (H3K4) trimethylation is an important mark of actively transcribed genes and it has been shown that *Hox* genes are the predominant targets of MLL1 (Mishra et al., 2009; Wang et al., 2009a; Yu et al., 1995). The SET domain in MLL1, similar to trithorax, is important for this function (Milne et al., 2002; Terranova et al., 2006).

Mice that are homozygous null for *Mll1* show embryonic lethality. In adults *Mll1* plays a predominant role in hematopoiesis (Hess et al., 1997; Yagi et al., 1998; Yu et al., 1995) $Mll1^{+/-}$ mice show disruptions in axial patterning due to the deregulation of *Hox* genes (Yu et al., 1995). $Mll1^{+/-}$ mice also display hematopoietic malignancies and display reduced amounts of hematopoietic precursors (Yagi et al., 1998). Supporting this role in blood cell production, Gan et al (2010) have also shown that a conditional hematopoietic lineage specific knockout of *Mll1* in mice shows defects in adult hematopoiesis (Gan et al., 2010). Furthermore *Mll1* deficiency leads to decreased cell proliferation and cell death in tumors in mice. MLL1, like MEIS1 regulates *Hif1α* expression which is important for maintaining the hypoxic niche of HSCs and hypoxic tumor growth (Ansari et al., 2012; Heddleston et al., 2012)

MLL1 is predominantly localized to the nucleus and is expressed ubiquitously in most tissues of the body and in leukemic cells (Butler et al., 1997; Ennas et al., 1997). The full-length MLL1 is a big protein consisting of 3969 amino acids that undergoes post-translational cleavage by Taspase1 enzyme into N- and C-terminal fragments. The N (320 kD) and C-terminal (180 kD) fragments then interact with each other through their FYR (phenylalanine-tyrosine rich) domains and their interaction is needed for nuclear localization (Hsieh et al., 2003a; Hsieh et al., 2003b; Nakamura et al., 2002; Yokoyama et al., 2002).

1.10.1. Interactions of MLL1:

MLL1 interacts with many proteins to perform its functions. To date, over 30 different interacting partners have been identified (Nakamura et al., 2002). Some of the important interacting proteins are WDR5, RBBP5, ASH2L, DPY30, MENIN, LEDGF (PSIP), MOF, HCFC1 and 2, HDAC1, CBP and p300. WDR5, RBBP5, ASH2L and DPY30 (together referred to as the WRAD complex) constitute the core interaction complex of MLL1 (Figure 1.6). The WRAD complex is indispensable for the SET domain-mediated methyltransferase function of MLL1. WDR5 is necessary for the recognition and recruitment of MLL1 to histone H3 (Cao et al., 2010; Crawford and Hess, 2006; Dou et al., 2006; Patel et al., 2009; Ruthenburg et al., 2006; Wysocka et al., 2005). Interaction with members of the core complex is a shared feature of some other MLL family members like MLL2 and MLL3 (Dou et al., 2006).

MLL1 gene translocations play a causative role in the induction of mixed lineage leukemia (MLL) in humans. The *MLL1* translocations are also implicated as a major cause for leukemia recurrence seen in patients treated with Topoisomerase II inhibitors like etoposides (Bigoni et al., 1999; Ernst et al., 2002; Felix et al., 1995a; Felix et al., 1995b; Felix et al., 1998; Hess, 2004). The translocation fuses the *MLL1* with over 60 different partners resulting in the production of fusion proteins. The predominant fusion partners include AF4, AF9, AF6, AF10, ENL and ELL which together account for 80% of all MLL in patients (Ernst et al., 2002; Meyer et al., 2009). MLL1 wild-type and fusion proteins activate similar target genes including *Hox* genes and *Meis1*. The recruitment of MLL1 wild-type and fusion proteins to *Hox* gene loci is dependent upon its interaction with the PAF elongation complex (consisting of LEO1, PAF1, CTR9, CDC73 and RTF1) and the recognition of H3K4 dimethylation marks. These interactions are mediated by the CXXC and PHD finger domains of MLL1. Since these domains are absent in the fusion proteins they require the presence of wild-type MLL1 to localize to the Hox locus (Milne et al., 2010; Muntean et al., 2010).

MLL1 structure and interaction partners

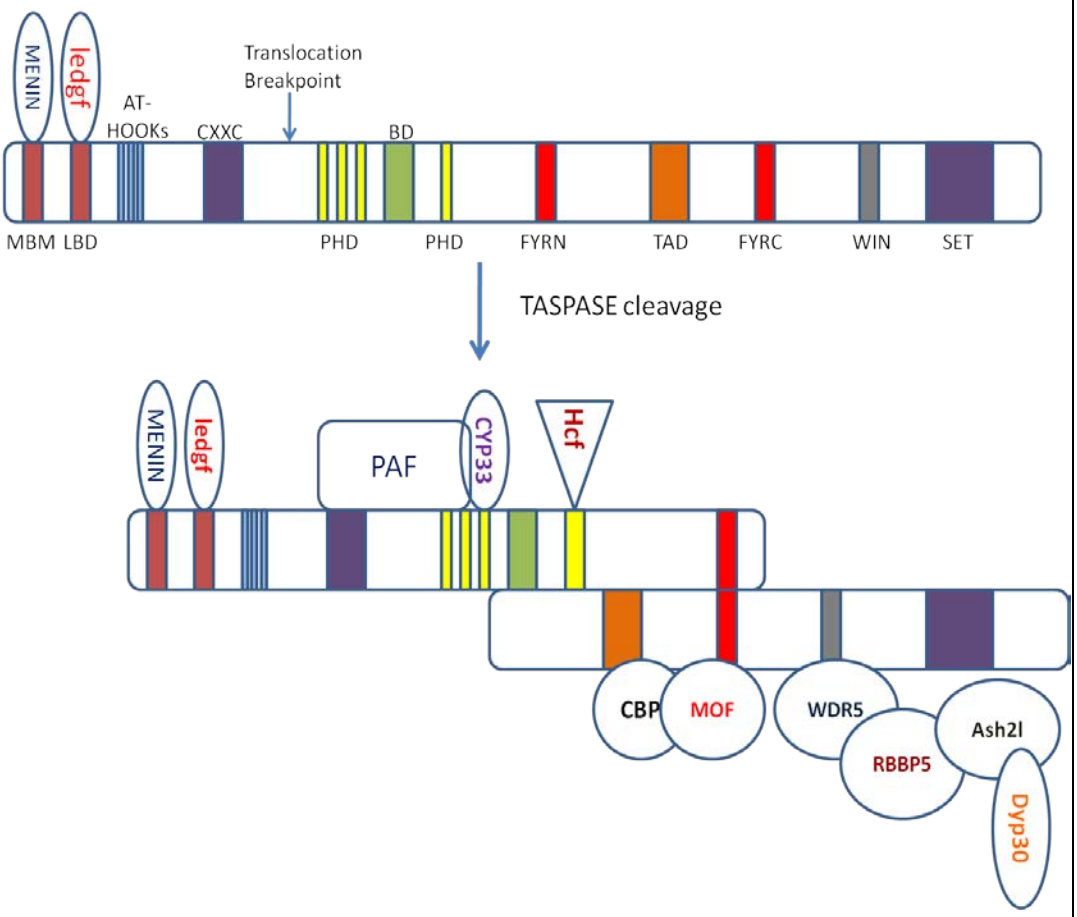


Figure 1.6: MLL1 has many conserved domains including the SET domain which mediates the methyltransferase function, 4 plant homeodomain fingers (PHD), an AT-hook DNA binding motif, a transcriptional activation domain which binds CBP and MOF, a cyclophilin CyP33 interaction motif at PHD3, a CXXC motif which mediates the interaction with PAF elongation complex, and the MENIN and LEDGF interaction motifs at the N-terminus.

MLL1 is cleaved by the threonine-aspartase protease TASPASE1. The cleaved proteins assemble into a macromolecular complex with other proteins.

In 11q23 translocations, MLL1 is expressed as a fusion protein with over 60 different partners. The most common partners are AF4, ENL, AF9, ELL, and AF10. In these fusion proteins a 1400 amino acid N-terminal region of MLL1 is fused with the translocation partners. Only the MENIN/LEDGF interaction motifs, AT-hooks and the CXXC domains are retained in the fusion proteins. Adapted from (S.Cosgrove, 2011)

The third PHD finger (PHD3) binds the cyclophilin protein CyP33 which attenuates the gene activation function of MLL1. CyP33 has been shown to recruit HDAC1 to mediate this repressive effect (Chen et al., 2008; Fair et al., 2001; Wang et al., 2010a; Xia et al., 2003). CREB binding protein (CBP), a histone acetyltransferase, mediates the transactivation function of MLL1. Both MLL1 and CREB have been shown to bind co-operatively to the KIX domain of CBP (Goto et al., 2002). Similarly, MOF, a H4K16 acetyltransferase, also interacts with MLL1 to confer transactivation function. MOF-MLL1 interaction is needed for the activation of *Hox* gene expression by MLL1 (Dou et al., 2005)

The extreme N-terminus of MLL1 exhibits binding with Menin (MEN1), a tumor suppressor protein (Milne et al., 2005b; Yokoyama et al., 2005; Yokoyama et al., 2004), and the interaction is essential for the activation of target *Hox* genes and induction of leukemia by wild-type and fusion proteins of MLL1 (Caslini et al., 2007; Grembecka et al., 2012; Yokoyama et al., 2005). Menin is also important for the recruitment of LEDGF to the N-terminus of MLL1. LEDGF is also important for the oncogenic functions of MLL1. Importantly it might also function at the crossroad between Menin-mediated tumor suppression and oncogenesis (Yokoyama and Cleary, 2008). Menin also tethers the C-MYB oncoprotein to MLL1. C-MYB recruitments is important for the MLL1-mediated H3K4 methylation at *HoxA9* and *Meis1* loci. In this regard it is interesting to note that c-myb itself is a downstream target of *HoxA9* and *Meis1* in leukemia (Jin et al., 2010).

Recently, MLL1 was shown to possess an E3 ubiquitin ligase activity. The activity was mapped to the second PHD finger and mediated through interactions with the E2 ubiquitin ligase enzyme CDC34 (Wang et al., 2012a).

H3K4 trimethylation by MLL1 is involved in altering the chromatin architecture in response to changes in circadian rhythm. MLL1 has been shown to interact with the CLOCK1 protein and recruits it to target promoters (Katada and Sassone-Corsi, 2010). Another physiological process regulated by *Mll1* is the (i) neuronal differentiation of neural stem cells in mouse (Lim et al., 2009).

1.11. CBX3 or HP1γ:

Heterchromatin protein 1 (HP1) is a complex of three proteins involved in heterochomatin formation and epigenetic silencing. HP1α (*Cbx5*), HP1β (*Cbx1*) and HP1γ (*Cbx3)* contain a unique chromatin modifying domain called the chromodomain. They bind specifically to H3K9 methylated histone and modify the chromatin (as reviewed by (Fanti and Pimpinelli, 2008; Lomberk et al., 2006a)). Contrary to the cellular localization of HP1α and HP1β, both of which are primarily localized to heterochromatin, HP1γ localizes to both euchromatin and heterochromatin (Minc et al., 2000; Minc et al., 1999). Not surprisingly, HP1γ is found to be associated with actively transcribed genes through an interaction with elongating phosphorylated RNA polymerase II and H3K9 trimethylation marks (Vakoc et al., 2005). Phosphorylation at the Ser 83 position by PKA has been found to induce this euchromatin localization (Lomberk et al., 2006b).

HP1γ has been shown to have dual functions as both a repressor and activator of transcription in different instances (Hsieh et al., 2006; Rastogi et al., 2006; Vakoc et al., 2005). Transcriptional activation function has been attributed to HP1γ leading to the stimulated expression of Hsp70 genes in cancer cell lines (Kim et al., 2011). HP1γ itself has been shown to be over-expressed in cancer cell lines (Takanashi et al., 2009). On the other hand, HP1γ has been shown to repress transcription in such instances as (i) BRCA-1 mediated transcription by direct binding to BRCA1 (Choi et al., 2012). During embryogenesis, HP1γ may function to repress *Sox2* expression in the developing neural plate of the chick embryo through an association with ERNI protein (Papanayotou et al., 2008). HP1γ and the demethylase KDM4 have complementing functions during the cell cycle. Whereas KDM4 is needed for inducing DNA replication and maintaining chromatin accessibility, HP1γ inhibits replication and closes chromatin (Black et al., 2010).

HP1γ also plays an important role during meiotic recombination to facilitate homologous chromosomal joining. It has been shown to accumulate at the centromeric heterochromatin in response to H3K9 methylation marks along with the H3K9 histone methyltransferase G9a, and mediates centromere clustering during meiosis (Takada et al., 2011). HP1γ and G9a are also needed for rDNA transcription and they accumulate near active genes (Yuan et al., 2007).

The HP1 proteins also regulate the transcriptional activity of HIV1 virus. They were found to bind the LTR of HIV1 in response to H3K9 methylation in a sequential manner. HP1β is bound during latency and maintains a repressive state. Upon proviral activation, it is replaced by HP1γ (Mateescu et al., 2008)

HP1γ shows interaction with many proteins including 1) $hTAF_{II}130$ (TAF4) which is a subunit of the TFIID transcription factor (Vassallo and Tanese, 2002), PIM1 serine-threonine kinase (Koike et al., 2000), transcription factor TIF1δ (Khetchoumian et al., 2004), tumor suppressor L3MBT1 (Trojer et al., 2007) nuclear argonaute protein AGO-2 (Ameyar-Zazoua et al., 2012) to regulate their function. HP1γ binding to H3K9 methylation marks near splice sites have been shown to promote alternative splicing and HP1γ/AGO2 interaction is important for this process (Ameyar-Zazoua et al., 2012; Saint-Andre et al., 2011) HP1γ also interacts with TIN2 at the telomeres and this interaction is needed for the sister chromatin cohesion and organization (Canudas et al., 2011)

1.12. NONO ($p54^{nrb}$):

Nono (non-POU domain containing, octamer-binding protein, otherwise called $p54^{nrb}$ nuclear RNA binding protein; molecular weight 54 kD) was identified initially to encode an RNA-binding protein homologous to the *Drosophila* NONA protein. It also shows high similarity to a mammalian splicing factor called PSF. NONO was found to bind an octamer sequence found in the promoters of some immunoglobulin genes. (Dong et al., 1993; Yang et al., 1993). NONO has been shown to be a multifunctional protein involved in RNA binding and processing (Buxade et al., 2008; Dong et al., 2007; Kaneko et al., 2007; Sunwoo et al., 2009; Zhang and Carmichael, 2001), transcriptional activation and repression (Dong et al., 2009; Zhang et al., 2008) and other cellular processes. NONO has been shown to interact with many proteins including PSF (Dong et al., 2009), CRTC1/2(Amelio et al., 2007), RNF43 (Miyamoto et al., 2008), SOX9 (Hata et al., 2008) and SOCS3 (Song et al., 2008).

NONO was shown to bind and induce the DNA relaxing activity of DNA topoisomerase I indirectly through association with PSF (Straub et al., 2000; Straub et al., 1998). It induces the DNA-binding activity and hence the transactivation of many

transcription factors including E47, OTF1 and OTF2 (Yang et al., 1997). NONO/PSF heterodimers were found to interact with the DBD of nuclear receptors like thyroid hormone receptor and retinoic acid receptor and induce transcriptional repression of their target genes in the absence of ligand. This repression is mediated by the binding of SIN3A by PSF and the subsequent recruitment of HDACs (Mathur et al., 2001).

Similar transcriptional repression function of the NONO/PSF complex was observed in the case of the bovine *cyp17* gene which encodes a cytochrome P450 protein. NONO binds to the *cyp17* enhancer in association with the SF-1 protein in response to phosphorylation through PKA signaling (Sewer and Waterman, 2002; Sewer et al., 2002). Interestingly, MEIS1/PBX complexes were also found to bind to the enhancer and activate cyp17 transcription (Bischof et al., 1998a; Bischof et al., 1998b). PKA signaling is also essential for the interaction between NONO and CRTCs and subsequently the activation of CREB/CRTC regulated genes (Amelio et al., 2007). In this case, NONO functions as an intermediate between the CRTCs and RNA polymerase II (Amelio et al., 2007) by virtue of its interaction with the latter's (C-terminal domain (CTD) (Amelio et al., 2007; Emili et al., 2002) Hence, NONO functions both as a repressor or an activator in response to PKA signaling depending upon its protein partner.

NONO was found to contain putative RNA recognition motifs (RRMs), basic helix-loop-helix (bHLH) and N- and C-terminal transcriptional activation domains based on protein sequence analysis and homolog to PSF (Dong et al., 1993; Yang et al., 1993). NONO as a complex with PSF and MATRIN3 was found to bind to adenosine deaminase (ADAR) modified dsRNA in the nucleus and promote their nuclear retention. ADARs convert adenines to inosines and the promiscuous RNAs produced by such modification are prevented from exiting the nucleus and being translated by their interaction with the NONO complex (Zhang and Carmichael, 2001). NONO and PSF play important roles during splicing and polyadenylation of pre-mRNA transcripts through their interaction with a specific sequence in the U5 snRNA and the U1 spliceosome protein. The mechanisms for NONO involvement in both these process remain to be elucidated (Liang and Lutz, 2006; Peng et al., 2002). XRN2, a protein involved in mRNA processing, has been shown to associate with NONO/PSF and lead to transcriptional termination (Kaneko et al., 2007). Another study showed that the transcriptional activation and splicing functions of NONO are

coupled. During chondrogenesis in mice, NONO binds with SOX9 and regulates the expression and alternative splicing of the *Col2a1* gene (Hata et al., 2008).

1.13. NOP56/58:

NOP56 and NOP58 are snoRNA-binding proteins playing an important role in snoRNA maturation and ribosome biogenesis. Both NOP56 and NOP58 were first identified in yeast as proteins interacting with Fibrillarin (NOP1p/ FBL). NOP56 and NOP58 were found to associate with FBL in the box C/D snoRNA complex (Gautier et al., 1997; Lafontaine and Tollervey, 2000; Lyman et al., 1999; Newman et al., 2000; Yang et al., 2000a). NOP58 is needed for the nucleolar localization of FBL (Lyman et al., 1999). Both NOP56/58 proteins were also found to be essential for nucleolar pre-rRNA processing. They contain KKE/D repeats at their C-terminus found in many microtubule associated proteins (Gautier et al., 1997)

Nop58 gene expression has been found to be activated by PDGF in human fibroblast cell lines (Nelson et al., 2000). A proteomics study to identify proteins interacting with hNOP56, apart from identifying known partners like NOP58 and FBL, also identified TCOF1, a protein involved in Treacher-Collins syndrome (Hayano et al., 2003). Detailed studies about the regulation of their expression and their functional roles have not been done. Tissue specific and Extraribosomal functions of NOP56/58 cannot be excluded. Recent studies have shown that NOP56 and NOP58 might interact with transcription factors like OCT-4, TIP48 and TIP49. Hence these proteins might be involved in transcriptional regulation as well (Cheong et al., 2011; McKeegan et al., 2009).

1.14. Biotin-tagging and purification of protein complexes:

Purification of a protein interactome predominantly involves affinity-based isolation of protein complexes that interact with the protein of interest. For this purpose the protein of interest to one or more affinity tags (e.g. FLAG, 6X-histidine (HIS), glutathione S-transferase (GST), maltose binding protein (MBP) calmodulin binding peptide (CBD), Hemagglutinin (HA), etc.). Alternatively, one can employ specific antibodies against the protein of interest. One of the main disadvantages of using specific antibodies is that the antibody might block interaction site. Affinity tags circumvent this problem because the proteins are purified using antibodies or other molecules specific for the tag which do not interfere with the interactions of the protein. Each of the commonly used affinity tags has limitations. Some tags like GST and MBP are large in size and hence might interfere with the natural conformation of the protein of interest and may also contribute to non-specific binding. Some tags bind relatively weakly to the antibodies or the resins resulting in significant contamination by non-specific peptides (as reviewed by (Arnau et al., 2006; Chang, 2006; Fritze and Anderson, 2000; Jarvik and Telmer, 1998; Kimple and Sondek, 2004; Sadaghiani et al., 2007)).

Biotin is a naturally occurring co-factor for some enzymes present in most organisms and it binds with high affinity to a protein called streptavidin which was isolated from the bacteria, *Streptomyces avidinii*. Streptavidin-coated matrices are commercially available and can be used to purify biotin-labelled macromolecules. However, most proteins are not biotinylated and mammalian biotin ligases are very inefficient and require long target peptides. By contrast, the *E. coli* BirA enzyme specifically and efficiently biotinylates the lysine (K) residue within the short peptide sequence shown in Figure 1.7. This biotin acceptor peptide can therefore be placed at either the N- or C-terminus of the protein of interest. Following co-expression with the BirA enzyme, the biotinylated fusion protein complexed to partner proteins can be efficiently purified over streptavidin-coated beads.

A 23 amino acid peptide was recently shown to be biotinylated efficiently in mammalian cells expressing BirA (Beckett et al., 1999; de Boer et al., 2003; Parrott and Barry, 2000; Schatz, 1993). Similar use of *in vivo* biotinylation and protein

purification has also been demonstrated in mouse embryonic stem cells (Kim et al., 2009).

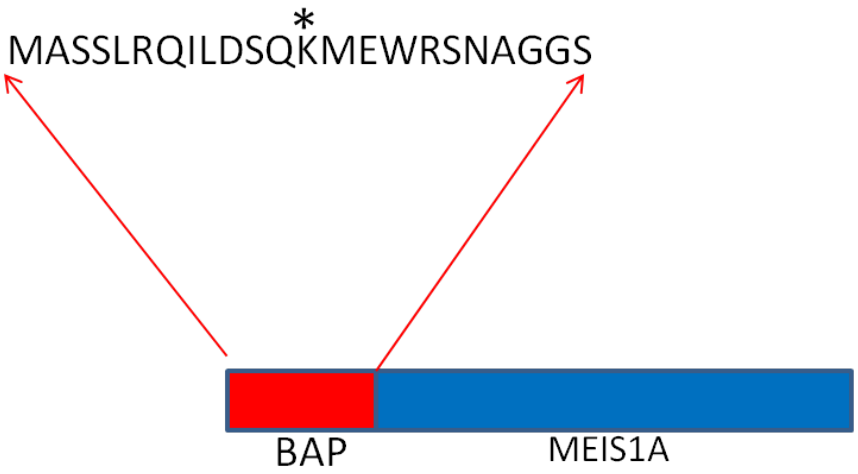


Fig 1.7: Biotin acceptor peptide (BAP) tagged-MEIS1A

The 23 amino acid Biotin acceptor peptide (BAP) was cloned N-terminus to MEIS1A. Asterix shows the lysine residue which is specifically biolinylated by BirA. Diagram not to scale.

In vivo biotinylation has been used to study the interactome of many nuclear and cytoplasmic proteins. The method has identified new interacting partners of transcription factors like GATA1, FOXP3 and LDB1 (de Boer et al., 2003; Grosveld et al., 2005; He et al., 2009; Meier et al., 2006; Rudra et al., 2012; Sanchez et al., 2007).

Rationale

The MEIS1 transcription factor is essential in a range of physiological processes both during embryogenesis and adult life. The mechanism by which it exerts its function is by the transcriptional regulation of downstream genes. The main partners of MEIS1 described to date are members of the HOX and PBX families. Recently, CRTC and CREB proteins have been added to the list of MEIS1-interactors. Taking into consideration the range of the physiological and oncological processes regulated by MEIS1, I hypothesized that MEIS1 could interact with many other proteins for its activity. Hence, the rationale for the present study was to identify novel interaction partners of MEIS1 which could help us better understand the mechanisms of MEIS1 function. In the succeeding chapters, I present the results of my findings. Firstly, I show the results of a recently described technique for the efficient and specific purification of MEIS1 interacting proteins followed by their identification by mass spectrometry. I then present data for the validation of some of these new interactions and their functional significance. Lastly, I have also interpreted the results in view of the existing knowledge and discussed the significance of the present work. I have also suggested new experiments for further studies.

Chapter 2
Materials and Methods

2.1. Plasmid constructs:

pcDNA3.1 Flag-CRTC1, pCS2+ Meis1a, pCS2+ *Pbx1*a, pHAv-Pknox2 have been described by us previously (Goh et al., 2009; Haller et al., 2004; Huang et al., 2005; Shanmugam et al., 1999). pFlag-Cbx3, pFlag-Mll1, pFlag-Mll-Af4, pcDNA-HA-Meis2 have also been described previously by others (Halder et al., 2011; Hiragami-Hamada et al., 2011; Milne et al., 2002). The cDNAs for human Nop56 and Nop58, Nono(p54nrb) were obtained as follows:

(i) The FLAG-tagged versions of Nop56, Nop58 and Nono were constructed by excising the Crtc1 coding region from the pcDNA3.1 Flag-CRTC1 vector and replacing it with the PCR amplified cDNAs for the respective proteins between the BamHI and XhoI sites. The cDNAs thus cloned are in frame with the N-terminal FLAG coding region. The plasmids thus generated are named pFlag-Nop56, pFlag-Nop58, pFlag-Nono, respectively. pHAv-Pknox1 was created by cloning the *Pknox1* cDNA between the NotI and XbaI sites in the pHAv vector in proper reading frame with the N-terminal 3X HA tag. Similarly, pHAv-*Meis1a* was created by cloning the *Meis1a* cDNA between the EcoRI and XbaI sites in the pHAv vector. All the plasmids were verified by sequencing.

(ii) *E. coli* genomic DNA was isolated using the Purelink Genomic DNA mini kit (Invitrogen). The *E. coli BirA* gene was amplified by PCR and cloned into the BamHI and XbaI sites of the pcDNA3.1/Hygro(+) vector. The BirA gene was modified to include a Kozak consensus sequence by replacing the adenine residue at the position immediately following the start codon to a guanine residue. This modification was included in the forward primer.

(iii) FLAG-BAP-MEIS1A (FBM) was cloned into the pLenti6 V5 DEST vector (Invitrogen) using a Gateway cloning kit according to the manufacturers' protocol.

Table 2.1: Primers used for cloning		
Name of cDNA	Forward Primer	Reverse Primer
BirA (pcDNA3.1 Hygro+)	5’GCTAGGATCCACCATGG AGGATAACACCGTGCCA 3’	5’GCATCTAGATTATTTTT CTGCACTACGCAG 3’
FBM (pLenti6-V5-Dest)	5’CACCATGGACTACAAA GACGATGACG 3’	5’ATCCTCGAGTTACATGT AGTGCCACTGC 3’
NOP56 (pcDNA3.1 Flag)	5'GACGGTACCGATGGTGC TGTTGCACGTG 3'	5'GACCTCGAGCTAATCTT CCTGGGATGCTTTATG 3'
NOP58 (pcDNA3.1 Flag)	5'GACGGATCCGATGTTGG TGCTGTTTG 3'	5'GACCTCGAGTTAATCCT CGTTCTCTC 3'
NONO (pcDNA3.1 Flag)	5’GCAGGATCCGATGCAG AG CAATAAAG 3’	5’GACCTCGAGCTAATATC G GCGGCGTTTA 3’
PKNOX1 (pHAv)	5'GACGCGGCCGCATGATG GCTACACAGACC 3'	5'GACTCTAGAACTACTGC AGGGAGTCACTG 3'
MEIS1A (pHAv)	5’GACATGGCGCAAAGGT ACGAC 3’	5’GACTCTAGATTACAT GTAGTGCCACTGCC 3’

2.2. Antibodies:

The following antibodies were used for analysis: Rabbit monoclonal anti-MEIS1 (Epitomics, Cat.No: 5194-1), rabbit polyclonal Anti-MEIS1 (Novus biologicals, Cat.No: H00004211-D01P), goat polyclonal Anti-MEIS1 (Novus biological, Cal.No: NBP1-06991), mouse monoclonal MLL N-terminus, clone N4.4 (Millipore, Cat.No: 05-764), mouse monoclonal Anti-p54nrb/NONO (Millipore, Cat.No: 05-950), mouse monoclonal Anti-FLAG Ab (Sigma, Cat.No: F3165), mouse monoclonal β-actin Ab (Sigma, Cat.No: A5316), mouse monoclonal Anti-Acetylated tubulin Ab (Sigma, Cat.No: T7451), rabbit polyclonal Anti Ki-67 Ab H-300 (Santa Cruz; Cat.No: sc-15402), chicken anti-BirA (Abcam, Cat.No: ab14002), anti-HA antibody (Covance, Cat.No: MMS-101P), Anti-Rabbit Ab (Dako, Cat.No: D0487), streptavidin-HRP conjugate (Invitrogen, SA10001), anti-mouse IgG (Sigma, Cat.No: A9044) and anti-goat IgG (Sigma, Cat.No: A5420).

For immunoblot probing in all experiments, primary antibodies were used at a concentration of 1:1000 diluted in 2.5% milk and secondary antibodies were used at a concentration of 1: 5000 in 2.5% milk.

2.3. Cell culture and transfections:

HEK293T cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1X minimum non-essential amino acids, 1 mM sodium pyruvate and penicillin/streptomycin. RS4;11 lymphoblast cells were maintained in RPMI-1640 medium (Gibco) supplemented with FBS, 2 mM L-glutamine, 25 mM D-glucose, 1 mM sodium pyruvate and 10 mM HEPES.

For co-immunoprecipitation experiments, cells were seeded at a concentration of 1×10^6 cells in 40 mm culture dishes. The cells were allowed to grow to 90% confluence and transfected using Lipofectamine-2000 (catalog no. 11668-019, Invitrogen) according to manufacturer's protocol with slight modifications. 8 µg of expression vectors for *Nop56, Nop58, Nono, Cbx3, Mll1* and *Mll-af4* and 6 µg expression vectors for *Meis1, Meis2, Pknox1 and Pknox2* were used for the co-IP assays.

For stable transfections, the cells were seeded at a concentration of 5×10^5 cells in 60 mm culture dishes. The cells were allowed to attach overnight and the plasmid was transfected with 8 µg DNA using lipofectamine 2000 reagent. For producing a stable cell line expressing *E. coli* BirA (HEK293T/BirA), the pcDNA3.1/Hygro(+) vector carrying the *BirA* gene was linearized by digestion with BglII enzyme. The DNA was run on a 1% agarose gel and purified by gel extraction (Qiagen) before transfection. After 24 hours the media was removed, followed by one wash with PBS and addition of fresh media supplemented with 400 µg/ml hygromycin B. Cells were allowed to grow for approximately 5 days with replacement of media every 24 hours, and then trypsinized and seeded at a very low concentration (1×10^3 cells) in a 14 cm culture plate. After visible colonies formed from single cells, the clones were isolated by the use of cloning cylinders and seeded on fresh culture dishes.

For producing FBM stable cell lines, the pLenti6/V5-Dest vector carrying the FBM/MBF construct was linearized by digestion with KasI. The DNA was run on an agarose gel and purified using gel extraction. The purified DNA was used to transfect the HEK293T/BirA cell line. The cells were selected with 10 µg/ml blasticidin S using a procedure similar to the one used for producing BirA stable cell lines.

2.4. Preparation of samples for Mass Spectrometry:

95% confluent cells from ten 10cm cell culture dishes each for control as well as the test samples were harvested using lysis buffer 1 (20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 150 mM KCl, 0.5% Triton X-100, 0.5% NP-40 and protease inhibitor cocktail). The harvested cells were then freeze thawed for a minimum of 3 times to rupture the cell membrane. The cells were then incubated on ice for 1 hr with intermittent vortexing. To ensure lysis of the nucleus, the cells were then dounce homogenized (20 times) and sonicated using a Bioruptor™ apparatus at maximum intensity for 3 cycles. One cycle consists of a 20 sec sonication followed by a 20 sec pause. The cell debris was removed by centrifugation at 12000 rpm for 15 min and the supernatant protein fraction was collected. The protein extract was then incubated with 200 µl of streptavidin-coupled Dynabeads™ (Invitrogen, Cat.No: 65601) overnight at 4°C. After 6 washes of 10 minutes each with Lysis buffer 1, the bound

proteins were then extracted by boiling with 200 µl of 2X Laemmli sample loading buffer. The eluate was run on a 10% SDS polyacrylamide gel and stained using the silver staining system (Biorad; Cat.No: 161-0449) as per the vendor's protocol

2.4.1. In-Gel Digestion:

The protein bands were diced and pooled together in a test tube. The gel slices were washed with freshly made 25 mM NH_4HCO_3 (Buffer A) and 25mM NH_4HCO_3/ 50% ACN (Buffer B) one after the other for 2 times, 5 mins each. They were then treated with a destaining solution from the SilverQuest kit (Invitrogen) for 10 min with intermittent vortexing. After washing again with buffers A and B five more times, the gel pieces were treated with 100% ACN in order to dehydrate the gel. The pieces were then centrifuged in vacuum to complete dryness.

To the dried gel pieces sufficient volume of 10 mM DTT in Buffer A was added and incubated at 60 ºC for 30 mins to disrupt the disulphide bonds. The pieces were then incubated with 55 mM iodoacetamide, an alkylating agent for 30 min in the dark at room temperature. The gel pieces were washed again with buffers A and B. Following this, once the pieces were completely dry, sequence grade modified trypsin solution (Promega) was added just enough to cover the gel and incubated at 37 °C, overnight.

The gel sample was then incubated with 50% ACN/2% acetic acid for 30 min with intermittent vortexing in order to extract the peptides from the gel. This step was repeated 2-3 times and the supernatants from each step were pooled. The extracted peptides were dried by centrifuging under vacuum using a Speedvac. The dried peptides were then diluted in about 120 µl of 0.1% formic acid. After vigorous vortexing the samples were chilled on ice for about 10 min and centrifuged at high speed for 10 min. The supernatant was then used for HPLC.

2.4.2. Mass Spectrometry protocol:

The samples were analyzed by a LC-MS/MS system including a Shimadzu micro HPLC system coupled online to a LTQ-FT ultra linear ion trap mass spectrometer (Thermo Electron, Bremem, Germany) fitted with a nanospray source. Injected peptides were trapped and desalted in a Zorvax 300SB-C18 enrichment column (5 mm × 03 mm, Agilent Technologies, Germany) and eluted into a nano-bored C18 packed column (75 μm × 100Å, Michrom Bioresources, Auburn, CA USA). A 60 min gradient at a constant flow rate of 20 μl/min with a splitter to an effective flow rate of 300 nl/min was used to elute the peptides into the mass spectrometer. The LTQ was operated in a data-dependent mode by performing MS/MS scans for 8 of the most intense peaks from each MS scan in the FTMS.

2.4.3. Protein identification:

Protein identification was achieved by searching the combined data against the IPI human protein database via an in-house Mascot server (Matrix Science, UK). The search parameters were a maximum of 2 missed cleavages using trypsin; fixed modification was carbamidomethylation of cysteine and variable modification was oxidation of methionine. The mass tolerances were set to 10 ppm and 0.8 Da for peptide precursor and fragment ions, respectively. Protein identification was accepted as true positive if two different peptides were found to have scores greater than the homology scores.

2.5. Co-immunoprecipitation (co-IP) protocol:

The cells were harvested in 200 ul of lysis buffer 1 containing 20 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 150 mM KCl, 0.1% NP-40, 0.2% Triton X-100 along with 2X protease inhibitor cocktail. The cells were freeze-thawed 3 times followed by incubation on ice for 1 hr with intermittent vortexing. The lysate was then centrifuged at 14000 rpm for 10 min. The supernatant containing the protein extract was used for further analysis. Briefly, after removing 10 ul of extract for use as input control, the remainder was added to FLAG M2 agarose beads (Sigma Aldrich, Cat.No: A2220). The proteins were allowed to bind the beads for 6-10 hours at 4 ^{o}C with gentle mixing

followed by 3 washes with the lysis buffer and a final wash with TBS buffer to remove non-specific binding. The bound proteins were then eluted using 5 ug/ul of 3X FLAG peptide in a volume of 75 ul TBS.

For the immunoprecipitation of endogenous MLL1 from RS4;11 cells, the cells were lysed using lysis buffer 1 using a protocol similar to that mentioned above. The protein lysate was then pre-cleared using protein G beads by incubating at 4^{o}C for 2 h. The protein extract was then incubated with MLL1 N-terminal antibody or anti-FLAG antibody (non-specific IgG control) at 4^{o}C for 6-7 h. The protein-antibody mixture was then bound to protein G beads for 2 h. The beads were washed with lysis buffer 1 for 3 times 10 min each. The bound proteins were then eluted by boiling in 2X Laemmli sample buffer at 95 ^{o}C for 5 mins.

All the co-immunoprecipitation experiments described in the thesis were performed at least twice and the blots shown are representative images.

2.6. SDS PAGE and Immunoblotting:

Whole cell protein extract or immunoprecipitation eluate was mixed with equal volumes of 2X Laemmli sample loading buffer containing 5% mercaptoethanol and boiled at 95°C for 5 min. The samples were then resolved on a 12% polyacrylamide gel. Prestained PageRuler^{TM} (Fermentas) was used as a molecular weight marker. The proteins were then transferred to a PVDF membrane using a tank transfer apparatus (Biorad). The membrane was blocked with 5% milk, washed briefly 2 times with TBST, and probed using appropriate primary antibody in 2.5% milk overnight at 4^{o}C. The membrane was washed with TBST for 4 times 10 min each and incubated with secondary antibody at room temperature for 2 h. After washing with TBST for 4 times 10 min each, the proteins were detected using a chemiluminescence kit (Immobilon, Millipore, Cat.No WBKLS0500).

2.7. Luciferase assay protocol:

HEK293T cells were seeded in 12 well plates on day 1 and allowed to attach. On day 2, the plasmids were transfected using Lipofectamine 2000 reagent. The following concentrations of the plasmids were used: 100 ng of pMLHoxb1ARE, 50 ng of pRL-Renilla, and 200 ng each of the expression vectors pFlag-Nono, pCS2+ Meis1a, pCS2+ Pbx1a, Hoxa1, pRsv-Pka, pFlag-Crtc1 and pFlag-Mll1. Media was changed every 24 h post-transfection. 2 days after transfection the cells in each well were harvested using 250 µl passive lysis buffer (PLB) from the Dual luciferase assay kit (Promega, Cat.No: E1960). The lysate was centrifuged at 12000 rpm for 10 min and the supernatant was collected. 50 µl of the lysate was transferred to a 96-well luminometer plate (Thermo scientific Cat.No: 9502887). The luminescence was quantified using an automated Fluoroskan Ascent FL luminometer (Thermo Scientific) which dispenses 100 µl of the firefly luciferase substrate and 100 µl of the Renilla luciferase substrate. Renilla luciferase plasmid was transfected to control for the transfection efficiency. Data was normalized to values from only the reporter plasmid transfection and compared as relative luciferase units. The error bars represent the standard deviation. Statistical analysis was done using the Student's *t* test.

2.8. Immunofluorescence Assay:

Cells seeded on poly-L-lysine-treated coverslips were washed with PBS followed by fixing with 4% paraformaldehyde for 20 minutes at rt. The cells were then permeabilized by incubating with 0.2% Triton X-100/PBS in PBS for 10 min at RT. Then, after blocking with 10% goat serum the cells were incubated with appropriate primary antibody diluted in 1% Triton X-100/PBS at 37ºC, overnight. The cells were then washed with 0.1% Triton X-100/PBS twice for 15 min followed by incubation with appropriate secondary antibody for 1 h in the dark at room temperature. The cells were washed again with 0.1% Triton X-100/PBS and dried. DAPI was used as a mounting solution to lay the coverslips on a glass slide.

2.9. siRNA knockdown:

For knock down of NONO (p54nrb) expression a commercially available ON-TARGETplus SMART pool siRNA (Thermo scientific; L-007756-01-0005, Human NONO) was used. The cocktail consists of 4 different siRNA which are all specific for NONO:

siRNA1: 5'-AAACAAACGUCGCCGAUAC-3',

siRNA2: 5'-GGAUGGGUCAGAUGGCUAU-3',

siRNA3: 5'-GUCAAUUCUGUGUGGUAUA-3',

siRNA4: 5'-CAAAGUGGAUCCAGUUAGA-3'

For transfections for luciferase assays, 2×10^5 HEK293T cells were seeded on day 1 and allowed to attach to the culture dish. On day 2, 100-200 pmol of NONO siRNA was transfected using Lipofectamine 2000 reagent. On day 3, plasmid constructs for the luciferase experiment were transfected. Medium was changed every 24 h. The cells were harvested on day 5.

Chapter 3
Results

3.1. PART 1

3.1.1. Creation of expression constructs for BAP tagged-MEIS1A:

We created two different constructs expressing tagged full-length MEIS1A. As shown in Fig. 3.1, the product of one construct is designated FBM and contains a FLAG tag and a biotin acceptor peptide (BAP) tag at the N-terminus of MEIS1A. The second construct expresses MBF which contains the FLAG and BAP tags at the C-terminus of MEIS1A. These variants were created because the location of tags have been shown to present inherent differences in the binding efficiency of the tag to affinity beads and the efficiency of immunodetection of the tag. The location of a tag within a protein can also interfere with macromolecular interactions. Normally, most tags function more efficiently at the termini rather than at internal sites (Jarvik and Telmer, 1998).

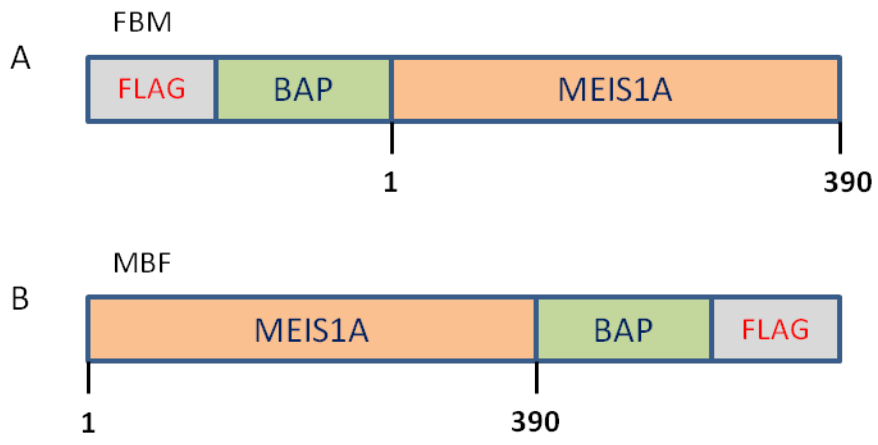


Figure 3.1: **FLAG and BAP-tagged MEIS1A constructs:** Top panel: FBM construct- FLAG and BAP tag at the N-terminus of MEIS1A; Bottom panel: MBF construct- FLAG and BAP tag the C-terminus of MEIS1A.

3.1.2. HEK293T cells as a system for the isolation of MEIS1A protein partners:

In the present study we have used human embryonic kidney 293T (HEK293T) cells as the model system to isolate interacting partners of MEIS1A. Apart from the fact that HEK293T cells are easy to culture and transfect, MEIS1 is endogenously expressed in these cells raising the likelihood of detecting biological relevant interactions in this cell line (Fig 3.2, lane 1).

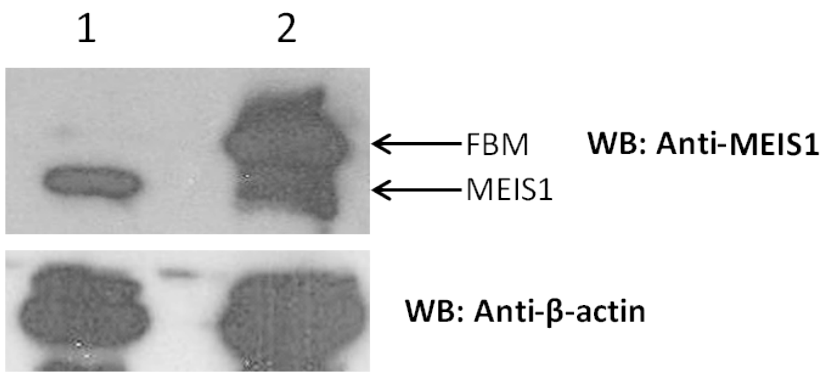


Figure 3.2: Endogenous expression of MEIS1A in HEK293T cells: 30% of whole cell extract was loaded for each lane. Upper panel: Lane 1 - Extract of un-transfected HEK293T cells. Lane 2 - Extract of HEK293T cells over-expressing FBM. Immunoblot was probed with Anti-MEIS1 antibody

Bottom panel: Equivalent amount of whole cell extract was probed with anti- β-actin antibody as a control.

3.1.3. Creation of BirA stable cell lines:

The *E. coli* BirA expression plasmid was linearized and transfected into HEK293T cells. Hygromycin-B was used to select cells that had stably incorporated the transfected vector. To isolate a population of cells showing uniform and stable BirA expression, the transfected cells were seeded at very low densities allowing the attachment of single cells in a highly dispersed manner. A few colonies were then isolated using cloning cylinders and were then allowed to grow to confluency. The individual colonies thus isolated were then screened for the BirA expression using immunoblotting and immunofluorescence assays as shown in figures 3.3 and 3.4. The stably transfected cells show clear expression of *E. coli* BirA protein while the un-transfected cells show no expression.

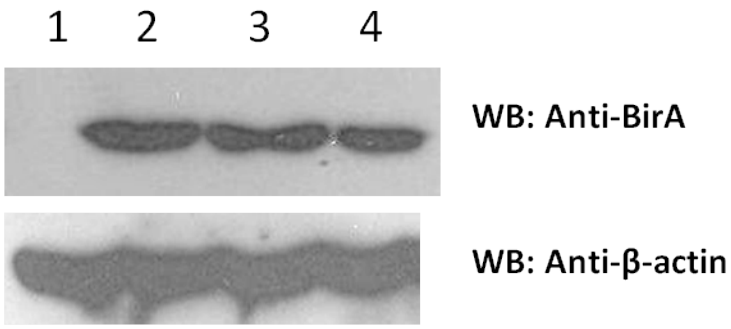


Figure 3.3: **Analysis of the stable expression of BirA in HEK293T cells by immunoblotting:** Upper panel: Lane 1 - wild type HEK293T cells, lanes 2-4 - HEK293T clones stably expressing BirA biotin ligase. Immunoblot was probed with Anti-BirA antibody.
Bottom panel: β-actin expression is shown as a control.

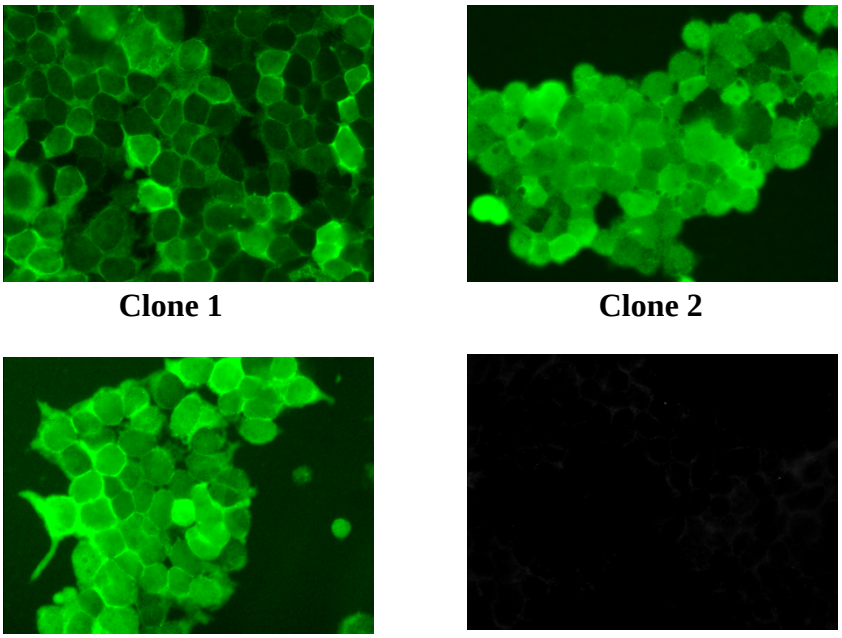


Figure 3.4: Immunofluorescence for BirA expression in stably transfected HEK293T cells: Three representative clones showing uniform and stable expression of BirA. In contrast, the un-transfected cells present background signal only. All images are at the same magnification and exposure.

3.1.4. *In vivo* biotinylation of FBM:

In order to determine whether the BAP-tagged MEIS1A protein was biotinylated *in vivo* by BirA, we expressed the protein in HEK293T cells stably expressing BirA. As a control, equal concentrations of the plasmids were transfected into wild type HEK293T cells that do not express BirA. Immunoblotting with streptavidin-HRP as a probe showed that FBM was biotinylated only in the HEK293T cells which stably express BirA and not in wild type HEK293T cells (figure 3.5). Even though biotin ligase enzymes are present in mammalian cells, these enzymes are unable to biotinylate the 23 amino acid biotin acceptor peptide.

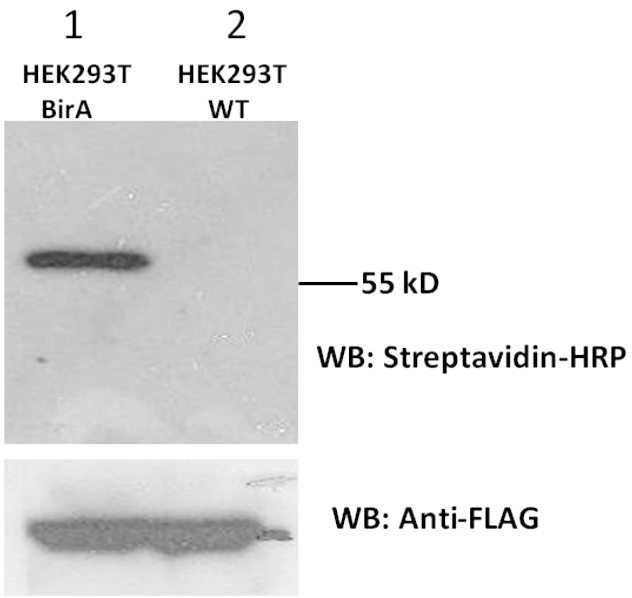


Figure 3.5: ***In vivo* biotinylation of FBM:** FBM is biotinylated only in those cells that have been stably transfected with BirA and not in un-transfected cells. Upper panel: Immunoblot of whole cell extracts from wild type (lane 2) and BirA stably expressing (lane1) HEK293T cells transfected with equimolar amounts of FBM plasmid. Immunoblotting was done using streptavidin-HRP.
Bottom panel: 10% of input was run as a control and the immunoblot was probed with anti-FLAG antibody which detects the expression of transfected FBM.

In order to ascertain the efficiency of biotinylation, biotin-tagged proteins (FBM and MBF) were subjected to immunoprecipitation using streptavidin-magnetic beads and subsequently detected by western blot using a pan MEIS1/2/3 antibody. The FBM protein is found to be biotinylated to a higher level than the MBF protein (Fig 3.6, left and right panels, respectively). More FBM was found in the bound fraction compared to the supernatant, whereas for the MBF protein equivalent amount of protein was observed in the bound fraction and the unbound fraction of the supernatant (Fig 3.6, left and right panels, respectively). It should be noted that the MBF protein could be biotinylated as efficiently as the FBM protein, but the C-terminal biotin could be inaccessible to the Streptavidin. Since the FBM protein was found to be biotinylated and bound Streptavidin beads more efficiently than the MBF protein, we used FBM for stable expression and further analysis.

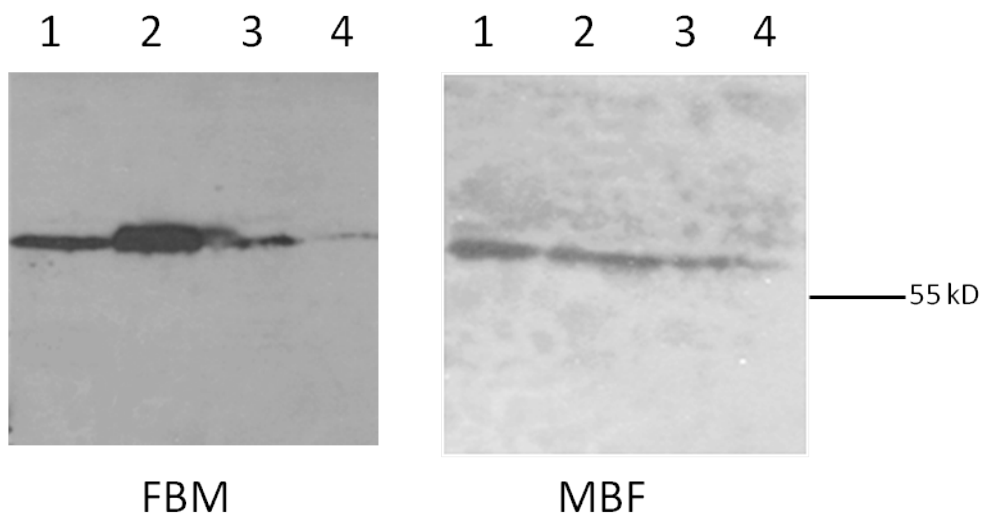


Figure 3.6: **Efficiency of biotinylation of BAP-tagged MEIS1A protein:** Left panel: Degree of biotinylation of the FBM fusion protein. Lane 1: 10% input. Lane 2: 1/3rd of the eluate. Lane 3: Unbound supernatant. Lane 4: 10% of 1st wash buffer. Right panel: Degree of biotinylation of the MBF fusion protein. Lane 1: 1/3rd of the eluate, Lane 2: 10% of input, Lane 3: Unbound supernatant, Lane 4: 10% of 1st wash buffer. Western blot was probed with a pan MEIS1/2/3 antibody.

3.1.5. Test for functionality- interaction with known partners:

One way to test whether a modified protein retains its activity and function is to check whether the protein is able to interact with known protein partners. MEIS1A interacts with PBX and CRTC1 and this interaction is important for many of its transcriptional activities. In order to test whether the FBM protein retains its interaction with PBX and CRTC1, we did co-immunoprecipitation assays. To test for the interaction between CRTC1 and FBM, Anti-CRTC1 antibody was used for immunoprecipitation and the western blot was probed with pan MEIS1/2/3 antibody. The FBM protein was found to interact with CRTC1 in cells transfected with FBM and FLAG-CRTC1 constructs (Fig 3.7, left panel, lane 2). Pulldown of CRTC1 was also observed from cells transfected only with FBM, showing that FBM is able to interact with endogenous CRTC1 as well (Fig 3.7, lane 1). Co-IP was not observed in cells transfected with CRTC1 alone (lane 3).

To test for the interaction between PBX1 and FBM, Streptavidin Agarose was used for the immunoprecipitation of Biotin-tagged FBM and the western blot was probed with Anti-PBX1 antibody. PBX1 was also found to interact with FBM in cells transfected with FBM alone or FBM with PBX1 (Fig 3.7, Right panel, lanes 2 and 3 respectively). As expected, there was no co-IP in cells transfected with PBX alone. Again, FBM was found to interact with endogenous PBX (lane 3).

This shows that the FLAG-BAP-tagged MEIS1A retains the structural configuration needed for interaction with its protein partners. This observation is very important because a native three-dimensional structure of the protein is always desired while trying to isolate its interaction partners. The results obtained prove that the biotin-acceptor peptide and the FLAG labels do not interfere with MEIS1A protein folding and hence its functionality.

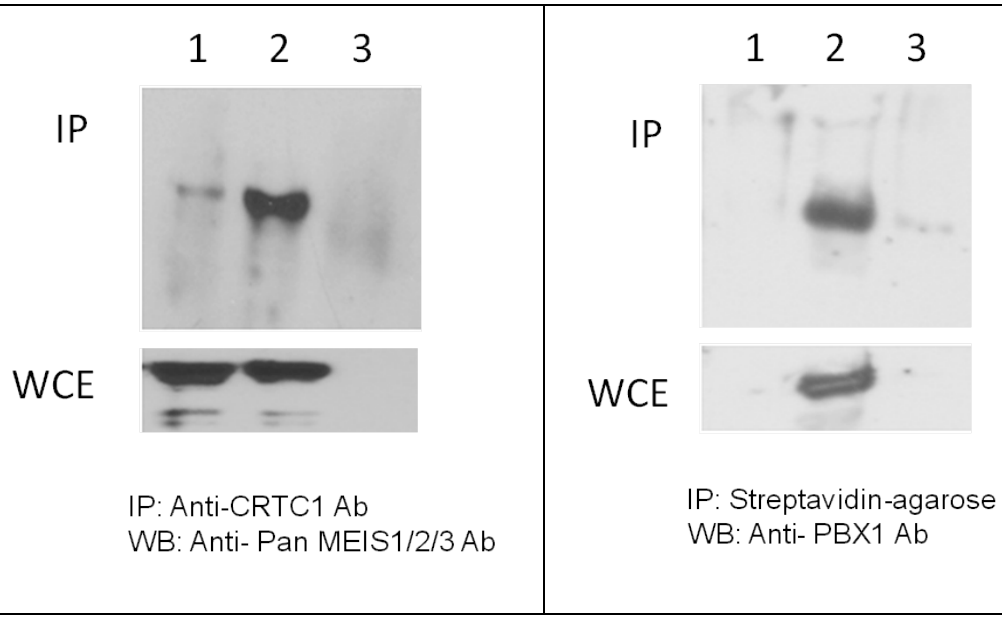
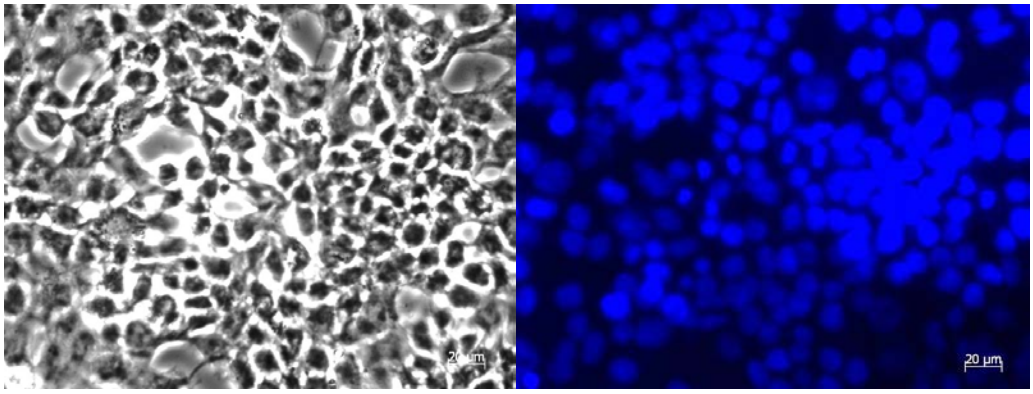


Figure 3.7: **Interaction of FBM with known partners:** Upper left panel: Co-immunoprecipitation of CRTC1 with FBM. Lane 1: Cells transfected with FBM only, lane 2: Cells transfected with CRTC1 and FBM, lane 3: Untransfected control. Lower left panel: 5% of the starting whole cell extracts (WCE) corresponding to lanes 1, 2 and 3 above. Right panel: Co-immunoprecipitation of PBX with FBM. Lane 1: Untransfected control, Lane 2: Cells transfected with PBX and FBM, Lane 3: Cells transfected with FBM only. Lower right panel: 5% of starting whole cell extracts (WCE) corresponding to lanes 1, 2 and 3 above. (IP: immunoprecipitation; WCE: 5% of input; WB: western blot).

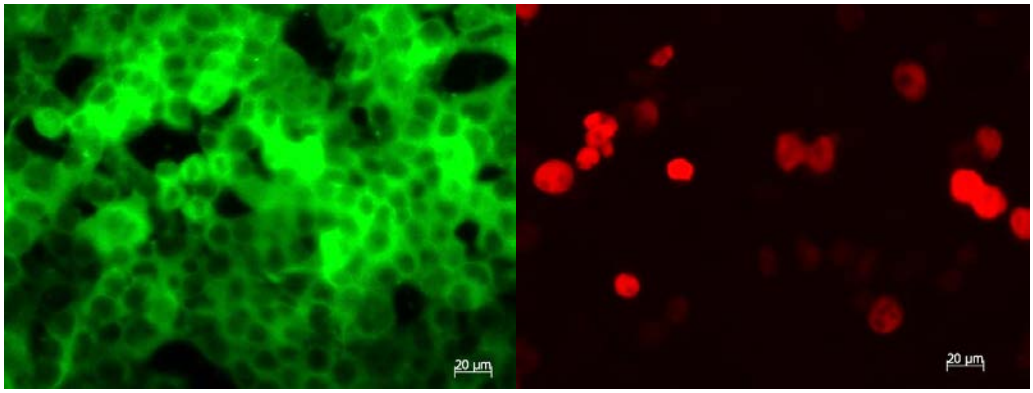
3.1.6. Intracellular localization of FBM:

Being a transcription factor MEIS1 is predominantly localized to the nucleus. To check whether the BAP- and FLAG-tagged FBM protein is localized to the correct intracellular compartment, immunofluorescence studies were performed on HEK293T/BirA cells transfected with FBM. After fixing, the cells were incubated with combinations of anti-BirA and either anti-FLAG or anti-MEIS 1, 2, 3 antibodies. FBM is localized to the nucleus in HEK293T/BirA cells (Fig 3.8 A). Images taken using the 100X objective clearly show the nuclear localization of FBM in the BirA stable cells. BirA itself is predominantly localized to the cytoplasm in these cells (Fig 3.8 B). In order to show that the intracellular localization of FBM is not modulated by the overexpression of BirA, immunofluorescence was done on wild-type HEK293T cells transfected with FBM. FBM was found to be localized to the nucleus in the absence of BirA as well (Fig 3.9). The intracellular localization of MEIS1A in wild-type and BirA over-expressing HEK293T cells is used as the control (Fig 3.9). In contrast to MEIS1A, BirA was present in both cellular compartments although a significant portion was observed in the cytoplasm.

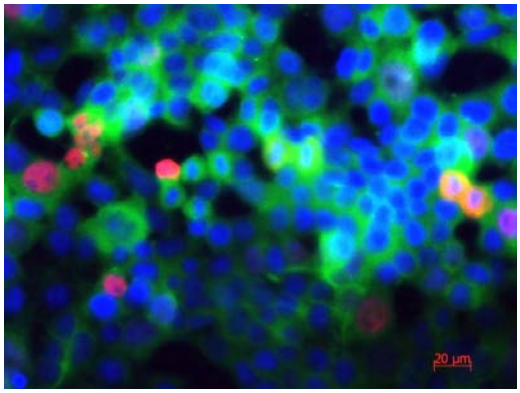
A)



Phase contrast DAPI



BirA FBM



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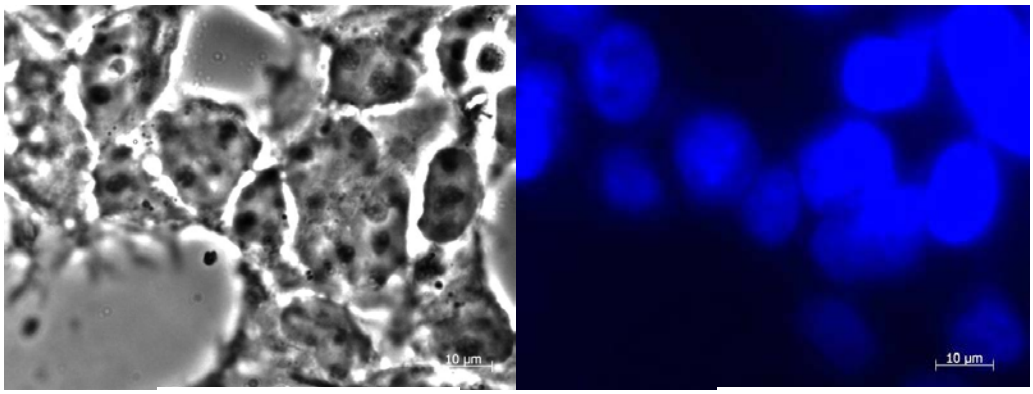
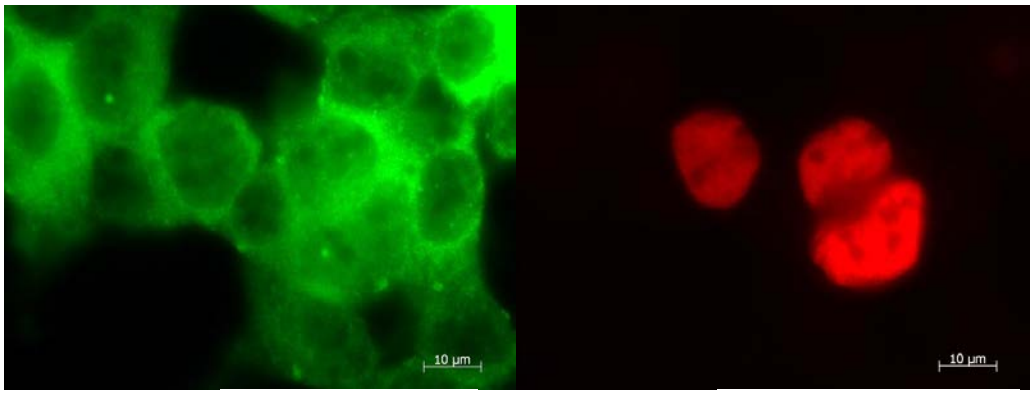
B)



Phase contrast DAPI



BirA FBM

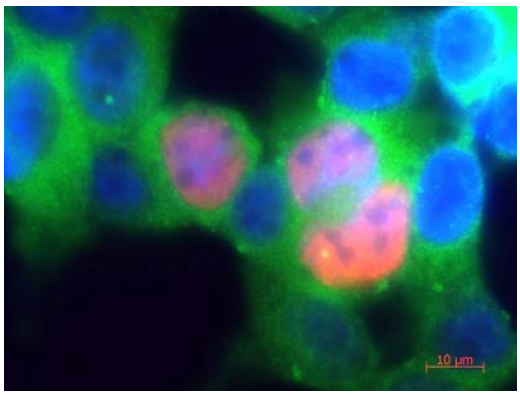


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Figure 3.8: Intracellular localization of FBM in the presence of co-expressed BirA: A) Immunofluorescence of HEK293T/BirA stable cells transfected with FBM. Cells were probed with Anti-BirA antibody and Anti-MEIS1 antibody (32X magnification). **B)** Immunofluorescence of HEK293T BirA stable cells transfected with FBM. Cells were probed with Anti-BirA antibody and Anti-MEIS1 antibody (100X magnification)

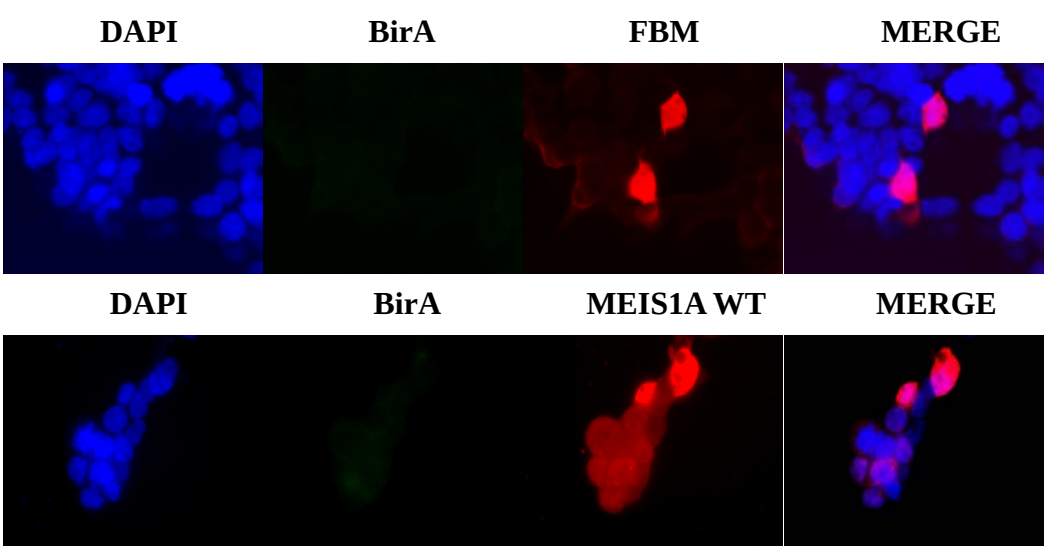


Figure 3.9: **Intracellular localization of FBM in the absence of BirA expression:** Top row: Immunofluorescence of wild type HEK293T cells transfected with FBM. Cells were probed with chicken Anti-*E. coli* BirA antibody and mouse Anti-FLAG antibody. Bottom row: Immunofluorescence of wild typeHEK293T cells transfected with untagged MEIS1A. Cells were probed with chicken Anti-*E. coli* BirA antibody and mouse Anti-MEIS1 antibody.

3.1.7. Stable expression of FBM in HEK293T/BirA cells:

The HEK293T which stably express *E. coli* BirA (HEK293T/BirA) cells were transfected with the linearized pFBM plasmid. Cells stably expressing FBM were selected using 10 µg/ml blasticidin. Individual clones which show uniform and stable expression were then isolated using colony cylinders as described for BirA stable cells (refer 1.3). Figure 3.10 shows the stable expression and biotinylation of FBM in a representative clone. No FBM expression is seen in untransfected HEK293T/BirA cells.

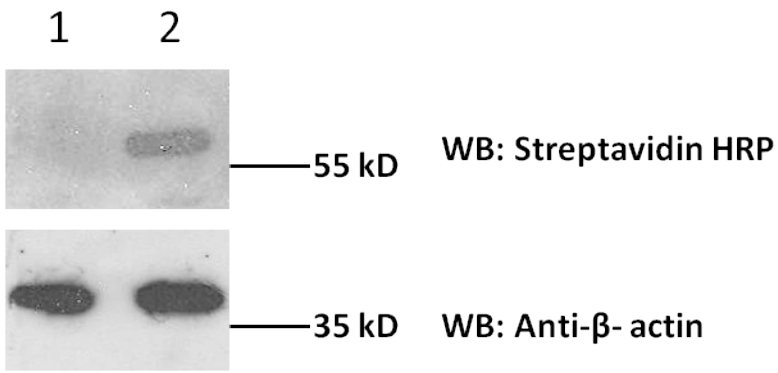


Figure 3.10: **Stable expression of FBM in HEK293T/BirA cells:** Upper panel – Immunoblot of whole cell extracts of untransfected HEK293T/BirA cells (lane 1) and HEK293T/BirA cells stably transfected with FBM (lane 2) probed with Streptavidin-HRP. Lower pannel: β- actin was run as a control.

3.2 PART 2:

3.2.1. Identification of MEIS1A interacting proteins:

In order to purify protein partners of MEIS1A, whole cell protein extract from HEK293T cells expressing FBM was used. The protein extract was incubated with streptavidin magnetic beads overnight and after washing at high stringency conditions of 1% detergent (0.5% NP40, 0.5% Triton X-100), the bound proteins were eluted. HEK293T cells expressing only BirA biotin ligase was used as the control. In order to achieve reproducible results and eliminate false positives three different biological replicates for both control and test samples were prepared. The eluates from both the control and the test samples were run on polyacrylamide gels, subjected to in-gel trypsin digestion and purified. The samples were then analysed by LC-MS/MS. The peptide spectra were then searched against the IPI database of human proteins to identify the proteins present in the sample.

Proteins identified in the control sample included highly abundant cytoskeletal proteins like actin, myosin, vimentin, filamin, spectrin, lamin, drebin etc and naturally biotinylated proteins like mitochondrial carboxylases and other catalytic enzymes, some ribosomal proteins and mRNA processing factors. All the proteins that were present significantly in the control samples were considered as background binding proteins and were disregarded for further analysis.

The proteins which were identified only in the test sample in all three biological replicates are listed in Table 3.1. The emPAI and the number of unique peptides identified are also mentioned for each protein. The emPAI score is the exponential of the Protein Abundance Index (PAI). This value gives an accurate quantification of the protein abundance in the sample compared with the observable abundance for the same protein in MS (Ishihama et al., 2005).

$$\text{PAI of a protein} = \frac{\textbf{Observed}\ \text{number of peptides for the protein}}{\textbf{Observable}\ \text{number of peptides for the protein in MS}}$$

$$\textbf{emPAI} = 10^{\text{PAI}} - 1$$

S.N	UNIPROTKB	GENE NAME	emPAI scores			Number of unique peptides identified		
			Ex1	Ex2	Ex3	Ex1	Ex2	Ex3
1.	Q9UJV9	MEIS1	0.34	0.22	0.34	5	3	4
2.	P40424	PBX1	0.31	0.27	0.31	5	4	3
3.	P09493	TPM1	0.93	0.21	0.12	7	2	1
4.	Q9UJV9	DDX41	0.38	0.22	0.15	7	7	3
5.	Q9Y2X3	NOP58	0.17	0.27	0.17	3	6	3
6.	O60784	TOM1	0.12	0.11	0.06	2	2	2
7.	O00567	NOP56	0.27	0.29	0.27	5	6	6
8.	P31943	HNRNPH1	0.29	0.19	0.29	4	3	4
9.	P11388	TOP2A	0.06	0.14	0.07	3	9	4
10.	P40426	PBX3	0.14	0.20	0.14	3	3	2
11.	Q7Z2W4	ZC3HAV1	0.08	0.07	0.14	2	2	3
12.	P40425	PBX2	0.15	0.13	0.07	2	3	2
13.	P46013	KI-67	0.04	0.13	0.08	4	16	11
14.	P35251	RFC1	0.05	0.02	0.05	2	1	2
15.	Q13185	CBX3	0.56	0.69	0.16	3	3	1
16.	Q13247	SFRS6	0.17	0.15	0.17	2	3	2
17.	Q03164	MLL	0.01	0.01	0.01	2	1	1
18.	P84090	ERH	0.63	0.54	0.63	2	2	2
19.	Q13242	SFRS9	0.28	0.24	0.28	2	2	3
20.	O00541	PES1	0.10	0.18	0.15	2	4	3
21.	P38919	EIF4A3	0.07	0.20	0.07	1	4	1
22.	Q02880	TOP2B	0.05	0.08	0.04	3	6	3
23.	P46777	RPL5	0.32	0.77	0.32	5	6	3
24.	Q01130	SFRS2	0.13	0.24	0.28	1	2	2

25.	Q53EZ4	CEP55	0.19	0.11	0.06	3	2	2
26.	P14866	HNRNPL	0.05	0.14	0.10	1	3	2
27.	P83916	CBX1	0.16	0.14	0.16	1	1	1
28.	P56537	EIF6	0.12	0.12	0.12	1	1	2
29.	Q9Y6M1	IGF2BP2	0.05	0.04	0.10	1	1	2
30.	Q9BZE4	GTPBP4	0.14	0.26	0.19	3	6	5
31.	P62266	RPS23	0.21	0.41	0.21	1	2	1
32.	P18077	RPL35A	0.27	0.90	0.27	2	4	2
33.	Q96ME7	ZNF512	0.05	0.14	0.16	1	3	3
34.	P42696	RBM34	0.07	0.42	0.14	1	6	2
35.	Q9UKM9	RALY	0.21	0.09	0.34	2	3	3
36.	Q15233	NONO	0.06	0.11	0.06	1	2	2
37.	Q8WWQ0	WDR11	0.02	0.03	0.05	1	2	3
38.	Q15029	EFTUD2	0.03	0.05	0.06	2	3	2
39.	Q13620	CUL4B	0.03	0.06	0.03	1	2	1
40.	Q9P0M6	H2AFY2	0.08	0.23	0.08	2	5	1
41.	Q9NVP1	DDX18	0.04	0.04	0.09	1	1	2

Table 3.1: Proteins identified in LC-MS/MS in FBM pulldown experiments: The table shows the proteins identified in 3 biological replicates of the test sample and not in the control. The emPAI scores and the number of unique peptides identified for each protein is mentioned.

About 40 proteins were identified reproducibly only in the test sample and not in the control. Firstly, all three PBX proteins, PBX1, 2 and 3 were identified in all three replicates only from cells expressing biotin-tagged MEIS1A and not in the control samples. PBX proteins, as discussed above, are the predominant binding partners of MEIS1 in many tissues both during embryogenesis and in cancer. Other transcription factors that were identified include ZNF512, CBX3, CBX1, NONO,

RBM34 and ERH. Apart from novel transcription factors, chromatin binding and modifying proteins like TOP2A, TOP2B and RFC1 were also identified in the experimental sample. Since MEIS1 is a transcription factor, the identification of these proteins involved in the transcriptional machinery further validates our approach since transcription and chromatin related proteins were not highly represented in the control purifications.

Figure 3.11 shows the classification of the identified proteins according to their molecular functions based on Gene Ontology (GO) analysis (http://www.geneontology.org/). The majority of the proteins identified are mRNA binding proteins including splicing factors like the Serine/Arginine-rich proteins and heterogeneous ribonucleoproteins. Together with the DEAD box RNA helicases they constitute about 30% of the total identified proteins. About 20% of the identified proteins comprise transcription factors and chromatin binding proteins. Interestingly, many proteins involved in rRNA processing and ribosome biogenesis were also reproducibly seen to co-purify with MEIS1A. These proteins include the snoRNA binding proteins like NOP56/58 and proteins involved in 60S ribosome biogenesis like PES1 and GTPBP4.

Although many ribosomal protein components were identified in the control purifications, 3 ribosomal proteins RPL5, RPL35A and RPS23 were identified specifically and reproducibly in the FBM pulldowns. Similarly, Tropomyosin 1 was the only actin cytoskeleton component to be reproducibly identified in the MEIS1A pulldowns. Cell cycle regulators like KI-67, ERH and CEP55 were also identified only in the experimental sample and not in the control. Other proteins like CUL4B, an E3 ubiquitin ligase; H2AFY2, a core histone protein; and TOM1, involved in intracellular protein transport were also identified reproducibly in the MEIS1A pull down.

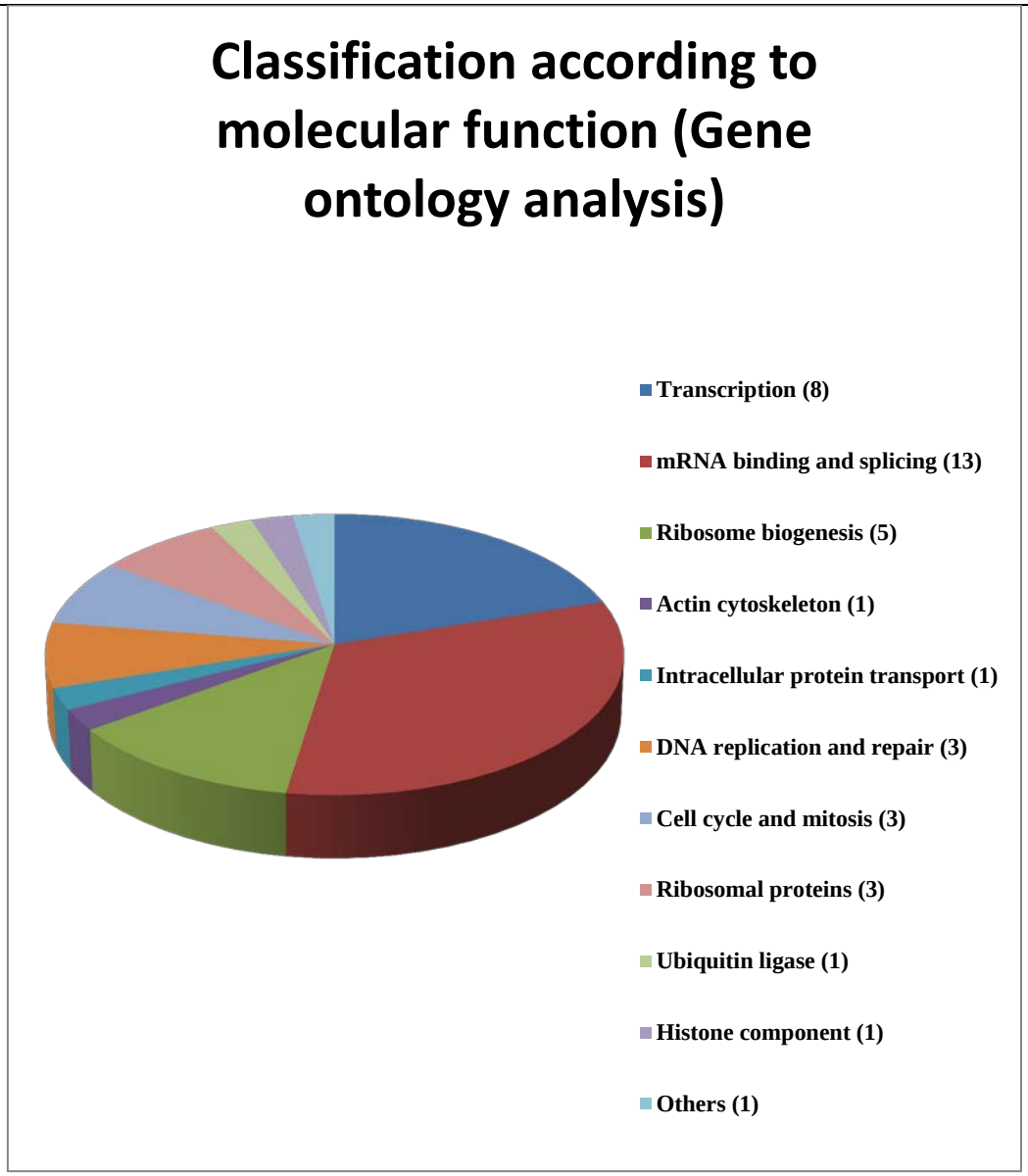


Figure 3.11. Pie chart of the identified proteins classified by function: The proteins have been classified by their molecular functions. The number of proteins identified in each category is given in parentheses. The proteins were organized using the Gene Ontology database (http://www.geneontology.org/).

Some HOX proteins, which are also known partners of MEIS1, were also found in the pull down experiments. HOXA5, HOXA10 and HOXC13 were identified

in the mass spectrometry results only in the test sample and not in the control. But unlike PBX proteins, these HOX proteins were not consistently identified in all the 3 replicate experiments. This could be due to the high stringency washing conditions used to purify the proteins. Table 3.2 shows some other important proteins which were identified less frequently in two of the three biological replicates.

S.No	GENE NAME	MOLECULAR FUNCTION
1.	UBTF	Nucleolar transcription factor
2.	PELOTA	Cell cycle associated protein
3.	CEBPZ	Transcription factor
4.	RPA1	DNA replication
5.	KPNA2	Nuclear import of proteins
6.	ATRX	Chromatin binding; Transciptional activation
7.	U2AF1	Splicing factor
8.	HMGA2	DNA binding; Transcription regulation
9.	SMARCA1	Chromatin modification

Table 3.2: Physiologically important proteins identified in 2 of the 3 independent experiments to co-purify with MEIS1A

3.3 PART 3:

3.3.1 Investigating the interaction between MEIS1A and NONO:

One of the initial targets that we selected for validation and functional analysis was NONO, also known as p54nrb.

The CREB co-activators CRTC1 and CRTC2 did not co-purify with MEIS1A in our pull-down experiments. This is surprising because both CRTCs have been shown to interact with MEIS1A via co-immunoprecipitation assays (Goh et al., 2009). Interestingly, NONO (p54nrb), which is a PKA-dependent transcription factor, has been shown to interact with CRTC1 and 2 and augment their transcriptional activation function (Amelio et al., 2007). We hypothesized that the CRTCs could interact with MEIS1A indirectly via NONO. Hence the primary reason for selecting NONO to validate the mass spectrometry results was to study its potential role as a mediator between CRTCs and MEIS1A and its role in the PKA-dependent transactivation function of MEIS1.

To this end, we did co-immunoprecipition assays to validate the interaction between MEIS1A and NONO. Following transient transfection, immunoprecipitation of ectopically expressed FLAG-NONO using FLAG M2 agarose beads resulted in the co-precipitation of HA-tagged MEIS1A (Figure. 3.12). HA-MEIS1A was unable to bind the beads in the absence of co-expressed FLAG-NONO. To test if the endogenous NONO and MEIS1 proteins could also interact, we used anti-NONO antibody to purify interacting proteins from 2×10^9 HEK293T cells and observed the interaction using anti-MEIS1 antibody on an immunoblot. Endogenous NONO was able to pull down endogenous MEIS1 expressed in HEK293T cells, whereas no MEIS1 protein was observed when the immunoprecipitation was done using anti-FLAG antibody as a non-specific control (Figure. 3.13). Hence, this proves that MEIS1 forms a complex with NONO endogenously.

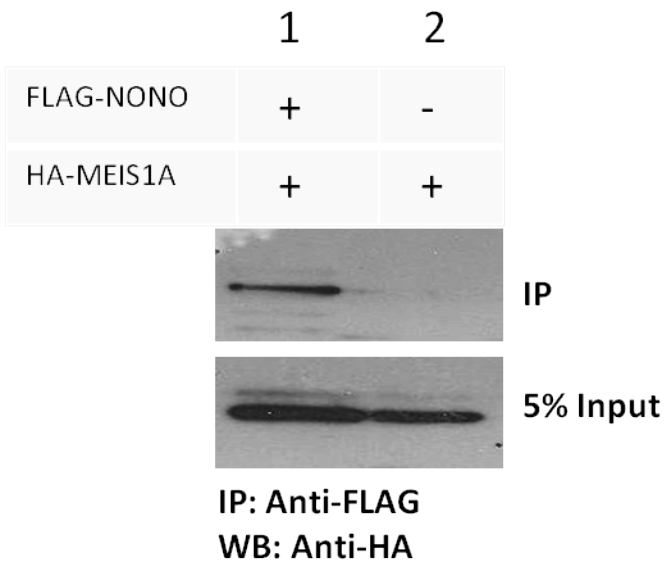


Figure 3.12: Over-expressed HA-tagged MEIS1A interacts with NONO: Figure shows the immunoblot of FLAG agarose immunoprecipitates from 293T cells overexpressing HA-MEIS1A, with (lane1) or without (lane 2) co-expressed FLAG-CRTC1. Western blot was done using Anti-HA antibody. 5% input was run as a control.

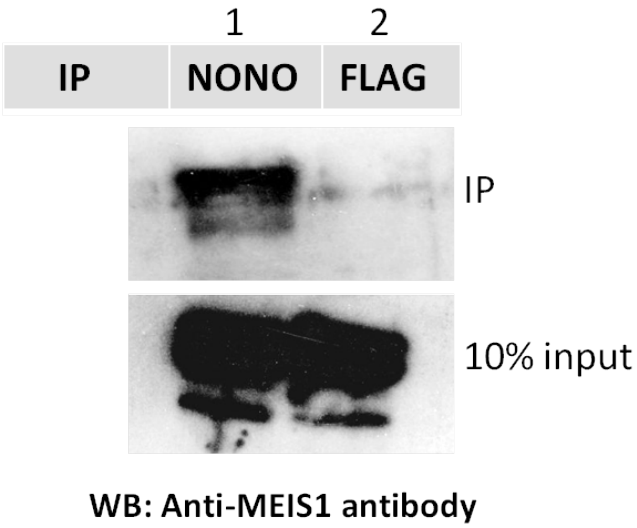


Figure 3.13: MEIS1A interacts with endogenous NONO: Equal quantities of HEK293T whole cell extracts were immunoprecipitated with either anti-NONO or non-specific IgG (anti-FLAG) antibodies as indicated. Western blot was done using anti-MEIS1 antibody. 10% input was run as a control.

3.3.2. Knockdown of endogenous NONO expression does not lead to a decrease in MEIS1-CRTC1 interaction:

In order to observe whether the knockdown of NONO expression would lead to a simultaneous reduction in the interaction between MEIS1 and CRTC1 we used a pool of four different siRNAs specifically targeted to the 3'UTR of the NONO mRNA (SMART pool, Thermo scientific). As it can be observed in figure 3.14 a starting concentration of 100 pmol lead to a considerable knockdown of endogenous NONO in HEK293T cells. As expected, a pool of control siRNAs (Thermo Scientific) did not have any effect on the endogenous expression of NONO. The knockdown efficiency was compared to equimolar amounts extracts from Untransfected HEK293T cells. β-actin expression used as a control was not affected the by the transfection of any of the siRNAs.

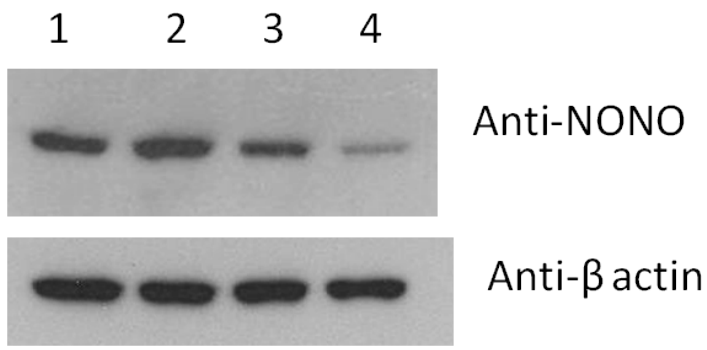


Figure 3.14: siRNA-mediated knockdown of NONO: Whole cell extracts of HEK293T cells treated with 100 pmol of control siRNA (lane 2), 100 pmol of NONO siRNA (lane 3), 200 pmol of NONO siRNA (lane 4) and untreated control (UT, lane 1), probed with anti-NONO antibody. Bottom panel: β-actin expression is shown as a loading control.

We next tested the effect of the knockdown of NONO expression on interaction between MEIS1A and CRTC1 in a co-immunoprecipitation assay. The reduction in expression of endogenous NONO had no effect on the interaction between FLAG-CRTC1 and HA-MEIS1A. Similar amounts of HA-MEIS1A are pulled down by CRTC1 both in the presence of 100 pmol and 200 pmol of NONO siRNA and in untransfected control (Figure 3.15).

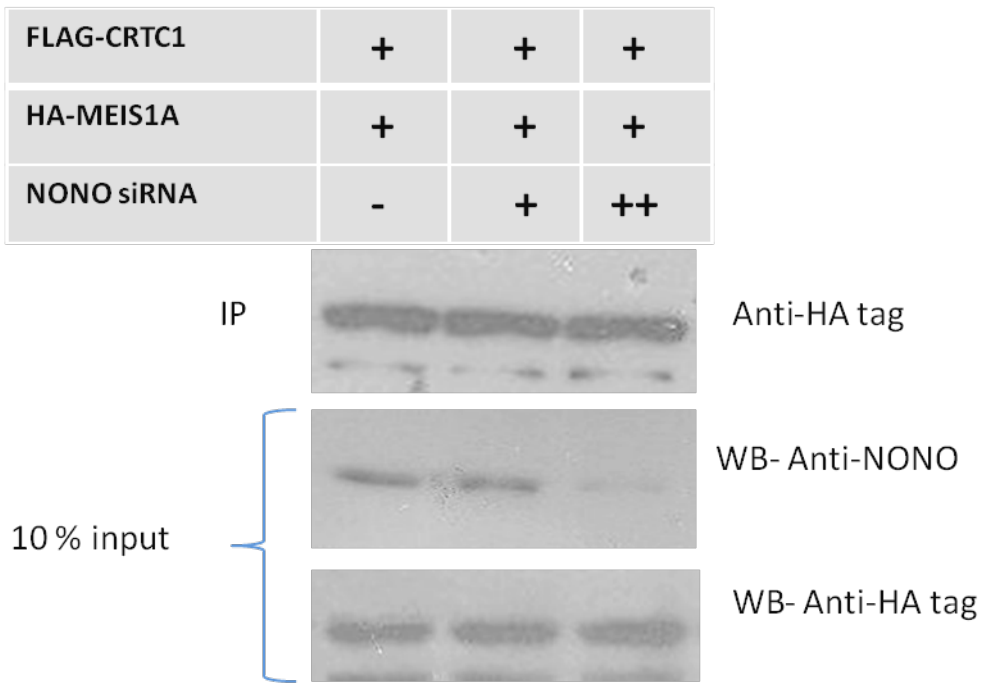


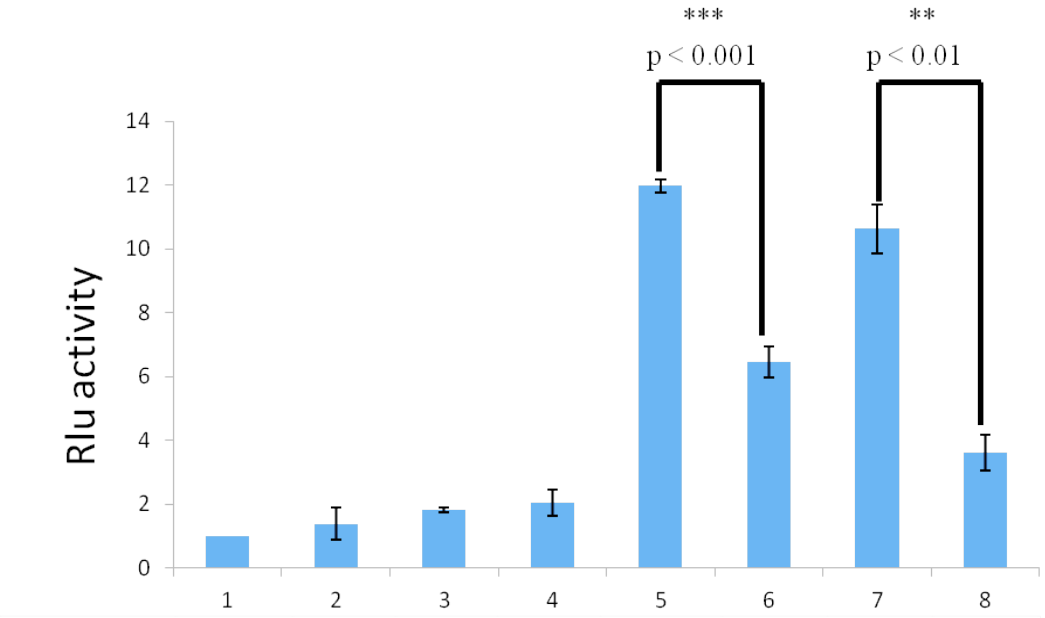
Figure 3.15: CRTC1-MEIS1A interaction is not mediated through NONO: Top panel: Co-immunoprecipitation of HA-tagged MEIS1A by FLAG-CRTC1 in the presence 100 pmol NONO siRNA (lane 2), 200 pmol NONO siRNA (lane 3) and Untransfected (lane 1) conditions. Middle panel: NONO expression is shown in normal and knockdown conditions. 10% input was probed with anti-MEIS1 antibody as a control.

3.3.3. NONO does not have any effect on MEIS1A transactivation, while have an inhibitory effect on PKA signaling:

We then asked whether NONO could have a role in regulating the transcriptional activation functions of MEIS1A. The *Hoxb1* auto-regulatory element (ARE) contains three binding sites for HOX-PBX complexes. MEIS1 binds with PBX and HOX partners to this regulatory element to augment transcriptional activation. The HOX-PBX-MEIS complex alone cannot activate transcription from this enhancer element in a luciferase reporter assay (Saleh et al., 2000b). In the presence of PKA-signaling or upon over-expression of CRTC1, the HOX-PBX-MEIS complex induces transcription from the *Hoxb1* ARE.

I tested the contribution of NONO to transcriptional activation by HOX-PBX-MEIS complexes of a luciferase reporter driven by the *Hoxb1* ARE. Transfection of expression vectors of *Hoxa1, Pbx1* and *Meis1a* did not increase the transcriptional activity of the *Hoxb1* ARE (Fig. 3.16). The addition of CRTC1 or PKA increased transcriptional activation through the *Hoxb1* ARE by several fold. The presence of NONO did not augment or decrease the transcriptional activity of the HOX-PBX-MEIS complex alone. Hence NONO by itself does not regulate the transcriptional activity of HOX-PBX-MEIS complexes. However, NONO did reduce the transcriptional activation of MEIS1 in response to PKA signaling. NONO has been previously shown to respond to PKA signaling and appears to have dual functions both as a repressor and activator of the PKA pathway. Hence, it is not possible to conclude whether the NONO-mediated repression of PKA signaling observed in this experiment is independently mediated by NONO or through its interaction with MEIS1A. Furthermore, the siRNA mediated knock down of NONO again did not show any effect on MEIS1 transcriptional activation (Fig. 3.17).

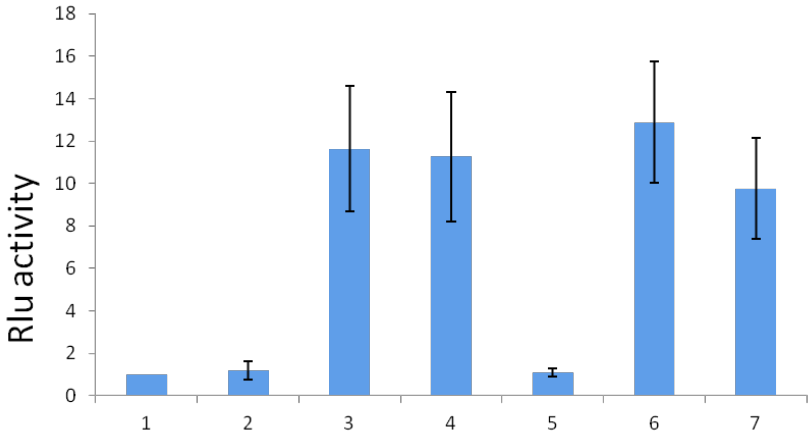
In summary, the luciferase assay experiments show that NONO does not contribute to the transcriptional activation functions of MEIS1 although it might indirectly affect the PKA signaling mediated transactivation function of MEIS1A.



Hoxb1 ARE Luc Reporter	+	+	+	+	+	+	+	+
HoxA1	-	-	+	+	+	+	+	+
Pbx1	-	-	+	+	+	+	+	+
Meis1A	-	-	+	+	+	+	+	+
NONO	-	+	-	+	-	+	-	+
CRTC1	-	-	-	-	+	+	-	-
PKA	-	-	-	-	-	-	+	+

Figure 3.16: NONO does not contribute to MEIS1A transcriptional activity while mildly decreasing the transactivation function of CRTC1 and PKA: Luciferase assay was performed using Hoxb1 ARE- LUC as the reporter. Other expression plasmids tested were transfected as indicated. A plasmid expressing Renilla luciferase was also included in all the transfections to normalize the transfection efficiency. Error bars represent the standard deviation (n=3). Rlu: Relative luciferase activity.

A



Hoxb1 ARE Luc Reporter	+	+	+	+	+	+	+
HoxA1	-	+	+	+	+	+	+
Pbx1	-	+	+	+	+	+	+
Meis1A	-	+	+	+	+	+	+
NONO siRNA	-	-	-	-	+	+	+
CRTC1	-	-	+	-	-	+	-
PKA	-	-	-	+	-	-	+

B

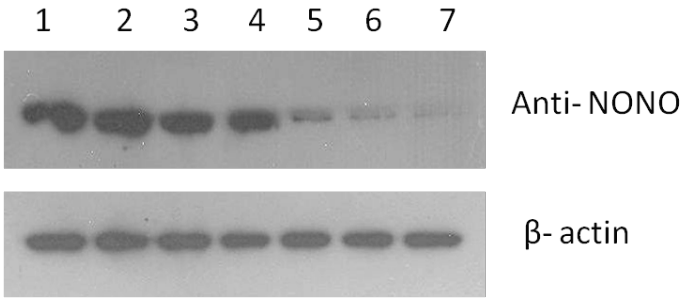


Figure 3.17: NONO siRNA transfection has no effect on the transcriptional activities of MEIS1A or CRTC1: Luciferase assay was performed using Hoxb1 ARE- LUC as the reporter. Other expression plasmids tested were transfected as indicated. A plasmid expressing Renilla luciferase was also included in all the transfections to normalize the transfection efficiency. Error bars represent the standard deviation (N=6). Rlu: Relative luciferase activity. B. NONO protein levels were assessed by western blot using the same protein extracts which were used for the luciferase assay. Lanes 1-7 in the western blot correspond with the transfections shown for the luciferase assay (A)

3.3.4. Other members of the MEIS family also interact with NONO:

Apart from MEIS1, the MEIS/PKNOX family consists of the highly similar MEIS2 and MEIS3 proteins and less similar PKNOX 1 and 2 proteins. This conservation at the level of primary amino acid sequence suggested the possibility that interactions with protein partners were also conserved. Previous studies have shown that all the members of the MEIS/PKNOX family show a conserved interaction with PBX proteins. This led us to examine whether MEIS2 and PKNOX1 could also interact with NONO.

FLAG tagged NONO was able to co-precipitate both HA-tagged MEIS2 and PREP1 (Figure 3.18). Hence we hypothesize that the interaction with NONO is conserved between all the members of the MEIS/PKNOX family.

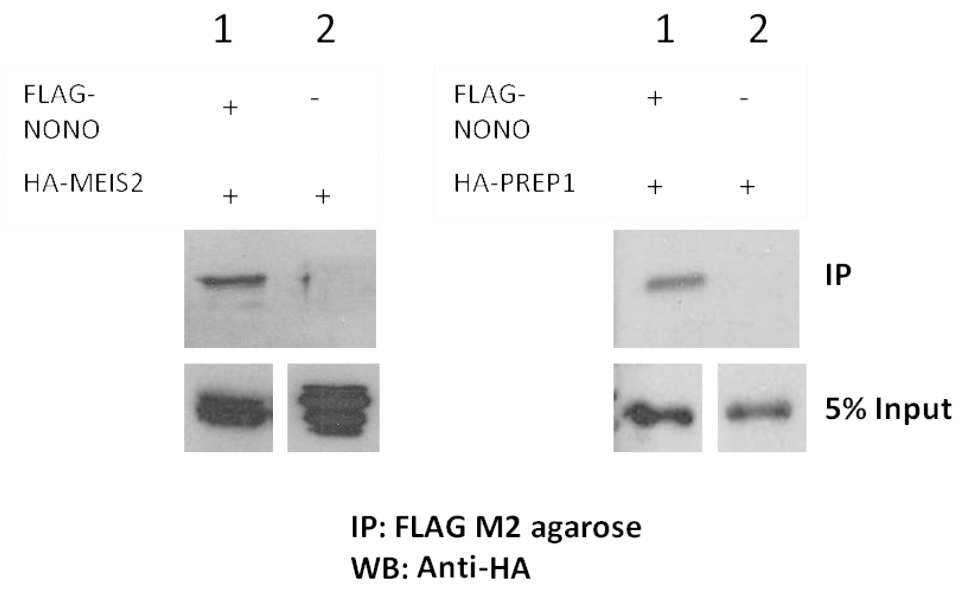


Figure 3.18: MEIS2 and PREP1 also interact with NONO: Anti-FLAG immunoprecipitates from cells transfected with HA-MEIS2+FLAG-NONO (left panel, lane1) and HA-MEIS2 alone (left panel, lane 2); HA-PREP1+FLAG-NONO (right panel, lane 1) and HA-PREP1 alone (right panel, lane 2). Western blotting was done with anti-HA antibody. 5% input is loaded as a control.

3.4 PART 4:

3.4.1 MEIS1A interacts with NOP56, NOP58, CBX3 and MLL1:

We did co-immunoprecipitation experiments to validate the interaction between MEIS1A and four other proteins which were identified in the mass spectrometry data of the experimental pulldown. NOP56 and NOP58 are snoRNA binding proteins involved in ribosomal biogenesis. As described previously, recent evidences indicate a role for many ribosomal biosynthesis proteins in tissue specific and extra ribosomal functions. Specifically, NOP56 and NOP58 proteins have been shown to interact with OCT4 transcription factor in a recent study (Cheong et al., 2011). There are no studies to date indicating an interaction of MEIS1 with proteins involved ribosomal biogenesis. Therefore we selected the NOP56 and NOP58 proteins for further validation since the interaction with these proteins could indicate novel pathways through which MEIS1 regulates cellular function.

MEIS1 has been implicated as an important downstream gene activated by MLL1-fusion proteins in mixed lineage leukemia (MLL). It has been shown to be a rate limiting factor and a stemness factor in MLL (Kumar et al., 2010; Wong et al., 2007). The identification that MLL1 is able to co-purify with MEIS1A is very interesting in this regard. CBX3 which primarily functions as a heterochromatin protein has been recently shown to function also as a transcriptional activator (Lomberk et al., 2006a; Vakoc et al., 2005). Hence it could perform both transcriptional silencing and activating function *in vivo*. MLL1 and CBX3 were selected for further validation since they both are important transcription factors and chromatin modifying proteins performing essential roles in many stages of animal development and cancer.

We carried out co-immunoprecipitation with FLAG-tagged NOP56, NOP58, MLL1 and CBX3 using FLAG M2 agarose beads. Whole cell extracts from HEK293T cells over-expressing the above mentioned proteins together with HA-tagged MEIS1A were incubated with the FLAG beads. After washing, the bound proteins were eluted by using saturating concentration of 3X FLAG peptide. MEIS1A was found in the eluate only in the presence of the aforementioned FLAG-tagged proteins (Figure 3.19, lanes 1-4). MEIS1A alone did not bind FLAG beads efficiently

(Figure 3.19, lane 5). Hence this experiment validates the interaction observed between MEIS1 and NOP56, NOP58, MLL1 and CBX3 proteins as revealed in the mass spectrometry data.

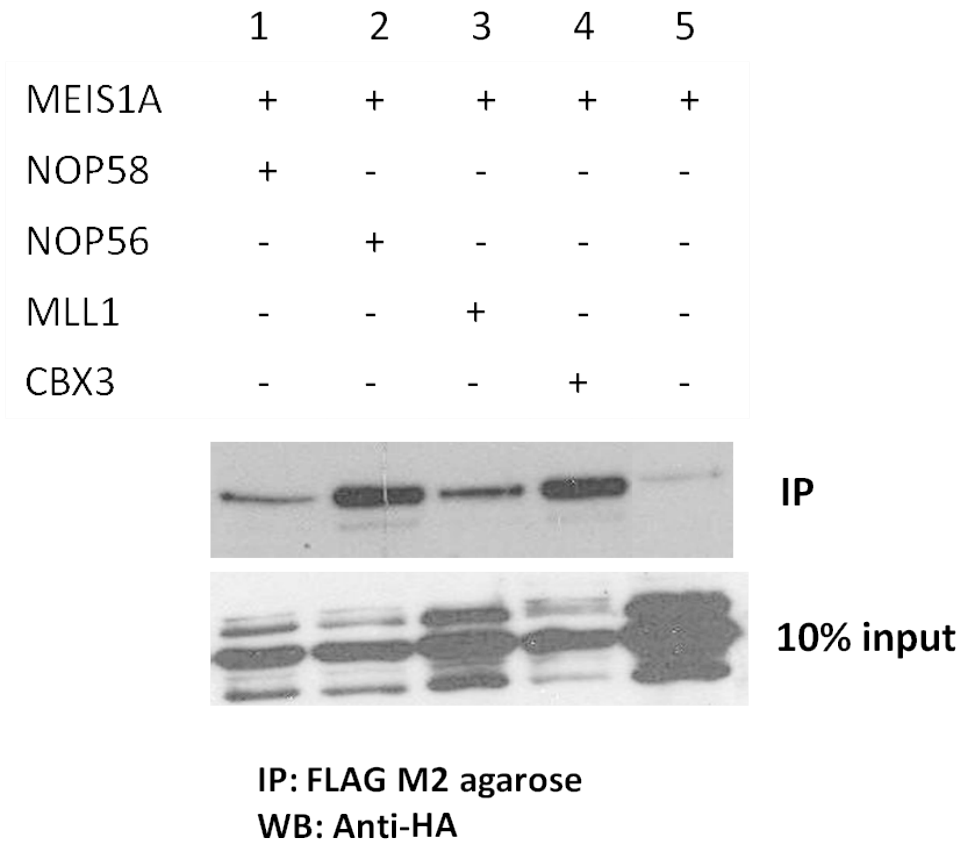


Figure 3.19: MEIS1A interacts with NOP56/58, CBX3 and MLL1: Top panel: Co-immunoprecipitation of HA-tagged MEIS1A by FLAG-NOP58 (lane 1), FLAG-NOP56 (lane 2), FLAG-MLL1 (lane 3), FLAG-CBX3 (lane 4) using FLAG M2 agarose beads. HA-MEIS1A alone did not bind to the FLAG M2 beads (lane 5). Bottom panel: 10% percent of the whole cell extracts (Input). The immunoblotting was done with Anti-HA antibody.

3.4.2. PKNOX1 and PKNOX2 also interact with NOP56 and NOP58 but do not interact with MLL1 and CBX3:

Next we tested whether PKNOX1 and PKNOX2, members of the MEIS/PREP family could also interact with the selected proteins. FLAG-tagged NOP56 and NOP58 were also found to co-immunoprecipitate HA-tagged PKNOX1 and PKNOX2 (Figures 3.20 and 3.21, respectively, lanes 1 and 2 in both figures).

But interestingly, MLL1 and CBX3 did not interact with the PKNOX proteins as observed in Figures 3.20 and 3.21, lanes 3 and 4, respectively.

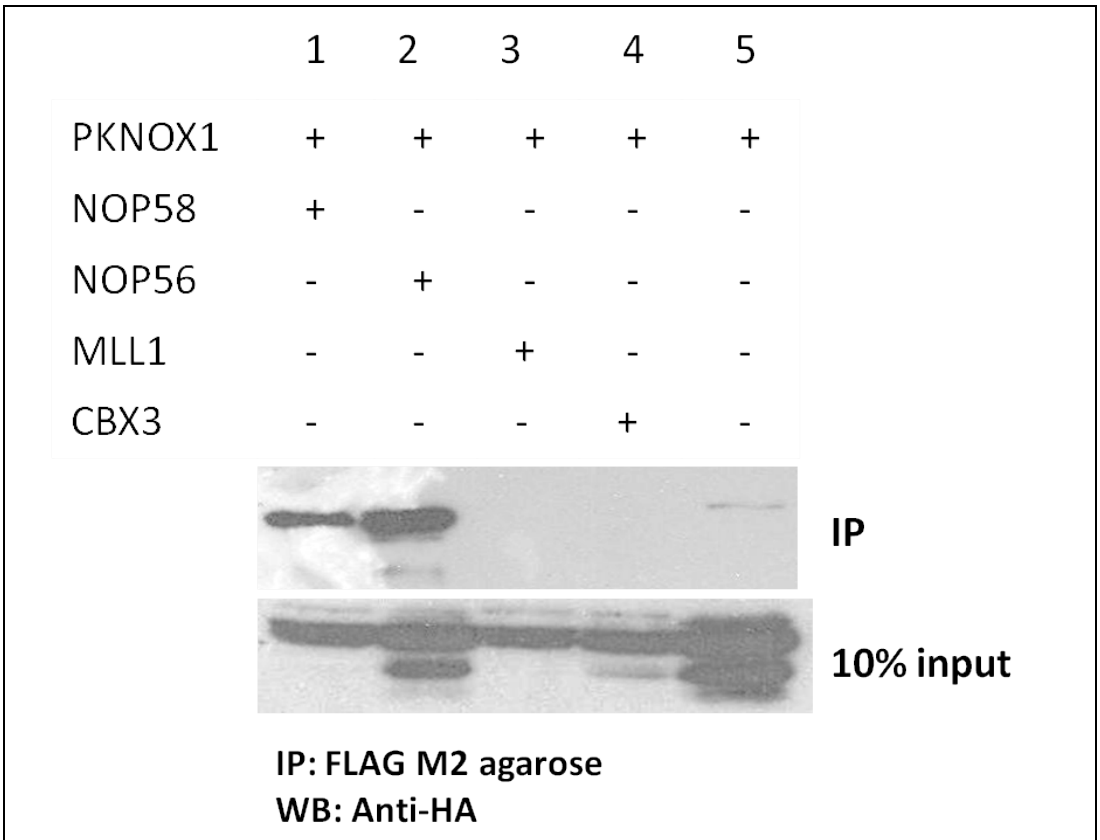


Figure 3.20: PKNOX1 interactions: Top panel: Co-immunoprecipitation of HA-tagged PKNOX1 by FLAG-NOP58 (lane 1) and FLAG-NOP56 (lane 2). FLAG-MLL1 (lane 3) and FLAG-CBX3 (lane 4) were unable to co-immunoprecipitate HA-PKNOX1. HA-PKNOX1 alone did not bind to the FLAG M2 beads (lane 5). Bottom panel: 10% percent of the whole cell extracts (input). The immunoblotting was done with anti-HA antibody.

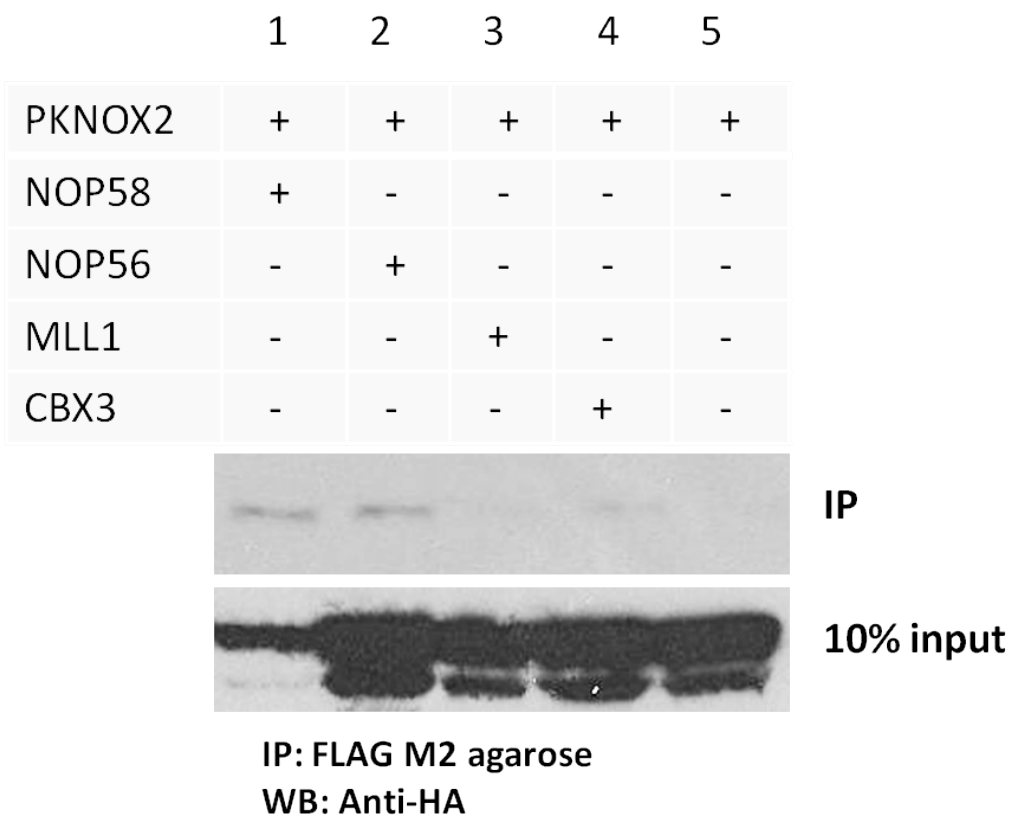


Figure 3.21: PKNOX2 interactions: Top panel: Co-immunoprecipitation of HA-tagged PKNOX2 by FLAG-NOP58 (lane 1) and FLAG-NOP56 (lane 2). FLAG-MLL1 (lane 3), FLAG-CBX3 (lane 4) were unable to co-immunoprecipitate HA-PKNOX2. HA-PKNOX2 alone did not bind to the FLAG M2 beads (lane 5). Bottom panel: 10% percent of the whole cell extracts (Input). The immunoblotting was done with Anti-HA antibody.

3.4.3. MLL-AF4 fusion protein also interacts with MEIS1A:

After validating the MLL1-MEIS1A interaction, we were interested to understand whether this interaction is retained for the fusion products of *Mll1* gene as well. *Mll1* tanslocation is the initiating event in mixed lineage leukemia and the resulting MLL1 fusion proteins function as the primary oncoproteins in MLL. MLL1 forms fusion proteins with over 30 different partners and the primary members which contribute to about 80% of all described fusions in human leukemia are AF4, AF9, AF10, AF6, ENL and ELL. *Meis1* along with some *HoxA* class genes is an important downstream target of these MLL1 fusions (Milne et al., 2005a; Milne et al., 2002; Milne et al., 2010; Zeisig et al., 2004). We did a co-immunoprecipitation assay to understand whether the MLL-AF4 fusion protein could also interact with MEIS1A. FLAG-tagged MLL-AF4 was able to interact with HA-tagged MEIS1A (Figure 3.22, lane 2). On the contrary, HA-tagged PKNOX1 was not able to interact with FLAG MLL-AF4 (Figure 3.22, lane 1). This is as expected since PKNOX1 was not able to interact with wild-type MLL1 as shown in figure 3.20.

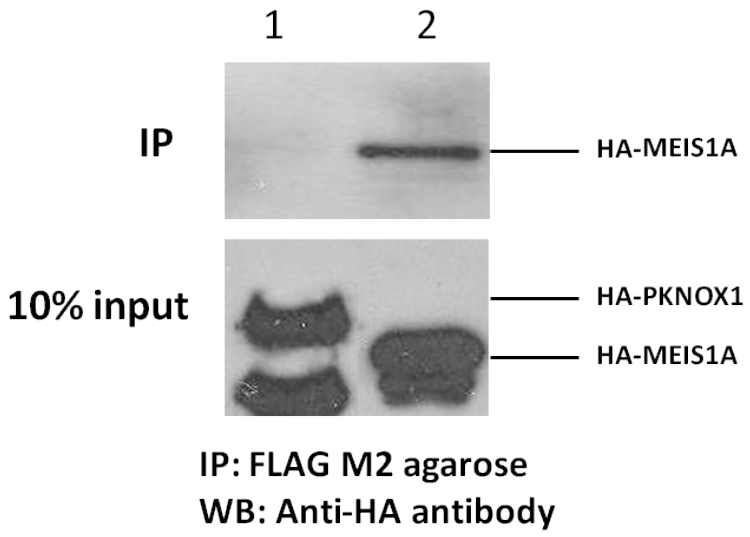


Figure 3.22: MLL-AF4 fusion protein interacts with MEIS1A. Top panel: Co-immunoprecipitation of HA-MEIS1A by FLAG-MLL-AF4 (lane 2). PKNOX1 did not co-immunoprecipitate with FLAG-MLL-AF4 (lane 1). Bottom panel: 10% input sample was loaded as control. The immunoblotting was done with Anti-HA antibody.

3.4.4. Endogenous MLL1 interacts with MEIS1 in RS4;11 lymphoblast cells:

Next to understand whether the interaction between MLL1 and MEIS1A could be observed endogenously, we used RS4;11 lymphoblast cells which express endogenous MLL-AF4 fusion protein as well as wild-type MLL1. We used anti-MLL1 N-terminus antibody to immunoprecipitate endogenously expressed MLL1/MLL-AF4 from whole cell protein extracts of RS4;11 cells. Endogenous MEIS1 was able to co-immunoprecipitate with endogenously expressed MLL1 or MLL-AF4 (Figure 3.23, lane 2). A control immunoprecipitation with non-specific anti-FLAG antibody did not pulldown endogenous MEIS1 in RS4,11 cells (Figure 3.23, lane 1).

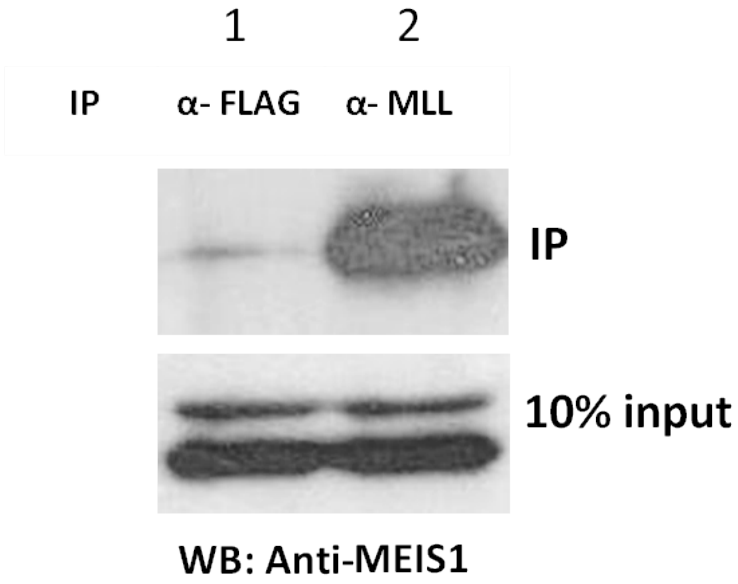
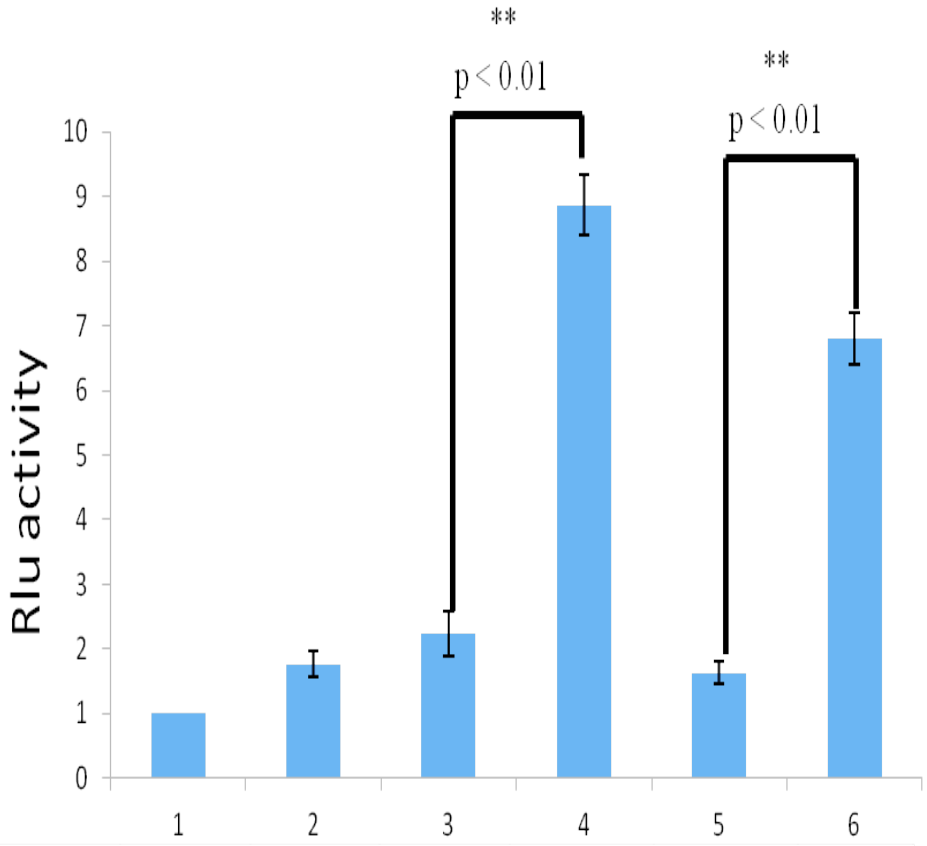


Figure 3.23: Endogenous interaction of MEIS1 with MLL1 in RS4;11 lymphoblast cells: Top panel: Lane 1: Control immunoprecipitation of the whole cell protein extract from RS4;11 cells with anti-FLAG antibody (non-specific). Lane 2: Immunoprecipitation of equivalent amounts of the whole cell protein extract as the control, with Anti-MLL1 N-term antibody. Bottom panel: 10% percent of the input was loaded as a control. The immunoblotting was done with Anti-MEIS1 antibody.

3.4.5. MLL1 positively contributes to the MEIS1A-mediated transactivation:

In order to understand the functional role for the MLL1-MEIS1A interaction, we performed luciferase assays using a luciferase reporter driven by the *Hoxb1* ARE as described in section 3.11. As was expected, the expression of HOXA1, PBX1 and HA-tagged MEIS1A alone does not increase the transcriptional activity of the *Hoxb1* ARE. The expression of FLAG–MLL1 alone also did not increase the reporter activity. But when FLAG–MLL1 was transfected together with expression vectors for HOXA1, PBX1 and HA-tagged MEIS1A, there was a threefold increase in reporter activity. The expression of FLAG–MLL1 together with HOXA1 and PBX1 in the absence of HA-MEIS1A also led to an increase in reporter activity by upto twofold. This could be due to the interaction of MLL1 with the endogenously expressed MEIS1 in HEK293T cells (Figure 3.24).



HoxB1 ARE-LUC	+	+	+	+	+	+
HOXA1	-	-	+	+	+	+
PBX1A	-	-	+	+	+	+
MEIS1A	-	-	+	+	-	-
MLL-AF4	-	+	-	+	-	+

Figure 3.24: MLL1 induces the transactivation function of MEIS1A: Luciferase assay was done with a firefly luciferase reporter driven by the *Hoxb1* ARE . The other plasmids to be tested were transfected as indicated. The error bars represent standard deviation (N=6). Rlu – Relative light units.

3.4.6. MLL1 interacts with the C-terminal region of MEIS1A:

In order to map the interaction domain of MLL1 on MEIS1A, we used two different truncation versions of MEIS1A. GAL4 DNA binding domain (GAL4 DBD) was tagged at the N-terminus of both truncated versions. GAL4 DBD-tagged MEIS1 N terminus encompasses the N-terminal 231 amino acids of MEIS1A containing the PBX interaction domains. GAL4 DBD-tagged MEIS1A C-terminal region encompasses the region between 232-390 amino acids containing the homeodomain and the transcriptional activation domain. FLAG-MLL1 was able to co-immunoprecipitate the MEIS1 C-terminal region (MEIS1 232-390) (Figure 3.25, lane 3). By contrast, the MEIS1 N-terminal region did not interact with MLL1 (Figure 3.25, lane 4). The transcriptional activation domain present in the C-terminus of MEIS1A shows high sequence variability with the related PKNOX proteins. The mapping of the MLL1 interaction to a region encompassing this domain could explain the inability of the PKNOX proteins to interact with MLL1.

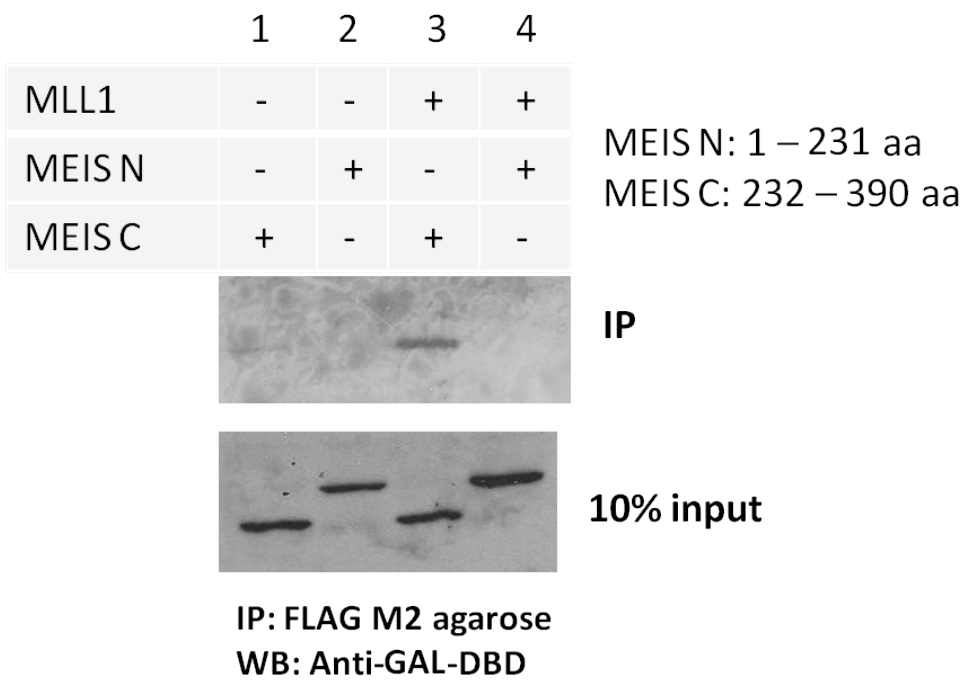


Figure 3.25: Mapping of the MLL1 binding region on MEIS1A to its C-terminus: Top panel: Co-immunoprecipitation of GAL-DBD tagged MEIS1A C-terminal region encompassing the homeodomain and the transcriptional activation domain (GAL-DBD MEIS-C), by FLAG-MLL1 (lane 3). GAL-DBD MEIS1A N-terminal region (GAL-DBD MEIS-N) did not interact with FLAG-MLL1 (lane 4). Both GAL-DBD MEIS-C and GAL-DBD MEIS-N did not bind the beads in the absence of FLAG-MLL1 (lanes 1 and 2, respectively).

Bottom panel: 10% input was loaded as a control. The immunoprecipitation was done with FLAG M2 agarose and the immunoblotting was done with anti-GAL-DBD antibody.

Chapter 4
Discussion

MEIS1 is an important transcription factor exercizing roles in many tissues. It is essential for the normal physiological functions of many organs and tissues (Hisa et al., 2004). It is expressed very early during development and plays both HOX-dependent and HOX-independent roles during embryogenesis. Furthermore, it is an oncogene in many human cancers especially in leukemia. Studies done in recent years have helped enumerate the target genes regulated by MEIS1. But the mechanisms by which MEIS1 regulates these targets, especially the protein partners with which it collaborates, have not been studied in detail. To date, HOX and PBX proteins are the only partners of MEIS1 studied extensively (Schnabel et al., 2000; Shen et al., 1997b). Recently however new studies have shown that MEIS1 might be directly regulated by cell signaling pathways. MEIS1 has been shown to possess a C-terminal transcriptional activation domain which responds to PKA signaling. Importantly, MEIS1 has been shown to interact with CRTC and CREB transcription factors to regulate transcription downstream of PKA (Goh et al., 2009; Huang et al., 2005; Wang et al., 2010b). These studies indicate new ways by which MEIS1 function could be regulated in the cell.

The elucidation of new interaction partners and novel pathways could help us better understand the molecular functions of MEIS1 and the roles it plays in different cellular processes. The identification of novel interaction partners of MEIS1 is also of immense interest to understand its role as an oncoprotein in leukemia. Possible new MEIS1 interactions could also serve as drug targets for combating leukemia and other cancers.

4.1. Identification of novel interaction partners of MEIS1A:

In vivo biotin tagging of proteins followed by streptavidin-mediated pulldown has been recently used for the identification of novel interaction partners for many proteins. Since biotin-streptavidin interactions are extremely strong ($K_d = 10^{-15}$), high stringency washing conditions can be used to reduce the binding of background

proteins and help identify true interaction partners. There are very few naturally biotinylated proteins in mammalian cells and hence they do not contribute heavily to background contamination. Hence the *in vivo* biotin tagging and streptavidin purification is a robust and efficient system for the isolation of novel partners of a protein.

We have shown that MEIS1A is expressed endogenously in HEK293T cells. Even though some of the tissue specific partners of MEIS1A might not be expressed in these cells and hence would not be identified in the mass spectrometry results, we expected to identify those partners needed for the normal functions of MEIS1A from this cell line. Furthermore, known partners of MEIS1 including PBX and HOX proteins are expressed in 293T cells, suggesting that additional novel partners should also be expressed. We stably transfected *E. coli* BirA and BAP-tagged MEIS1A in HEK293T cells and verified their stable expression. Wermuth et al (2005) had shown that the overexpression of MEIS1 in different lymphoblastic and fibroblast cell lines led to their apoptotic cell death (Wermuth and Buchberg, 2005). But we did not observe high rates of apoptosis in HEK293T cells overexpressing MEIS1A. One explanation could be that we inadvertently selected those colonies which express moderate amounts of MEIS1 that are below a threshold for inducing apoptosis. It is also possible that MEIS1-mediated apoptosis is cell-line-dependent and 293T cells are resistant to this process.

We verified that BAP-tagged MEIS1A was biotinylated efficiently in these stably transfected cells. Furthermore, the BAP-tagged MEIS1A was functional in the sense that it localized to the nucleus and interacted with known partners like PBX1 and CRTC1 in co-immunoprecipitation experiments. Cells expressing only BirA served as a control in the pull down experiments.

The mass spectrometry results showed that MEIS1A was able to pull down about 40 different proteins specifically. These proteins were absent in the control immunoprecipitations. All the pulldown experiments were done in triplicate to assess reproducibility. All three PBX proteins which are already known interaction partners of MEIS1 were present in the test samples but not in the controls, increasing our confidence in the results. Some HOX proteins like HOXA5, HOXA10 and HOXC13 which are also known interaction partners of MEIS1 were also identified only in the

test sample. But these HOX proteins were identified only in two of the three experiments. This could be due to their low endogenous expression levels in HEK293T cells or could also be due to the high stringency washing conditions used. Other transcription factors like MLL1, NONO and CBX3 were also found to interact with MEIS1. The current study involved the *in toto* purification and identification of interaction partners of MEIS1A. Hence it is likely that all endogenous interaction partners of MEIS1A in HEK293T cells co-purified with MEIS1A in the pull-down experiment. However it is possible that all proteins co-precipitating with MEIS1A may not have been positively identified in the mass spectrometry analysis. Only the demonstration of interaction between purified recombinant proteins can exclude indirect interactions.The likelihood of heteromeric complexes among the proteins identified in this study also cannot be excluded.

Interestingly some ribosomal proteins like RPL5, RPL35A, and RPS23; splicing factors including SFRS6, SFRS2 and SFRS9; translation proteins like DDX41, DDX18, EIF4A3 and EIF6; and proteins involved in ribosomal biogenesis like NOP56, NOP58 and PES1 were also found to co-purify with MEIS1. This is not surprising since many ribosomal proteins and splicing factors have been previously shown to interact with different transcription factors to regulate distinct physiological process. For example, recently NOP56 and 58 nucleolar proteins were found to interact with OCT4 (Cheong et al., 2011). TBX5 transcription factor and RNA Pol II have been shown to interact with SFRS2, although this interaction is RNA-mediated (Fan et al., 2009). RPL11 interacts with PPAR-α and c-MYC. It binds to PPAR-α and augments its transcriptional activation function. RPL11/c-MYC interaction on the other hand is required for the repression of c-MYC transcriptional activity (Dai et al., 2007; Gray et al., 2006). The GFI1 transcription factor which is essential for normal hematopoiesis has been shown to directly interact with U2AF26 splicing factor and regulate the splicing of downstream genes (Heyd et al., 2006). PU.1, another hematopoietic transcription factor, interacts with FUS splicing factor and this interaction is needed for the transcriptional function of PU.1 (Hallier et al., 1998). PKNOX1, a relative of MEIS1, has been shown to bind to ribosomal protein S3A (RPS3A) (Diaz et al., 2007a).

4.1.1 Ribosomal proteins and splicing factors as interaction partners of a transcription factor - Why they should not be disregarded:

Many recent reviews have provided a detailed perspective on the tissue-specific and extra-ribosomal roles of ribosomal proteins. Extra-ribosomal functions and tissue-specific expression have been described for ribosomal proteins in many organisms ranging from plants to humans (Lindstrom, 2009; Warner and McIntosh, 2009; Xue and Barna, 2012). Growing evidence contradicts the conventional thinking that ribosomal proteins and splicing factors perform normal housekeeping functions and that they are expressed ubiquitously, having no tissue-specific role. RPL38, for example, has been shown to selectively regulate the post-transcriptional expression of a select group of Hox genes (Kondrashov et al., 2011). The mouse *Bellyspot and tail (Bst)* phenotype, which shows decreased pigmentation, shorter tail and extra digits, is caused by a mutation which affects the expression of RPL24. RPL29 knockout mice show defects in osteogenesis and skeletal malformations (Kirn-Safran et al., 2007; Oristian et al., 2009). Furthermore, recently it has been shown that RPL29 is important for normal and cancer angiogenesis in mice (Jones et al., 2013). Mutations in *Rps6*, *Rps19* and *Rps20* have been shown to be the cause behind the Dark skin (*Dsk*) phenotype observed in mouse. Mutations in these ribosomal proteins lead to the accumulation of p53 and downstream effects like increased keratinocyte proliferation and decreased erythrocyte production (McGowan et al., 2008).

RACK1, a downstream effector of PKC signaling, is primarily a ribosomal protein (Adams et al., 2011; Warner and McIntosh, 2009). Extra-ribosomal functions have also been described for RPL13A which functions as a component of an mRNA binding complex called GAIT to repress translation of specific mRNAs (Mazumder et al., 2003; Mukhopadhyay et al., 2008; Ray and Fox, 2007).

An extensive study of the effects of morpholino-mediated knockdown of an array of different ribosomal proteins showed tissue specific effects for many of the proteins (Uechi et al., 2006). Recent studies have also shown that many ribosomal protein genes are tumor suppressor genes in zebrafish since mutations in these genes lead to the spontaneous development of different types of cancers (Amsterdam et al.,

2004; Lai et al., 2009). These studies show that the deficiency of ribosomal proteins leads to the accumulation of p53 and subsequent cell cycle arrest and apoptosis. In *Drosophila*, the *Minute* mutations lead to a multitude of morphological and physiological changes like shorter life span, abnormal wings, eye defects, sterility and also patterning defects. All the *Minute* phenotypes to date have been attributed to mutations in ribosomal proteins (Marygold et al., 2007). Surprisingly, mutations in *Rpl38* and *Rpl5* have also been implicated in the *Minute* phenotype in *Drosophila*. (Coelho et al., 2005; Marygold et al., 2005).

Both RPL5 and RPL35, which have been found to co-purify with MEIS1 in this study, have been implicated in Diamond-Blackfan anemia, a congenital condition with severe anemia due to reduced erythroid progenitors. Craniofacial defects have also been shown to accompany anemia (Ball, 2011; Cmejla et al., 2009; Farrar et al., 2011; Farrar et al., 2008; Gazda et al., 2008; Konno et al., 2010; Kuramitsu et al., 2012; Quarello et al., 2010). More recently, mutations in RPL5 have been implicated in T-cell acute lymphoblastic leukemia (T-ALL) as well (De Keersmaecker et al., 2013). RPL5 along with RPL11 is the primary ribosomal protein involved in the activation of p53 mediated cell cycle arrest in response to perturbations in ribosomal biogenesis. RPL5 has been shown to bind MDM2 (HDM2) and attenuate its inactivation of p53 (Fumagalli et al., 2012; Macias et al., 2010; Miliani de Marval and Zhang, 2011).

Some splicing factors including the Serine-Arginine rich (SR) splicing proteins, the heterogeneous nuclear ribonucleoproteins (HNRNPs) and the DEAD box proteins have also been shown to have tissue-specific expression patterns and phenotypes. Mutations in DDX18 and DDX41 have been implicated in human acute myeloid leukemia (Payne et al., 2011). HNRNPH1 has been found to undergo post-translation modifications like O-linked glycosylation specifically in the MLL1-mediated leukemia 11q23 (Balkhi et al., 2006). Very interestingly, SF3B1 splicing factor has been shown to regulate the expression of *Hox* genes in conjunction with Polycomb proteins in mice. SF3B1 interacts with class II Polycomb proteins and leads to a global repression of *Hox* gene expression. *Sf3b1* knockout mice concomitantly show axial patterning defects in mice (Isono et al., 2005). In this regard, it is interesting to note that PSIP1 (LEDGF) which is known to regulate *Hox* gene expression in conjunction with MLL1, interacts with many splicing factors which are

important for its functions. Hence, it could be possible that axial patterning defects observed in *Psip1* knockout mouse could be indirectly mediated by splicing factors (Pradeepa et al., 2012; Sutherland et al., 2006). Mutations in splicing factor genes have been recently implicated in hematopoietic malignancies including acute myeloid leukemia, myelomonocytic leukemia, chronic lymphocytic leukemia and myelodysplastic syndromes (Dolnik et al., 2012; Graubert et al., 2012; Meggendorfer et al., 2012; Ogawa, 2012; Quesada et al., 2012; Rossi et al., 2011; Takita et al., 2012; Yoshida et al., 2011; Zhang et al., 2012)

The helicase DDX18 which was identified in this study to interact with MEIS1 has previously been shown to be important in hematopoiesis in zebrafish. DDX18 is required for the erythroid and the myeloid progenitor development in zebrafish (Payne et al., 2011). DDX41, another helicase, is important for the immune response against foreign viral and bacterial DNA and second messengers (Parvatiyar et al., 2012; Zhang et al., 2011b; Zhang et al., 2013).

B52/SRp55, which is the homolog of SFRS6 in *Drosophila*, plays an indispensable role during insect development (Ring and Lis, 1994). It is involved in the alternative splicing of specific target genes like *eyeless* (ortholog of *Pax6*) to regulate eye development in *Drosophila* (Fic et al., 2007; Gabut et al., 2007). SRp55 also performs important roles during cell cycle progression and initiation of RNA pol II transcription in flies. SRp55 regulates the alternative splicing of E2F transcription factor to control the G1/S phase progression. Its interaction with topoisomerase I is required for the latter's recruitment to transcription start sites and the initiation of transcription (Juge et al., 2010; Rasheva et al., 2006). In humans, *Sfrs6* has been shown to be an important oncogene in lung and colon cancers (Cohen-Eliav et al., 2013). SC35/SFRS2 is very important for the transcription elongation of RNA polymerase II (Lin et al., 2008). SC35 foci in the nucleus have been shown to mark transcriptionally active genes in conjunction with RNA pol II and H3K4 trimethylation marks (Collas et al., 1999).

Plentiful evidence in recent years strongly suggests a role of ribosomal proteins and splicing factors in processes outside their traditionally recognized functions. As discussed before, many of these proteins show tissue-specific expression, highly specific knockout phenotypes and mutations which lead to organ-

specific disorders. Growing evidence also shows that some of these functions are mediated through the interaction with cross-functional proteins including transcription factors. Hence the identification of ribosomal proteins and splicing factors as novel potential interaction partners of MEIS1 could indicate new pathways through which MEIS1 could regulate normal physiological cell function and oncogenesis.

4.2. NONO-MEIS1A interaction:

We had previously identified the CREB-regulated transcriptional co-activators CRTC1 and CRTC2 as binding partners of MEIS1A. CRTC1/2 functions in a PKA-dependent pathway to associate with MEIS1A and cooperate in its transcriptional activation functions (Goh et al., 2009). Implicit evidence for this cooperation between CRTCs and MEIS1A in transcriptional activation is also demonstrated in GSK-3 kinase-mediated MLL. GSK-3 has been shown to promote the oncogenesis caused by the HOX-MEIS transcriptional program by inducing the interaction between CRTC and CREB with MEIS1 (Wang et al., 2010b). Considering these observations we were surprised to find that neither CRTCs nor CREB were identified in the mass spectrometry data. This could be due to the presence of very low amounts of endogenous CRTC in HEK293T cells which failed to co-immunoprecipitate with MEIS1 due to the strong washing conditions used in the pull-down procedure. Alternatively, the CRTC-MEIS interaction could be indirectly mediated by another protein.

It was therefore interesting to note that NONO (p54nrb) was identified in a ‘co-activator trap’ experiment to interact with CRTCs and this interaction is responsive to cAMP signaling (Amelio et al., 2007). NONO was identified in our LC-MS/MS results to be a potential partner of MEIS1A. Using transient transfection of plasmids for FLAG-NONO and MEIS1A in HEK293T cells followed by co-immunoprecipitation, we also validated the interaction. Furthermore, NONO was able to pull-down endogenous MEIS1 from HEK293T cells. To understand whether NONO could mediate the interaction between MEIS and CRTC1, we used siRNAs to knockdown NONO and observed its effects on the ability of MEIS1A to interact with CRTC1 in a co-immunoprecipitation assay. The pool of siRNAs used was able to

efficiently knockdown endogenous NONO as observed by the decrease in protein levels on a western blot. But the knockdown of NONO had no effect on the extent of MEIS1-CRTC1 interaction. Luciferase assays done to assess whether NONO could regulate the transcriptional activation function of MEIS1 showed no effect except for a decrease in the transcription activation by MEIS1 under the control of PKA signaling.

PREP1 which is a MEIS family member has been previously shown to interact with NONO and induce nuclear β-actin mediated regulation of *Hoxb* genes in response to RA signalling. NONO was found to bind as a complex along with other proteins like PSF and WASP to regulate this process. But the role of NONO and PSF splicing factor has not been clearly elucidated in this process (Ferrai et al., 2009). In this study, we showed that MEIS2, another MEIS1 related protein also interacts with NONO.

NONO is also a splicing protein containing many RNA binding RRM domains. The interaction between MEIS1 and NONO instead of having transcriptional regulatory functions could be involved in the regulation of alternative splicing. In fact, the PU.1 hematopoietic transcription factor has been shown to interact with NONO and regulate splicing *in vitro*. Surprisingly, PU.1 also bound single-stranded RNA through its DNA binding domain (Hallier et al., 1996). MEIS1 has not been shown to bind RNA or regulate alterative splicing to date. It would therefore be interesting to understand whether the MEIS family proteins which have been shown to interact with NONO could also regulate alternative splicing.

4.3. MEIS1A interaction with NOP56, NOP58, CBX3 and MLL1:

We have validated the interaction between the nucleolar proteins NOP56 and NOP58 as well as the transcription factors MLL1 and CBX3 with MEIS1A. FLAG-tagged NOP56, NOP58, CBX3 and MLL1 were found to interact with HA-tagged MEIS1A in a co-immunoprecipitation experiment.

4.3.1. NOP56/58-MEIS1A interaction:

NOP56 and NOP58 are snoRNA binding proteins which are involved in 60S ribosome biogenesis. They bind C/D box containing snoRNAs and direct 2'-*O*-methylation of pre-rRNAs (Gautier et al., 1997; Lyman et al., 1999; Tran et al., 2003). TIP49 (PONTIN or RuvBL1) and TIP48 (REPTIN or RuvBL2), which are ATPases involved in DNA repair processes, have been recently shown to bind C/D snoRNAs. Here, both TIP48 and TIP49 interact with the NOP56/58 proteins to regulate rRNA processing (Bauer et al., 2000; Kanemaki et al., 1999; McKeegan et al., 2009; Newman et al., 2000). The TIP48/49 proteins are involved in a many cellular processes ranging from DNA repair and recombination to transcriptional regulation. Both TIP48 and TIP49 were first identified as TATA-box binding protein (TBP) interacting proteins (Bauer et al., 2000; Kanemaki et al., 1999). They have also been shown to interact with β-catenin and c-MYC to regulate downstream transcription (Bellosta et al., 2005; Etard et al., 2005; Gallant, 2007; Weiske and Huber, 2005). Very interestingly, TIP48/49 proteins have been shown to regulate *Hox* gene expression in collaboration with *Polycomb* and *Trithorax* group proteins (Diop et al., 2008). Hence, through their interaction with TIP48/49, the NOP56 and NOP58 proteins may also function in transcriptional regulation, although such pathways have not been described to date. In a similar context it is interesting to note that recently, both NOP56 and NOP58 were shown to interact with OCT4 transcription factor (Cheong et al., 2011).

In this study, we have identified that both TIP49 and TIP48 also co-purify with MEIS1A, albeit with lesser efficiency. Both the proteins were identified in one of the three independent experiments only in the experimental samples. We hypothesize that the TIP48/49 pull down by MEIS1A might be indirectly mediated by NOP56/58. The interaction between NOP56/58 proteins and MEIS1A described in this study might have diverse roles in the cell. Whether the NOP56/58-MEIS1A interaction plays a role in the regulation of gene expression or ribosome biogenesis is not known and is an interesting pathway to be explored.

4.3.2. CBX3-MEIS1A interaction:

CBX3 also known as HP1γ is a heterochromatin binding protein found as a complex with HP1α and HP1β. The complex called heterochromatin protein 1 (HP1) specifically interacts with H3K9 methylated histones and induces gene silencing (as reviewed by (Fanti and Pimpinelli, 2008; Zeng et al., 2010). Unlike the other members, HP1γ localizes to the euchromatin as well. Recent evidence indicates an important role for HP1γ in transcription elongation of RNA pol II in these transcriptionally active regions. Phosphorylation of HP1γ on Ser 83 position is important for this role of HP1γ (Butcher et al., 2005; Kim et al., 2011; Lomberk et al., 2006b; Minc et al., 2000; Minc et al., 2001; Vakoc et al., 2005). Hence HP1γ can function as both a transcription repressor and an activator depending upon post-translation modifications. In this context, HP1γ interacts with many transcription factors like MECP2 (Agarwal et al., 2007), TAF4 (Vassallo and Tanese, 2002) and TIF1δ (Khetchoumian et al., 2004) to regulate downstream transcription. Apart from transcription regulation recent evidence also indicates a role for HP1γ in the regulation of alternative splicing (Ameyar-Zazoua et al., 2012; Saint-Andre et al., 2011; Smallwood et al., 2012). A role for HP1γ in maintaining the telomere length has also been proposed (Canudas et al., 2011). HP1γ is thus a ubiquitously expressed multifunctional protein involved in different cellular processes performing indispensable functions.

We have validated the interaction between HP1γ and MEIS1A through co-immunoprecipitation assay. Apart from HP1γ, HP1α (CBX5) and HP1β (CBX1) were also found to co-purify with MEIS1A albeit with lesser efficiency. Although CBX1 was reproducibly isolated in three independent experiments like CBX3, more number of unique peptides was isolated for CBX3 compared to CBX1. In comparison, CBX5 was found in two of the three independent experiments. MEIS1 thus co-purifies all the members of the HP1 complex with HP1γ being the predominant interaction partner. The domains needed for the CBX3-MEIS1A interaction and the functional role for this interaction are yet to be understood. Recent studies have shown that the leucine and arginine rich (LR) domain of KI-67 cell proliferation protein interacts with all members of the HP1 complex (Kametaka et al., 2002; Scholzen et al., 2002). Protein KI-67 was also found in this study to co-purify with MEIS1A. Hence it could be

possible that the CBX3-MEIS1A interaction described here could be mediated by KI-67.

4.3.3. MLL1-MEIS1A interaction:

The identification that MLL1 interacts with MEIS1 is very interesting since both these proteins are implicated in hematopoiesis and leukemia (Ansari et al., 2012; Gan et al., 2010; Hess et al., 1997; Hisa et al., 2004; Wan et al., 2011; Yagi et al., 1998). It is known that *Mll1* functions upstream of *Meis1* and many *Hox* genes during embryonic development, adult hematopoiesis and leukemogenesis (Ferrando et al., 2003; Milne et al., 2005a; Milne et al., 2002; Wong et al., 2007; Yu et al., 1995; Zeisig et al., 2004). MEIS1 along with HOXA9, HOXA7 and HOXA10 is implicated in mixed lineage leukemia which is caused by MLL1-fusion proteins. MLL1 interacts with many proteins which regulate its function (refer figure 1.6). Here we identified that many interaction partners of MLL1 like RBBP5, PSIP and HCFC1 also co-purify with MEIS1A. These proteins were isolated at lower frequency, in one or two of the three independent experiments compared to MLL1 implying an indirect interaction with MEIS1A. CUL4B, a E3 ubiquitin ligase which was identified to co-purify with MEIS1 in all three independent experiments functions in a common pathway with MLL1 to activate downstream genes (Kotake et al., 2009).

Chromosomal translocations partner MLL with over 30 different proteins and MLL-AF4 is one of the predominant fusion proteins causing ALL. MLL1 wild-type and fusion proteins have been show to directly regulate the transcription of *MEIS1* (Kumar et al., 2010; Kumar et al., 2009; Wang et al., 2011b; Zeisig et al., 2004). In this study, we show that MLL-AF4 fusion protein also interacts with MEIS1. In MLL-AF4, the N-terminal ~ 1400 amino acids of MLL1 are fused to the C-terminus of AF4 (AFF1) protein. Hence the identification that MLL-AF4 is able to interact with MEIS1 shows that this interaction is mediated by the N-terminal fragment of the MLL1 protein. This fragment consists of the Menin binding domain, LEDGF interaction domain and the DNA binding AT-hooks and CXXC domain. Neither AF4 nor any of its known interaction partners were identified in the mass spectrometry data excluding the possibility that the MLL-AF4 and MEIS1 interaction could be

mediated by AF4. Luciferase assay experiments performed to understand the functional role of MLL1-MEIS1 interaction revealed that MLL1 is able to induce MEIS1-mediated transcription by up to three folds in the presence of HA-MEIS1A on a *Hoxb1*ARE enhancer element. MLL1 thus contributes to the transactivation of MEIS1 on a naturally occurring enhancer. Therefore this experiment indicates a probable role for MLL1 in regulating MEIS1 mediated transcription of many downstream genes.

We also show that MLL1 and MEIS1 interact endogenously in RS4;11 and HEK293T cells. Anti-MLL1 antibody but not the non-specific Anti-FLAG antibody, was to immunoprecipitate MLL1 or MLL-AF4 from these cell lines and the presence of MEIS1 in the eluate validate the endogenous interaction between these proteins. The RS4;11 cell line is a lymphoblastic cell line which was derived from an ALL patient and carries the t(4;11) translocation (Stong et al., 1985). The cell line thus endogenously expresses the MLL-AF4 (der4 translocation product) protein. Due to reciprocal translocation, recently it has been shown that the RS4;11 cell line also express the der11 translocation product AF4-MLL. In the AF4-MLL fusion the N-terminal region of the AF4 protein is fused with the C-terminal fragment of MLL1, which consists of the region between the PHD finger and the C-termini. Hence, the AF4-MLL fusion lacks the N-terminal 1400 amino acids of MLL1 thought to be important for MEIS1A interaction (refer figure 1.6) (Bursen et al., 2004; Kumar et al., 2011; Marschalek et al., 1995). Apart from these fusion proteins RS4;11 cell line also expresses wild-type MLL1 protein. Hence the endogenous interaction observed in the RS4;11 cell line could be mediated by both MLL-AF4 and wild type MLL1 but not by AF4-MLL.

We have mapped the interaction domain of MLL1 on MEIS1A to the C-terminal half of MEIS1 consisting the homeodomain and the transcriptional activation domain. The DNA homeodomain region is highly conserved between all MEIS/PREP family members. The C-terminal transcriptional activation domain which shows high sequence variability between the MEIS/PREP members is important for transcription and leukemogenic properties of MEIS1 (Huang et al., 2005; Mamo et al., 2006; Wang et al., 2005). The MEIS1 TAD is important for transcriptional activation in response to PKA signaling. Goh et al (2009) have shown that this PKA responsiveness is mediated by the interaction of MEIS1A with the CRTC co-activators. Concomitantly,

the TAD is indispensable for the CRTC mediated transactivation properties of MEIS1 (Goh et al., 2009; Huang et al., 2005). GSK3 is an oncogene in mixed lineage leukemia and the mechanism by which it promotes leukemia is by inducing the interaction between CREB and CRTC proteins with MEIS1 (Wang et al., 2010b). Considering the importance of the MEIS1 TAD for its functions, the mapping of the MLL1-MEIS1 interaction to this region is very interesting. Whether the MLL1-MEIS1 interaction is mediated by factors like PKA or GSK3 remains to be understood.

Taken together all these experiments conclusively validate the interaction between MLL1 and MEIS1A and indicate a functional role for this interaction in MEIS1A transactivation. Further experiments need to be done to produce an even finer map of the interaction domains between both the proteins. Mutational studies of these regions and functional characterization could further help understand the physiological importance of the MLL1-MEIS1A interaction.

4.4. Interactions of the PKNOX proteins:

The MEIS and PKNOX proteins share high sequence homology in their PBX interaction domains and the homeodomain. Like MEIS1, both PKNOX1 and PKNOX2 have also been shown to form co-operative interactions with PBX and HOX proteins. These complexes have been shown to perform physiologically relevant functions in many tissue types. The C-terminal region which contains the TAD in MEIS1 shows high variability between MEIS1 and the PKNOX proteins. MEIS1 known to accelerate the onset of HOX-mediated leukemia and has been identified as an essential factor in MLL. The CTD of MEIS1 is essential for its transcriptional activation function and its transforming properties (Huang et al., 2005; Mamo et al., 2006). Despite sharing sequence similarities with MEIS1, PKNOX1 does not have the ability to cause leukemia with or without HOX proteins. In fact, it has been shown that the over-expression of PKNOX1 delays the onset of HOXA9-mediated leukemia (Thorsteinsdottir et al., 2001). Interestingly, recent evidence points towards a tumor suppressor role for PKNOX1 in cancers (Iotti et al., 2011; Longobardi and Blasi, 2003; Longobardi et al., 2010).

In this regard it is interesting to note that PKNOX1 could be converted into an oncoprotein having the ability to accelerate HOXA9-mediated leukemia when its C-terminus was replaced with that of MEIS1A. A PKNOX1 chimera carrying a VP16 activation domain also gains similar leukemogenic properties. Interestingly, wild-type MEIS1 and the PKNOX1-MEIS1 CTD chimera activate similar downstream genes during leukemogenesis (Bisaillon et al., 2011).

We have explored whether the potential MEIS1 interaction partners identified in this study could also interact with the related PKNOX proteins. An earlier proteomics study identified many interaction partners of PKNOX1 but except for the PBX proteins many novel MEIS1 partners described here were not identified as part of that study (Diaz et al., 2007a). We hypothesized that since the MEIS and the PKNOX proteins share extensive sequence similarity, many partners identified in this study could also interact with PKNOX1. Through co-immunoprecipitation assay we demonstrated that like MEIS1, both the PKNOX proteins also interact with NOP56 and NOP58. This finding reveals a novel pathway through which MEIS1 and the PKNOX proteins could regulate similar cellular functions.

Here we show that MEIS1 is able to interact with MLL1 and CBX3 transcription factors. Furthermore, the MLL1-MEIS1A interaction has been mapped to the C-terminal region comprising the homeodomain and the transcriptional activation domain in MEIS1. Neither PKNOX1 nor PKNOX2 interact with MLL1 or CBX3 in our co-immunoprecipitation experiments. We hypothesize that this could be due to the differences in the CTD between the PKNOX and MEIS1 proteins. Therefore the MLL1, CBX3 interactions observed in this study and possibly other interactions could mediate the functional differences observed between MEIS1 and the PKNOX proteins.

Future directions

The present work has expanded the knowledge about the functions of MEIS1 and has opened new avenues for future studies. The primary course of future work would be to study in detail the functional relevance of those interactions which have been validated through this work. As discussed above, with respect to the MLL1-MEIS1A interaction, the next step would be to produce a finer map of the interaction domains between these proteins. Mutational studies on these possible novel domains could help in understanding the nature of the interaction. Transgenic animals carrying these mutations could be created and studied to assess the functional role of the MLL1-MEIS1A interaction.

Since both MEIS1 and MLL1 are transcriptional regulators, the next logical step would be to identify the downstream targets regulated by the interaction. Conventional ChIP assay could be performed on known targets of MEIS1 to understand whether MLL1 wild-type and fusion proteins could bind co-operatively with MEIS1 to the enhancer regions of these targets. Some of the targets which could be analyzed first are the Hypoxia inducible genes, *Hif1α* and *Hif2α*, since both MEIS1 and MLL1 have been shown to transcriptionally regulate their expression. MEIS1 directly binds to the enhancers of both the genes (Ansari et al., 2012; Kocabas et al., 2012; Simsek et al., 2010; Unnisa et al., 2012). Additionally, experiments have shown that the knockdown of *Mll1* expression results in the reduction of H3K4 methylation marks in the enhancer regions of the *Hif* genes (Ansari et al., 2012; Heddleston et al., 2012). Hence it would be interesting to explore the co-operative role of the MLL1-MEIS1A interaction in the regulation of *Hif* gene expression. Modern techniques like ChIP-Seq could also be used to identify novel downstream targets of the interaction. As previously discussed, the MLL1-MEIS1A interaction has potential pathogenic roles in many hematopoietic malignancies. Structural studies could be done to reveal the binding properties and the dynamics of the interaction which could further be used to develop drugs which could disrupt the interaction.

Since CBX3 is also a transcription regulator, similar experiments as described for MLL1-MEIS1A interaction could be carried out to understand the role of the CBX3-MEIS1A interaction. Since the PKNOX proteins do not interact with either

MLL1 or CBX3, the functional characterization of their interactions with MEIS1 would help to delineate the physiological roles of these two related proteins.

Many proteins involved in ribosomal biogenesis and ribosome core components have been identified to co-purify with MEIS1 indicating a possible role for MEIS1 in the global synthesis of ribosomes. The primary site for ribosomal biogenesis is the nucleolus and hence the preliminary experiment would be to perform confocal microscopy to see whether MEIS1 is localized to the nucleoli. siRNA-mediated knockdown experiments could then be performed to see whether ribosomal rRNA synthesis and processing is affected. After MEIS1A knockdown the efficiency of pre-rRNA processing could be examined by northern blotting. NOP56 and NOP58, validated to interact with MEIS1A in this study are snoRNA modifying proteins. Mutational studies on binding domains as described above could be done to identify the role of these interactions in snoRNA processing.

The helicases DDX18 and DDX41, which have dual roles in both ribosome biogenesis and splicing, are other important partners which could be taken up for further studies. DDX18 is interesting since it has been implicated in hematopoiesis and leukemia (Payne et al., 2011), key processes in which MEIS1 also plays a fundamental role. The ribosomal proteins RPL5 and RPL35 both of which have been implicated in Diamond-Blackfan anemia (Farrar et al., 2008; Gazda et al., 2008; Quarello et al., 2010) could be other important targets for further studies. With increasing evidence suggesting gene specific post-transcriptional regulatory roles for ribosomal proteins (Kondrashov et al., 2011; Xue and Barna, 2012), similar functions could be studied for the MEIS1-RPL5/RPL35A interactions. After the characterization of the binding domains between MEIS1 and RPL5/RPL35A, downstream targets could be identified. *Hox* genes could be the primary targets to be examined since some of these genes have been recently shown to be regulated in a gene-specific manner by the ribosomal protein RPL38 (Kondrashov et al., 2011). Mutations which disrupt MEIS1-RPL5/RPL35A binding could be used to study global *Hox* gene expression by RT-PCR.

The potential interaction between MEIS1 and the splicing factors which have been identified in this study is also an interesting area for further studies. Artificial minigene constructs could be used to study the role of MEIS1 in splicing. A minigene

construct contains a short fragment of a target mRNA (usually containing 1 or 2 exons with an intron) cloned in an expression vector. Non-specific minigenes composed of fragments of β-globin mRNA are commercially available. The protocol involves the incubation of the *in vitro* transcribed and radioactively labelled minigenes with HeLa nuclear extracts in the presence or absence of recombinant MEIS1. The potential activation or inhibition of splicing of the minigene by MEIS1can then be assessed by northern blotting. Potential mRNA binding roles for MEIS1 could also be assessed by northern blotting using radioactively labelled total RNA from cells. DDX18 along with the SR proteins SFRS2 and SFRS6 would be the initial targets for such studies because of roles in many physiologically important functions as described above. Since our initial efforts to characterize the role for NONO in MEIS1A transactivation gave negative results, the next step would be to see if the NONO-MEIS1A interaction has potential roles in splicing regulation. NONO is primarily an RNA binding protein which functions as a splicing factor along with PSF (Dong et al., 2007; Hallier et al., 1996; Kaneko et al., 2007; Peng et al., 2002). Splicing factor assays as described above could be used to study the splicing regulation by NONO-MEIS1A.

Conclusion

I have summarized the major findings of this study below:

1) My work involved the identification of novel interaction partners of MEIS1. We employed a recently described technique involving *in vivo* biotinylation of target proteins and subsequent streptavidin purification to isolate new partners of MEIS1A.

2) I have created HEK293T stable cell lines expressing E.coli Biotin ligase (BirA) enzyme and biotin acceptor peptide (BAP) tagged MEIS1A.

3) This cell was then used to purify new interaction partners of MEIS1 by Streptavidin affinity purification. The MEIS1A co-purifying proteins were then identified using mass spectrometry analysis. Cells expressing only BirA served as a control.

4) I have identified 40 novel proteins which co-purify with MEIS1A. PBX1/2 and 3 proteins which are known partners of MEIS1A were identified specifically in the experimental sample serving as a positive control.

5) Apart from transcription factors and chromatin modifying proteins the novel partners of MEIS1A include many mRNA binding proteins and ribosomal proteins.

6) I have validated the interaction between MEIS1A and NONO (p54nrb) a known PKA dependent transcription factor. Both transfected and endogenous NONO interact with MEIS1A.

7) NONO does not mediate the interaction between MEIS1A and CRTC1. The siRNA knockdown of NONO did not alter the MEIS1A-CRTC1 interaction.

8) NONO does not have any effect on the transactivation properties of MEIS1A on a *Hoxb1* ARE enhancer. NONO shows a mild inhibitory effect on the TORC and PKA mediated transactivation on the same enhancer.

9) The siRNA-mediated knockdown of NONO does not have any effect on MEIS1A transactivation.

10) I have validated the interaction between MEIS1A and the ribosomal biogenesis proteins NOP56 and NOP58; and the transcription factors MLL1 and CBX3.

11) NOP56 and NOP58 also interact with PKNOX1 and PKNOX2 which are related to MEIS1. MLL1 and CBX3 do not show any interaction with the PKNOX proteins.

12) Validated the endogenous interaction between MLL1 and MEIS1 in RS4;11 lymphoblast cells. MLL-AF4 fusion protein also interacts with MEIS1A indicating that the interaction is mediated by the N-terminal region of MLL1.

13) The MLL1-MEIS1A interaction is mediated by the C-terminal region of MEIS1A which consists of the homeodomain and the C-terminal transactivation domain.

PERSPECTIVE:

In conclusion, through this study we have expanded the repertoire of protein interaction partners of MEIS1A. This cohort of MEIS1A interactions might help understand the mechanisms by which MEIS1 regulates different physiological processes. The MEIS1-MLL1 interaction described in this study could play vital roles, especially in hematopoiesis. The interaction could also be pathogenically important in leukemia and could thus pose as important drug targets. Apart from transcription regulators, we have identified many proteins with diverse functions like splicing, ribosome biogenesis and structural components of ribosome. Exploring the functional relevance of these interactions could unravel novel and interesting pathways through which MEIS1 exerts its functions. In these regards this study is an essential tool for the better understanding of the activities of MEIS1 transcription factor.

Appendix 1

HEK293T cells used in the present study were recently shown to have a neuronal phenotype contrary to the kidney epithelial cell origin it was thought to possess. But the type of neuronal cell from which HEK293T has its origin is not known (Shaw et al., 2002). The role of MEIS1 in neuronal cell specification and function has also not been studied in detail. The importance of MEIS1 during hematopoiesis, especially its indispensable role in HSC cell survival and function is well established (Argiropoulos et al., 2007; Kocabas et al., 2012). Therefore I decided to use hematopoietic cell line which would express hematopoiesis-specific proteins in high levels thereby aiding in the identification of hematopoietic cell specific MEIS1 interactions apart from the generic interactions.

A.1 Creation of *Nup98-Cdx1* leukemic stem cells stably expressing FBM and BirA:

NUP98 protein is a component of the nuclear pore complex (NPC) which is involved in the translocation of polypeptides and ions across the nuclear membrane. Apart from its role in NPC function, recent studies have made the surprising observation that NUP98 is also involved in transcriptional regulation of the RNA polymerase II transcribed genes. As discussed above, the *NUP98* gene is involved in translocations which lead to AML and T-cell leukemia in humans. About 28 different fusion partners have been described so far. The primary partners for *NUP98* in these translocations are the *Hox* genes, mainly *HoxA9*. The mechanism by which the *NUP98*-fusions induced leukemia is by the transcriptional regulation of downstream target genes. The predominant targets of the *NUP98*-fusions are the *HoxA* class genes and *Meis1*. Mice transplanted with bone marrow cells transduced with *Nup98-Hox* fusions invariably develop leukemia (as reviewed by (Abramovich et al., 2005; Gough et al., 2011; Moore et al., 2007; Nakamura, 2005)).

Cdx genes are homologs of the *Drosophila caudal* gene and encode transcription factors containing a homeodomain. The *Cdx* genes, which include *Cdx1, Cdx2 and Cdx4* in vertebrates along with the *Gsx* and *Pdx* genes, are organized as a

cluster and are referred to as the *ParaHox* genes. The CDX proteins play vital roles during axial patterning primarily as upstream regulators of *Hox* gene expression. More recently, *Cdx* genes have also been shown to be important for adult hematopoiesis and in the induction of leukemia presumably by the deregulation of *Hox* gene expression (as reviewed by (Lengerke and Daley, 2012; Rawat et al., 2012; Young and Deschamps, 2009)).

Bone marrow cells transfected with a *Nup98-Cdx1* gene are able to repopulate the hematopoietic lineage in lethally irradiated mice. The *Nup98-Cdx1* fusion is able to convert the bone marrow progenitors into cells possessing HSC-like activity and reconstituted mice survive for up to 10 months normally without the development of leukemia (Wu Zhihao and Klaus Karjalainen, unpublished observations).

Meis1, as discussed above, is expressed highly in the hematopoietic system. *Meis1* plays important roles during HSC self-renewal and sustenance, and also in differentiated progenitors mainly in the myeloid lineage. *Meis1* is also vital for the survival of the megakaryocytes. *Meis1*-mediated AML and MLL has also been discussed above (Afonja et al., 2000; Argiropoulos et al., 2007; Azcoitia et al., 2005; Cvejic et al., 2011; Hisa et al., 2004; Kocabas et al., 2012; Pineault et al., 2004; Rosales-Avina et al., 2011; Thorsteinsdottir et al., 2001; Unnisa et al., 2012). Since *Meis1* plays physiologically relevant roles during hematopoiesis we decided to use the *Nup98-Cdx1* immortalized HSCs to purify and identify novel interaction partners of MEIS1. *Meis1* is endogenously expressed in bone marrow cells. NUP98-CDX1 fusion protein up regulates the transcription of *Meis1* whereas bone marrow cells transfected with neonatal HSC specific transcription factor *Sox17* do not overexpress *Meis1* (Figure A.1). Many hematopoietic cell-specific proteins would be expressed in these cells aiding in the identification of physiologically relevant interactions of MEIS1.

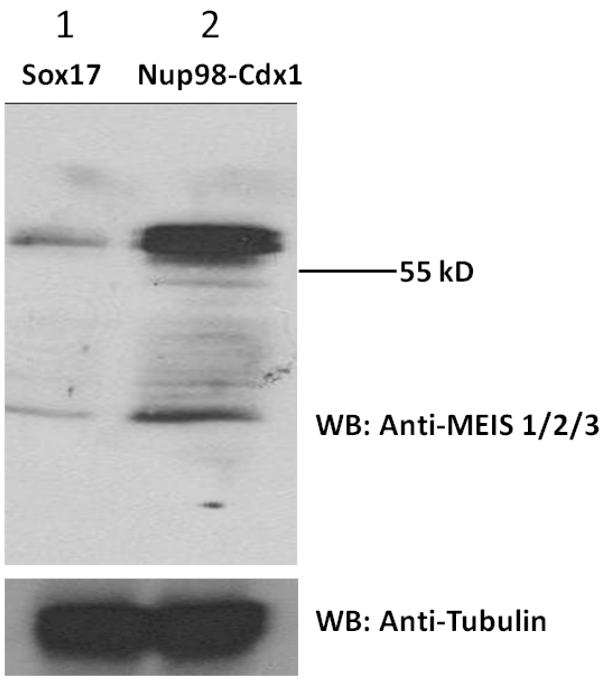


Figure A.1: Endogenous expression of MEIS1 in Nup98-Cdx1 immortalized bone marrow cells: Nup98-Cdx1 immortalized bone marrow cells over-express *Meis1* (lane 1). Bone marrow cells transduced with Sox17 gene do not overexpress *Meis1* expression (lane 1). Tubulin expression is shown as a control.

To achieve this we first created a multi-cistronic retroviral vector expressing FBM, *E.coli* BirA and EGFP. Instead of the commonly used IRES system we employed the FMDV virus 2A peptide based system (de Felipe et al., 2006) for the coordinate expression of these three proteins (Figure A.2). The FMDV 2A which is a short 18 amino acid peptide is able to undergo co-translational cleavage in mammalian cells. The N-terminus of the 2A peptide initiates the cleavage of its own C-terminus as indicated in Figure A.2. When the 2A sequence is placed between two cDNAs in a single large open reading frame (ORF), the auto-cleavage of the 2A peptide results in the production of two independent proteins. The protein upstream to 2A retains the 2A peptide (Figure A.2).

The cDNAs for EGFP, BirA and FBM were cloned in to the pMyc-Klf4-Sox2-Oct4 retroviral vector (courtesy of Prof. Klaus Karjalainen, NTU). The resulting plasmid hereafter referred to as pMyc-EBF would produce the EGFP and the BirA

proteins with a small 2A peptide overhang at their C-termini. Since there is no 2A peptide downstream of FBM it does not have the overhang (Figure A.2).

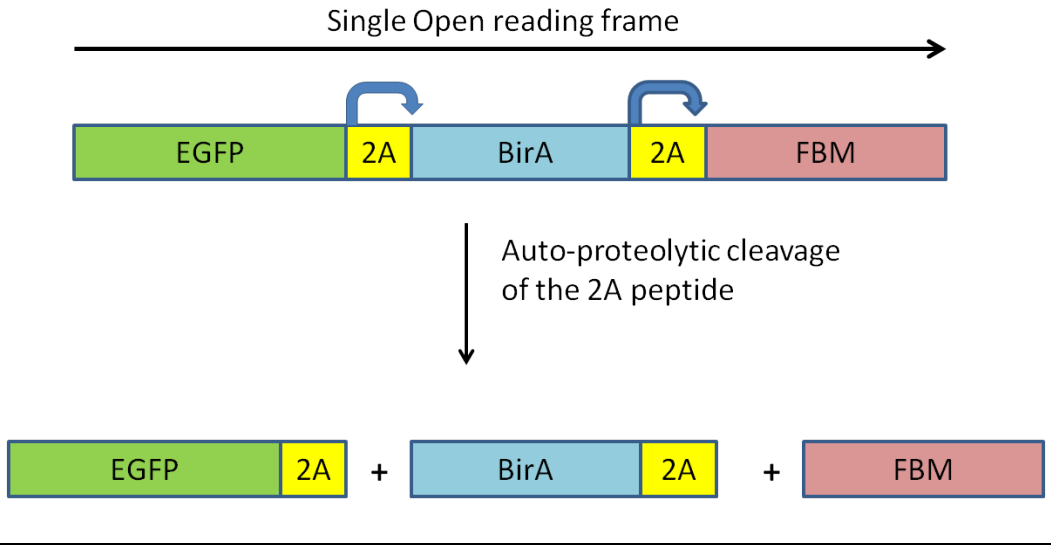


Figure A.2: Production of multiple proteins from a single open reading frame using FMDV 2A peptide: A short 18 amino acid peptide derived from the Foot and mouth disease virus (FMDV). This peptide is able to undergo auto-proteolytic cleavage to produce three independent proteins.

A.2. Gene transfer and the validation of protein expression:

Retroviruses containing the pMyc-EBF plasmid were generated by using the Plat/E packaging cell line. The retrovirus-containing supernatants (4-5ml) were transferred onto fibronectin-coated 60mm dishes. The dishes were spun at 4°C, 4000rpm for 30 min. The step was repeated once with more supernatant. The *Nup98-Cdx1* immortalized bone marrow cells were then added to the virus-coated dish and spun at 37°C, 750g for 45min. The culture dishes were then incubated at at 37°C to establish the cultures. This process was repeated if desired. Transduction frequency was routinely more than 50% based on EGFP expression. Transduced lines were maintained in S6 medium on Petri dishes at average cell density of 1×10^{6}cells/ml with regular counting and replating every 2 or 3 days. After many passages, the cells were sorted based on their EGFP expression using a FACSAria sorter. The bone marrow cells showing high EGFP expression were isolated and cultured further in S6 medium.

For immunoblotting, about 2×10^5cells cells were lysed by boiling in 2X laemmli sample buffer containing SDS at a final concentration of 4%. Both FBM (Left panel in Fig A.2) and BirA (Middle panel in Fig A.3) were expressed in the EGFP+ *Nup98-Cdx1*immortalized bone marrow cells. Both proteins were not expressed in un-transduced *Nup98-Cdx1* bone marrow cells (Figure A.3). Furthermore, FBM was endogenously biotinylated in the transduced *Nup98-Cdx1* cells (Figure A.3, right panel).

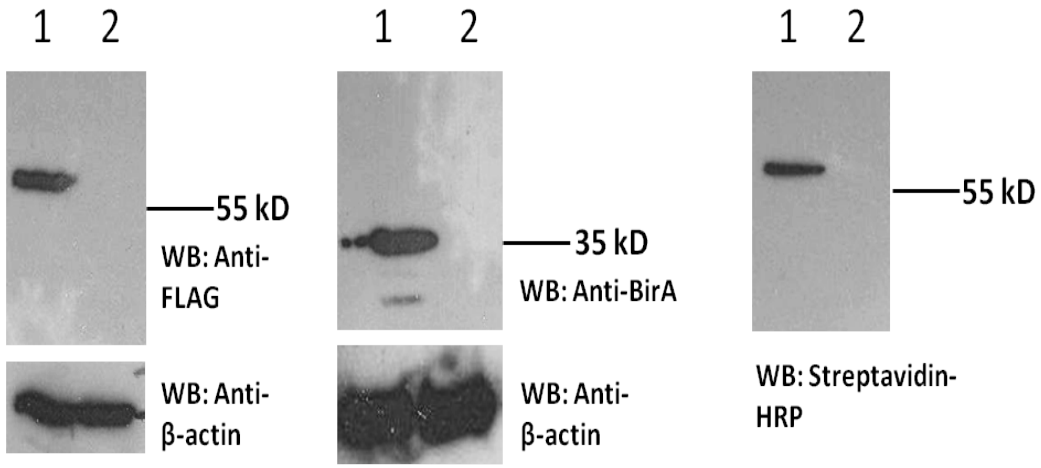


Figure A.3: Stable expression of FBM, BirA and the *in vivo* biotinylation of FBM in Nup98-Cdx1 cells: Expression of FBM (Left panel) and BirA (Middle panel). Whole cell extracts from pMyc-EBF transduced *Nup98-Cdx1* BM cells (lane 1 in both panels) and wild-type *Nup98-Cdx1* BM cells (lane 2 in both panels). Immunoblot was probed with anti-FLAG antibody (left panel) and anti-BirA antibody (Middle panel). β-actin expression is shown as a control for both the panels.

Right panel: The FBM protein is endogenously biotinylated in the pMyc-EBF transduced *Nup98-Cdx1*BM cells (lane 1) compared with the untransduced cells (lane 2). Immunoblot was probed with Streptavidin-HRP

A.3. Optimizing cell lysis conditions for the gentle lysis of the cells to preserve endogenous interactions:

Next, we tried to optimize the cell lysis buffers for the gentle and optimal lysis of these cells which would preserve the endogenous interactions of MEIS1. First, we tried using similar lysis conditions as described for HEK293T cells above. We were not able to detect MEIS1 when lysis buffer 1 (0.5% Triton X-100 and 0.5% NP-40, 150 mM KCl) was used to lyse the cells (data not shown). Increasing the concentration of NP-40 to up to 2% did not improve the conditions. We were able to detect β-actin expression but expression of both MEIS1 and PBX1 were not detected. This could possibly indicate that the lysis of the cytoplasmic membrane but the nuclear membrane could be resistant to lysis under these conditions (Figure A.4). Harsher methods of cell lysis like manual grinding of cell pellet after lyophilization using liquid nitrogen also were not able to lyse the nuclear membrane (data not shown).

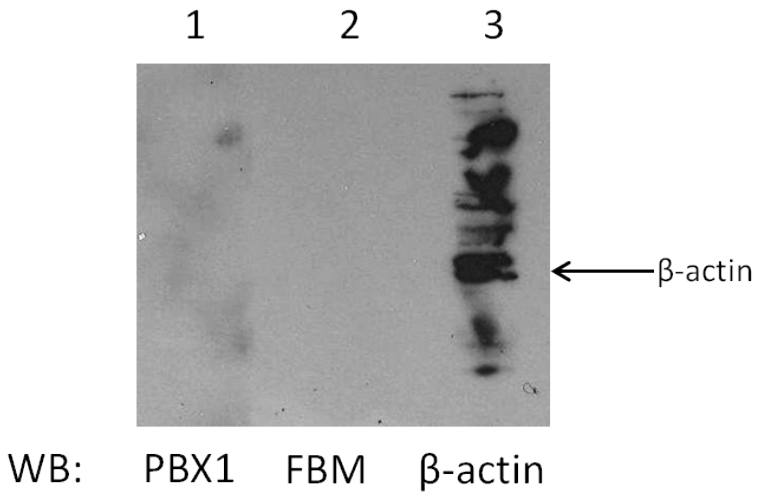


Figure A.4: Detection of β-actin but not FBM and PBX1 expression after lysis: Cells were lysed with a modified lysis buffer 1 containing 2% NP-40 using a similar protocol as mentioned for HEK293T cells. Immunoblotting showed that β-actin expression could be detected in the lysate (lane 3) but neither PBX1 (lane 1) nor FBM (anti-FLAG antibody) (lane 2) expression could be observed.

We hypothesize that the reason for the robustness of the nuclear membrane in these cells could be the high expression and localization of the NUP98-CDX1 fusion proteins to the nuclear envelope. It should be noted that NUP98 wild-type proteins normally localize to the nuclear membrane. The presence of the NUP98-CDX1 fusion protein may somehow modify the nuclear membrane to make it robust and impermeable to the conditions tested. Very high concentrations of detergent denature endogenous protein interactions. We did not try very high concentrations of SDS or Urea for this reason. Formaldehyde cross-linking followed by cell disruption using high concentrations of Urea can be used for lysis. But this procedure has the limitation of co-immunoprecipitating many non-specific protein interactions.

As far as we know, no interaction proteomics study has been reported with NUP98-fusion immortalized bone marrow cells similar to the one used here. Problems encountered with proper cell lysis could be a significant reason for the lack of studies.

Appendix 2

All the proteins identified in only 2 of the three individual experiments have been listed below

S.N	UNIPROT KB	GENE NAME	emPAI scores		No. of unique peptides identified	
			Ex1	Ex2	Ex1	Ex2
1.	P67936	TPM4	0.25	0.10	4	2
2.	Q9NZW5	MPP6	0.05	0.05	1	1
3.	O75683	SURF6	0.07	0.08	1	2
4.	Q3LXA3	DAK	0.06	0.05	1	1
5.	P17480	UBTF	0.04	0.03	2	1
6.	Q9BRX2	PELO	0.07	0.08	1	1
7.	P08708	RPS17	0.19	0.22	1	1
8.	Q03701	CEBPZ	0.02	0.03	1	1
9.	Q07065	CKAP4	0.05	0.04	1	1
10.	Q9BQ39	DDX50	0.07	0.04	3	1
11.	Q128690	CNTN1	0.03	0.03	1	1
12.	Q13601	KRR1	0.08	0.07	1	1
13.	P27694	RPA1	0.05	0.10	1	2
14.	Q9NW13	RBM28	0.04	0.07	1	2
15.	Q8WYP5	AHCTF1	0.01	0.01	2	1
16.	P52292	KPNA2	0.05	0.12	1	2
17.	Q6P158	DHX57	0.02	0.02	1	1
18.	Q9Y5J1	UTP18	0.09	0.05	2	1
19.	Q15007	WTAP	0.07	0.07	1	2

20.	Q8N9T8	KRI1	0.03	0.04	1	1
21.	Q9H6R4	NOL6	0.02	0.05	1	2
22.	Q01081	U2AF1	0.25	0.22	2	2
23.	P46100	ATRX	0.02	0.01	2	1
24.	P62910	RPL32	0.41	0.32	2	1
25.	P52926	HMGA2	0.57	0.29	1	1
26.	P28370	SMARCA1	0.05	0.03	3	1
27.	D55769	NHP2L1	0.24	0.45	1	2
28.	Q16629	SFRS7	0.12	0.11	1	1
29.	O66506	SYNCRIP	0.04	0.10	2	3

Figure A.5: Proteins which were identified to co-precipitate with MEIS1A in 2 of the three independent experiments.

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